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Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less

Jun Wu and Stephen M. Cohen*

European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany
Reprint requests to leillard@embl-heidelberg.de
*Author for correspondence (e-mail: scohen@embl-heidelberg.de)

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SUMMARY

The developing legs of *Drosophila* are subdivided into proximal and distal domains by the activity of the homeodomain proteins Homothorax (Hth) and Distal-less (Dll). The expression domains of Dll and Hth are initially reciprocal. Wingless and Dpp define both domains by activating Dll and by repressing Hth in the distal region of the disc. Wg and Dpp do not act through Dll to repress Hth. Hth functions to reduce the sensitivity of proximal cells to Wg and Dpp. This serves to limit the effective range of these signals in regulating later-acting genes such as Dac. We present evidence that proximal and distal cells tend to sort-out from one another. Cells forced to express Hth are unable to mix with distal cells. Likewise, cells forced to express Dll are unable to mix with proximal cells. Clones of cells unable to express Dll in the distal region sort-out from the disc. Clones of cells unable to express Hth lose the specialized population of cells at the interface between proximal and distal territories and cause fusion between body wall and leg segments. These observations suggest that sorting-out behavior of Hth- and Dll-expressing cells contributes to subdivision of the leg into proximal and distal domains.

Key words: *Drosophila*, Homothorax, Distal-less, Proximodistal axis, Leg

INTRODUCTION

The secreted signaling proteins Wingless (Wg) and Decapentaplegic (Dpp) play important roles in organizing the major axes of the developing legs and wings of *Drosophila*. Wg and Dpp act as concentration-dependent morphogens to pattern the dorsoventral (DV) and anteroposterior (AP) axes of the wing (reviewed in Lawrence and Struhl, 1996; Neumann and Cohen, 1997). Wg and Dpp also pattern the DV axis and proximodistal (PD) axis of the leg (Struhl and Basler, 1993; Díaz-Benjumea et al., 1994; Held et al., 1994; Lecuit and Cohen, 1997; González-Crespo et al., 1998).

Wg and Dpp act antagonistically through a process of mutual repression to define a stable DV subdivision in the leg primordium (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). Wingless is expressed in ventral cells and represses Dpp. Wg also specifies ventral cell fates (Couso et al., 1993; Struhl and Basler, 1993; Brook and Cohen, 1996; Jiang and Struhl, 1996). Dpp is expressed at elevated levels in dorsal cells, represses Wg and is required for specification of dorsal cell fates (Masucci et al., 1990; Held et al., 1994; Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). Wg and Dpp have been shown to act directly at a distance in a manner that suggests that they function as morphogens along the ventral and dorsal axes, respectively (Struhl and Basler, 1993; Lecuit and Cohen, 1997).

The combined action of Wg and Dpp triggers formation of the PD axis. During postembryonic limb development, Wg and Dpp expression domains meet at the center of the leg imaginal disc, corresponding to the most distal point of the presumptive limb (Campbell et al., 1993; Couso et al., 1993). The combined activity of both signals is required to define the spatial domains of target gene expression along the PD axis of the leg as the disc develops (Lecuit and Cohen, 1997; González-Crespo et al., 1998). Wg and Dpp act simultaneously to organize the DV and PD axes of the leg. They act in opposition to define the wedge-shaped expression domains of genes specific to dorsal or ventral axis and cooperatively to define the circular domains of gene expressed as rings along the PD axis (Lecuit and Cohen, 1997).

The targets of Wg and Dpp regulation define a series of discrete domains along the PD axis of the leg for the *Distal-less*, *dachshund* and *extradenticle* genes (Lecuit and Cohen, 1997; González-Crespo et al., 1998). *Distal-less* encodes a homeodomain protein (Dll) required for leg formation (Cohen et al., 1989). Distal-less activity is required during the early larval stages for development of the entire limb (Cohen and Jürgens, 1989). As the leg disc matures, Dll expression becomes restricted to the primordia of more distal leg segments (Díaz-Benjumea et al., 1994; Lecuit and Cohen, 1997) and clones induced at these stages show that the later requirement for Dll activity correlates with its expression in tibia and tarsal segments (Gorfinkiel et al., 1997). *dachshund* encodes a nuclear protein expressed in a domain overlapping Dll and is

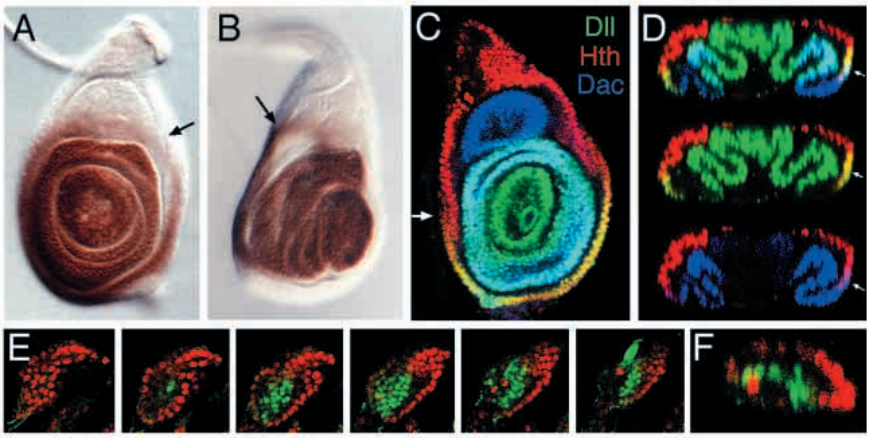

Fig. 1. Comparison of Dll and Hth expression at different stages of leg development. (A,B) Histochemical labeling to visualize Dll protein in mature third instar leg discs. (A) Frontal view showing expression in the tarsal and tibia segments. (B) Side view showing the folded disc epithelium. The arrows indicate a secondary ring of Dll expression at the level of the trochanter. (C,D) Optical sections of a mature third instar leg disc labeled to visualize Dll protein (green), dac-lacZ reporter protein (blue) and Hth protein (red). (C) Horizontal section; (D) optical cross-section projected from a series of horizontal sections of the disc in C. Dll is expressed in the center of the leg disc (green). Overlap of Dll and dac-lacZ appears light blue. dac-lacZ alone appears dark blue. All three labels are shown together in the upper portion of D. Dll and Hth are shown in the center portion. Overlap of the two expression domains at the outer ring of Dll expression appears yellow (arrow). dac-lacZ and Hth are shown in the lower portion. Overlap of the two expression domains at the edge of the two domains appears pink (arrow). (E) Series of optical sections through a second instar leg disc (labeled as in C,D). (F) Optical cross-section projected from the sections shown in E. Dll and Hth do not overlap. dac-lacZ is not expressed at this stage, indicated by the absence of blue labeling in the disc. dac-lacZ expression was seen in the CNS of the same animal (not shown).

required in an intermediate domain that corresponds to the femur and tibia (Mardon et al., 1994; Lecuit and Cohen, 1997).

The *extradenticle* (*exd*) and *homothorax* (*hth*) genes are expressed in a proximal domain corresponding to the body wall and proximal leg segments (Rauskolb et al., 1995; González-Crespo and Morata, 1995, 1996; Casares and Mann, 1998; Pai et al., 1998). Exd encodes a Pbx-like homeobox protein that functions as a cofactor for Hox proteins (Rauskolb et al., 1993; Chan et al., 1994; van Dijk and Murre, 1994). *homothorax* encodes a homeodomain protein (Hth) that binds to Extradenticle protein (Exd) and regulates its translocation into the nucleus, thus Hth is required for Exd activity (Rieckhof et al., 1997; Casares and Mann, 1998; Kurant et al., 1998; Pai et al., 1998). *exd* and *hth* are required for normal development of proximal leg segments, suggesting that the two proteins function together (Rauskolb et al., 1995; González-Crespo and Morata, 1995, 1996; Pai et al., 1998).

In this report, we address the functional relationship between

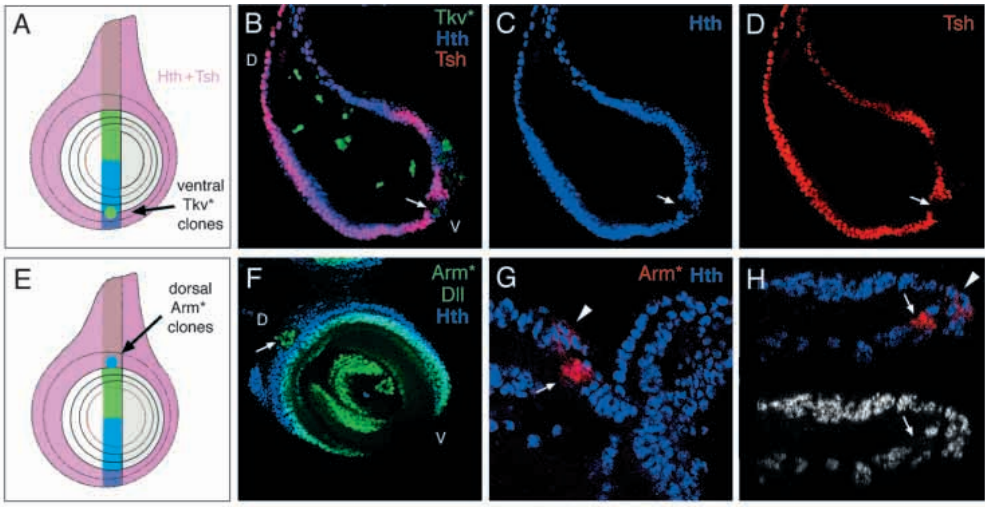

Fig. 2. Combined action of Wg and Dpp repress Hth and Tsh. (A) Schematic representation of a clone of cells expressing the activated Dpp receptor Tkv* in the ventral leg. The dorsal Dpp-expressing region is indicated by the green stripe. The ventral Wg-expressing domain is indicated by the blue stripe. The clone of Tkv*-expressing cells is represented as a green circle to indicate that these cells have the Dpp signal transduction pathway activated. The pink shading indicates the peripheral region of the disc where Tsh and Hth are expressed. (B-D) Disc containing multiple Tkv*-expressing clones. The disc is labeled to show Tkv*-expressing cells (green), Hth protein (blue, shown separately in C) and Tsh protein (red, shown separately in D). Overlap of Hth and Tsh appears pink. Tsh and Hth are repressed by a ventral clone (arrow). Note that the Tsh is also repressed in cells adjacent to the clone that continue to express Hth (blue only). D, dorsal; V, ventral. Clones were induced in larvae of genotype HSFlp/Act>CD2>Gal4; UAS-Tkv* UAS-GFP. (E) Schematic representation of a clone of cells expressing activated Armadillo (Arm*) in the dorsal leg. The clone is shaded blue to indicate activation of the Wg pathway. (F) Arm*-expressing clone in the dorsal portion of the leg disc (shown in a basal optical section). Arm* activity in dorsal cells induces Dll expression (green). Hth expression (blue) is repressed in the clone (arrow). The overlap of the proximal ring of Dll and the distal-most edge of the Hth domain appears light blue. (G,H) Arm*-expressing clone in the dorsal proximal part of a leg disc shown at higher magnification. Arm*-expressing cells are marked by antibody to the Flu-epitope tag on the Arm* protein (red). The disc contains two closely spaced patches of Arm* expression. Arm* is nuclear in one patch (arrow) and cytoplasmic in the second patch (arrowhead). It is not possible to be certain if these represent two clones in close proximity or one clone where Arm* expression is differentially translocated to the nucleus. Hth (blue) is repressed in the cells where Arm* is nuclear, but not where it is cytoplasmic. (G) A horizontal optical section; (H) a vertical optical section of the same disc. Hth expression is shown in black and white in the lower part of the panel. Many clones were observed that show one or the other type of Arm* localization with comparable effects of Hth expression.

Hth and Dll as primary readouts of the PD patterning system. We show that the combined action of Wg and Dpp defines the domain of Hth expression by repressing Hth in the distal region of the disc. In contrast to two recent reports (González-Crespo et al., 1998; Abu-Shaar and Mann, 1998), we find no evidence that Wg and Dpp act through Dll to repress Hth expression in the leg imaginal disc. Rather our results suggest that Wg and Dpp regulate Hth and Dll independently in early stages of leg disc development. Hth contributes to subsequent patterning of the PD axis by limiting the spatial domain in which Wg and Dpp can activate Dac expression. Hth therefore helps to distinguish limb and body wall territories, in part by limiting the effective range of Wg and Dpp. The subdivision into leg and body wall is not associated with a boundary of cell lineage restriction in wild-type discs. However, we present evidence that expression of Hth and Dll keeps cells from mixing at the interface between these two territories. This may be analogous to the subdivision of the DV axis of the leg into stable domains defined by gene expression without the need for a boundary of cell lineage restriction.

MATERIALS AND METHODS

Antibodies

dac-lacZ and anti-Dac (Mardon et al., 1994), anti-Dll (Vachon et al., 1992; Panganiban et al., 1994), anti-Hth (Kurant et al., 1998), and anti-Tsh (Ng et al., 1996) were used.

Fly strains

The following strains were employed: dpp^{Gal4} (Morimura et al., 1996), $UAS\text{-}Hth^{12}$ (Pai et al., 1998), *UAS-tkv** (Lecuit et al., 1996), *act>CD2>Gal4* (Pignoni and Zipursky, 1997), *UAS>CD2, y⁺>flu-Δarm* (Zecca et al., 1996), and $FRT82\ hth^{C1}$ (Rieckhof et al., 1997). *UAS-Dll* was constructed by cloning full-length cDNA (cDll7) into pUAST (Brand and Perrimon, 1993).

Clonal analysis

hth mutant clones were induced in larvae of genotype: *w HSFlp* f^{36a}*/+; FRT82 armlacZ Dp(f⁺) M(3R)W123 / FRT82* hth^{C1}. Clones were induced at 48-72 hours AEL, which corresponds to first or early second instar larvae due to the Minute developmental delay. *Dll* mutant clones were induced in *w HSFlpI; FRT42* Dll^{SA1} */FRT42 armlacZ* larvae. Clones were induced in 48-72 hour larvae (second instar) and examined in mid- to late-third instar. Control clones were induced in *w HSFlpI; FRT42 /FRT42 armlacZ* larvae.

Flip-out clones

GFP-expressing clones: *yw act>CD2>Gal4 / y HSflpI; UAS-GFP /+*. Clones were induced in 48-72 hour larvae.

Hth-expressing clones: *yw act>CD2>Gal4 / w HSflp22; UAS-GFP /* $UAS\text{-}Hth^{12}$. Clones were marked by coexpression of GFP and Hth. Clones were induced in 24-48 hour and 48-72 hour larvae.

Dll-expressing clones: *yw act>CD2>Gal4 / y HSflpI; UAS-GFP / UAS-Dll* Clones were marked by coexpression of GFP. Clones were induced in 72-96 hour larvae.

Tkv*-expressing clones: *yw act>CD2>Gal4 / y HSFlp; UAS-GFP / UAS-tkv**. UAS-Tkv* larvae were identified by the absence of the TM6B, Tb marker. Clones were marked by GFP expression. Clones were induced in 72-96 hour larvae.

Arm*-expressing clones: *yw HSFlp1; C765-GAL4 / UAS>CD2, y⁺>flu-Δarm*. Clones were induced in 72-96 hour larvae.

RESULTS

Dll and Hth expression in the leg imaginal disc

Fig. 1 summarizes the spatial relationship between Distal-less, Dachshund (Dac) and Homothorax expression in the developing leg disc. In the mature third instar disc, Dll is expressed in a large central domain that corresponds to the presumptive tarsus and distal tibia. In addition Dll is expressed

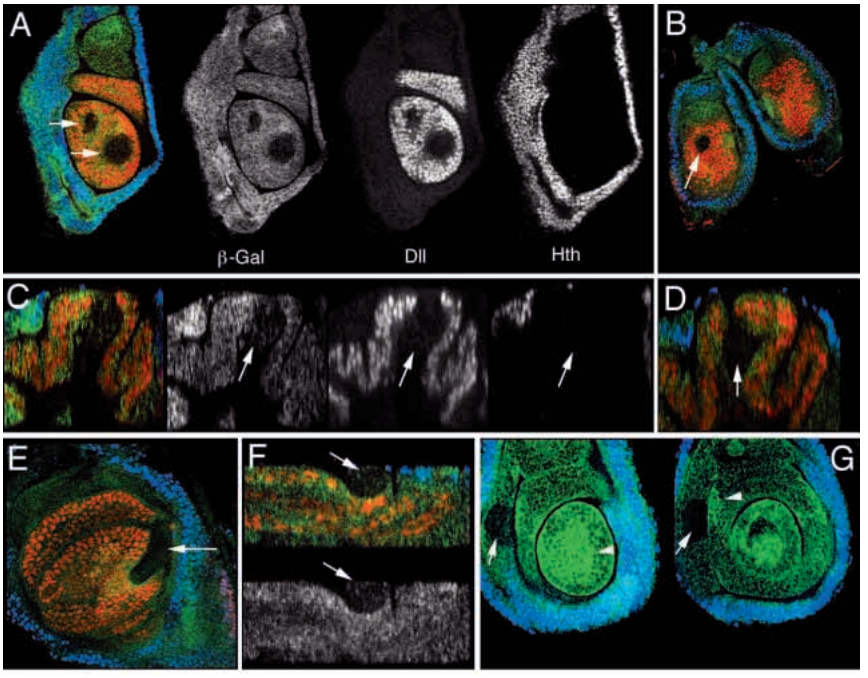

Fig. 3. Dll mutant clones in the distal leg do not express Hth. (A) Leg disc carrying two clones of *Dll* mutant cells in the tarsal segment (arrows, larval genotype: *HSFlpI; FRT42* Dll^{SA1}*/FRT42 armlacZ*). Clones are marked by the absence of β-gal (green) and by the absence of Dll (red). The clones do not express Hth (blue). The individual channels are shown separately in black and white. Several other Dll mutant clones are seen in the proximal Hth-expressing region. (B) Early-mid third instar disc showing a clone of *Dll* cells in the Dll domain (arrow). The clone is marked as in A. Hth is not expressed in the mutant cells. (C,D) Optical cross-sections through the two clones in A. Note the clones of cells lacking β-gal and Dll are mostly located below the plane of the epithelium (arrows); (C) the larger clone (merged image and separate channels are shown as in A); (D) the smaller of the two clones in A; note that the clone has almost completely segregated from the overlying epithelium. (E,F) Optical sections showing a clone of Dll mutant cells that has sorted-out above the disc epithelium. (E) Horizontal section through the tarsus and the clone (arrow). The clone does not express Hth. (F) Cross-sections showing the clone above the epithelium. The β-gal channel is shown separately below in black and white. (G) Two optical sections of a leg disc carrying a large Dll mutant clone in the femur (arrow). The clone does not express Hth. Note the presence of the twin spot adjacent to the clone (arrowhead). A second twin spot is seen in the tarsus (arrowhead) but is not associated with a Dll mutant clone.

in a secondary ring (Fig. 1A,B, arrows). X-gal staining of adult legs carrying a *Dll-lacZ* reporter gene shows that this ring is located at the proximal edge of the femur, possibly extending slightly into the distal trochanter (not shown). The central domain of Dll expression is controlled by Wg and Dpp. The proximal ring arises in third instar and does not depend on Wg or Dpp activity (see Díaz-Benjumea et al., 1994).

The leg disc is a continuous single-layered epithelial sheet which forms a series of folds as it grows. The peripheral region of the disc makes the proximal segments. This region is folded back over the central region where Dll is expressed. The topology of the disc and the expression patterns of Dll, dac-lacZ and Hth are shown in Fig. 1C,D. The domain of Hth expression (red) extends from the peripodial membrane at the top (Fig. 1D), through the coxa and trochanter segment primordia. The distalmost portion of the Hth domain overlaps the proximal part of the dac-lacZ domain (blue) within the proximal ring of Dll expression in the femur (green, arrows). Dll is expressed alone in the central folds of the disc (which correspond to tarsal segment primordia). In proximal tarsus and tibia Dll and Dac overlap (light blue). Dac is expressed alone in the presumptive femur (dark blue). Note that because the disc is highly folded, horizontal optical sections make proximal and distal regions of the disc appear to be closely apposed, although they are actually far apart along the PD axis in plane of the disc epithelium (compare Fig. 1C and D).

Fig. 1E shows that Hth (red) is expressed in the upper layer and around the lateral sides of the epithelial sac. Dll (green) is expressed in the center of the lower layer. In a vertical optical section through the same disc it is apparent that the two expression domains abut, but do not overlap (Fig. 1F). dac-lacZ is not detectably expressed at this stage (Fig. 1E,F), but can be reliably detected in slightly older discs at the transition from second to third instar (not shown). These observations suggest that the primary subdivision of the disc is into two domains: a central Dll-expressing domain and a proximal Hth-expressing domain.

Wg and Dpp repress Hth, but not through activation of Dll

Wg and Dpp act together to induce Dll and Dac in the center of the leg disc (Lecuit and Cohen, 1997). This suggested that Wg and Dpp might also be responsible for repressing Hth and Teashirt (Tsh), which is expressed in a similar domain. To ask whether cells receiving both signals are able to express Hth and Tsh, we produced clones of cells in which the Wg or Dpp signal transduction pathways were artificially activated at ectopic positions in the leg disc during third instar. The Dpp pathway was activated in clones of cells that express the ligand-independent activated form of the Dpp-receptor Thick veins (Tkv*, as depicted in Fig. 2A; Lecuit and Cohen, 1997). Tkv*-expressing clones on the ventral side of the leg disc repress Hth and Tsh expression (Fig. 2B-D). We note that repression of Hth is only seen in the Tkv*-expressing cells, but repression of Tsh extends one or two cell diameters outside the clone, suggesting the relay of another signal. Tkv* clones in dorsal or lateral positions do not affect Hth or Tsh (not shown). Clones expressing the activated form of the Wg signal-transducer Armadillo on the dorsal side of the disc repress Hth expression (Fig. 2E). The activated form of Armadillo is thought to translocate spontaneously to the nucleus in the absence of input from the Wg signal transduction system (Zecca et al., 1996). Armadillo* clones were examined in two ways. In one set of experiments clones were marked indirectly by their ability to induce Dll expression. These clones repress Hth expression (Fig. 2F). Similar results were obtained for repression of Tsh (data not shown). In a second set of experiments, Armadillo*-expressing clones were marked directly using the Flu epitope tag (Fig. 2G,H). We observed two types of clones. Clones where Armadillo* was nuclear showed repression of Hth, clones where Armadillo* was cytoplasmic did not repress Hth. We have no explanation for why Armadillo* is cytoplasmic in some clones and nuclear in others, but note that repression of Hth correlates with nuclear localization of Armadillo. Together these observations indicate that cells receiving both Wg and Dpp signals can repress expression of Hth and Tsh in the proximal segments of the leg.

The correlation between Dll expression and repression of Hth in proximal cells raised the possibility that Wg and Dpp act through Dll to repress Hth. This notion gains further support from the observation that Hth and Dll expression domains are reciprocal at early stages of disc development (i.e. before Dac is expressed, Fig. 1E). To ask whether Dll activity is required to repress Hth, clones of genetically marked Dll mutant cells were induced in second instar larvae and examined for Dll and Hth expression in third instar (genotype: *w HS Flp; FRT42 DllSA1/FRT42 arm-lacZ*). The majority of Dll mutant clones induced in second instar were recovered in the proximal, Hth-expressing region of the leg (126/159 clones examined). 7 clones were recovered in the tarsal region (e.g. Fig. 3A-E). Dll mutant clones were marked by the absence of the β-gal expression (green, directed by the arm-lacZ transgene) and by the absence of Dll protein (red). They do not show Hth expression (blue). 26 Dll^{SA1} clones were recovered in the femur and tibia. None of these clones show ectopic expression of Hth (e.g. Fig. 3G). Dll mutant clones examined in early third instar discs fail to show ectopic Hth expression (Fig. 3B). Our findings contrast with recent reports by Gonzalez-Crespo et al. (1998) and Abu-Shaar and Mann (1998) (see Discussion).

Sorting-out of *Dll* mutant clones

The distribution of Dll^{SA1} clones along the PD axis of the leg differs from the distribution of control clones. Only 20% of Dll^{SA1} clones were found distal to the outer ring of Dll expression (i.e. in femur, tibia or tarsal segments, see Fig. 1), compared to 65% of control clones (see Fig. 4 for numbers and clone locations). The ratio of distal-to-proximal clones is 2:1 for control clones and 1:4 for *Dll* mutant clones. If we assume that there is no proximodistal bias in the origin of clones, this suggests that most *Dll* mutant clones are lost distally. One reason for the low recovery of Dll^{SA1} clones in the tarsal segments is that the clones segregate out of the surrounding wild-type epithelium. Fig. 3C,D shows clones of Dll^{SA1} mutant cells in the process of invaginating below the plane of the disc epithelium, into what will be the interior of the leg when the disc everts. In other cases, the clones appear to extrude above the plane of the epithelium (Fig. 3E,F). Such clones would be lost when the disc evaginates. In many discs, wild-type twin spot clones were found in the tarsal segments, without an associated Dll^{SA1} mutant clone (e.g. Fig. 3G). It is likely that the corresponding mutant clones have segregated from the epithelium to form vesicles. We have observed vesicles of *Dll* mutant tissue inside the tarsal segments of the legs of adult flies

from the same experiment (data not shown; see also Gorfinkiel et al., 1997; Campbell and Tomlinson, 1998), indicating that the Dll^{SA1} clones survive until adult stages. Together these observations suggest that Dll^{SA1} clones are not lost from the distal part of the leg due to cell death, but that they can be lost due to sorting from the surrounding wild-type cells.

A second possible explanation for the poor recovery of mutant clones in the distal leg segments is suggested by comparing the distribution of mutant and wild-type clones. Of 126 proximally located Dll clones, 27 (21%) touch the outer ring of Dll expression. None of these clones enter the Dll ring. A similar proportion of control clones touch the Dll ring (7/35, or 20%), but about half of these clones were found either in the ring or crossing it (Fig. 4A). This suggests that the *Dll* mutant clones are unable to cross the outer Dll ring. In some cases, the Dll^{SA1} clones have grown adjacent to the ring for several cell diameters (e.g. Fig. 4B), suggesting that the mutant cells cannot mix with cells in the ring. This idea is supported by earlier observations that genetically marked Dll^{SA1} clones induced in first instar were limited to forming body wall and coxa and could not contribute to more distal leg segments in the adult (Cohen and Jürgens, 1989). In those experiments, the mutant cells were given a growth advantage. 70% of control clones contributed to both coxa and more distal leg segments while 30% were exclusively distal. In contrast, 100% of Dll^{SA1} clones were restricted to the coxa (Cohen and Jürgens, 1989). Together these observations suggest that cells must be able to express Dll in order for clones to cross the ring.

We have observed Dll^{SA1} clones interrupting the newly formed ring in early-mid third instar discs (2/10 clones, data not shown). The observations described above suggest that these clones must sort-out from the ring as the disc matures. In principle, clones could sort either distal to the ring or proximal to it. Those that sort proximally are likely to be recovered and contribute to formation of coxa and body wall. Some of the clones that sort distally can contribute to the femur but others appear to be lost from the disc epithelium (see also Campbell and Tomlinson, 1998).

Dll-expressing cells segregate from the Hth domain

Removing Dll expression causes cells to sort out from the distal region of the disc. We asked whether forced Dll expression would cause cells to sort out from the proximal, Hth domain. Clones of Dll-expressing cells were produced using Act>CD2>Gal4 to direct expression of UAS-Dll and UAS-GFP. Dll-expressing clones induced in second instar were recovered in the central region of the mature disc at high frequency, but were not recovered in the proximal Hth-expressing region (not shown). Clones expressing other genes do not show this distribution bias (see below). This suggests that Dll-expressing clones in the proximal region either sort out into the Dll-expressing domain or are lost from the disc.

Dll-expressing clones induced in early-mid third instar are also under-represented in the proximal region of the disc (Fig. 5). Most of the proximally located clones that were observed appeared to be in the process of segregating from the disc epithelium. Hth is expressed in Dll-expressing cells, though at somewhat reduced levels (confirming that these cells originated in the proximal epithelium). The fact that these cells express Hth indicates that they do not originate from the central region of the disc where most of the Dll-expressing clones are found.

Hth expression functionally separates proximal and distal domains

Clonal analysis suggests that there is no lineage restriction between proximal and distal domains in wild-type discs. For example, clones of cells marked by GFP expression (but otherwise wild-type) can cross from the Hth-expressing region through the Dll outer ring and into the Dac domain (Fig. 6A,B; Act>Gal4; UAS-GFP). In contrast, clones of cells expressing Hth appear to be unable to cross out of the endogenous Hth domain into the Dac domain (Fig. 6C-F; Act>Gal4; UAS-Hth + UAS-GFP). As shown in Fig. 1D, in wild-type discs cells expressing Hth overlap with cells expressing Dac only within the proximal ring of Dll expression. The proximal edge of the Dll ring is the limit of Dac expression and the distal edge of the ring is the limit of Hth expression. 12 out of 26 control GFP-expressing clones that touch the Dll ring extend distally to it compared with 0 out of 29 Hth-expressing clones. These observations suggest that Hth-expressing clones cannot mix with distal cells. Hth expression is not normally a clonally inherited property. Wg and Dpp can repress Hth expression in wild-type cells. In clones forced to express Hth, Wg and Dpp cannot repress Hth. These cells are therefore forced to respect a boundary between proximal and distal regions of the disc which normally does not serve as a lineage restriction.

These observations suggested that Hth expression may help to keep proximal and distal cells from mixing during normal development. In support of this proposal we noted that Hth-expressing clones located in the distal region of the disc segregate out of the epithelium (Fig. 7A-D). Fig. 7A and B show horizontal sections of a disc with a clone of Hth-expressing cells in the tarsus. In cross-section it is clear that the Hth-expressing clone lies beneath the tarsal epithelium (Fig. 7C, arrow). Hth expression does not repress Dll but causes ectopic expression of Dac in the central domain (Fig. 7D, overlap of Dll and Dac appears light blue). Expression of Dac in the central domain suggests that Wg and Dpp are unable to maintain repression of Dac in Hth-expressing cells (see Discussion). Hth-expressing clones were also marked with GFP expression. We observed vesicles of invaginated tissue in the tarsus and tibia in a high proportion of the adult legs examined in these experiments. These vesicles express GFP, suggesting that they derive from the invaginating Hth-expressing clones (Fig. 7E,F). Thus Hth-expression appears to make cells unable to mix with distal cells.

To investigate *hth* function further, we examined the effects of *hth* mutant clones on Dll and Dac expression. Clones of *hth* mutant cells induced in second instar do not affect Dll expression in its central domain, (Fig. 8A), but lead to loss of Dll expression in the outer ring (Fig. 8B). *hth* mutant clones can cause ectopic expression of Dac (Fig. 8C). The cross-section in Fig. 8D shows that the clone (arrow) is located in the presumptive dorsal coxa, which is folded back over the femur where Dac is normally expressed. Thus Hth represses Dac expression, but not Dll. We note that not all *hth* mutant cells show ectopic Dac expression suggesting that the ectopic activation of Dac depends on additional factors (see discussion). In the adult leg, *hth* mutant clones cause fusion of coxa, trochanter and femur segments but do not cause

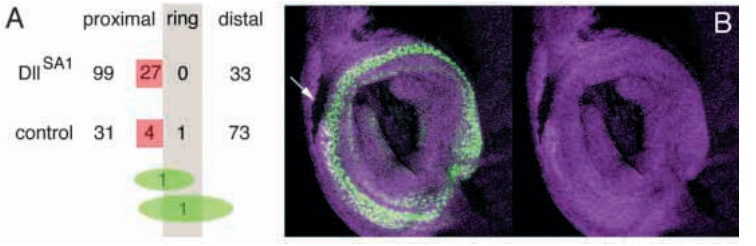

Fig. 4. Dll mutant clones in the proximal leg. (A) Summary of the distribution of marked Dll mutant and control clones in the leg disc. Dll clones were produced in *HSFlpI; FRT42 Dll^{SA1}/FRT42 armlacZ* larvae. Control clones were produced in *HSFlpI; FRT42 /FRT42 armlacZ* larvae. 'ring' indicates the proximal ring of Dll expression. 'distal' indicates clones in femur, tibia and tarsal segments. 'proximal' indicates clones in the Hth expression domain (excluding the Dll ring). Proximal clones are divided into two groups: those that do not touch the Dll ring and those that do. Proximal clones that touch the ring but do not enter it are shaded red, those that enter are shaded green. One clone was limited to the ring. (B) Leg disc carrying clones of Dll mutant cells marked by the absence of β-gal (purple) shown in a basal optical section. The position of the clone is indicated by the arrow. Wild-type twin spots are seen adjacent to the clone in the proximal and distal regions. The clone runs adjacent to the Dll ring (green) but does not enter it. This disc had several other proximal Dll mutant clones that are not visible in the basal section shown (data available on request). Dll mutant cells located outside the Dll ring express Hth (data available on request).

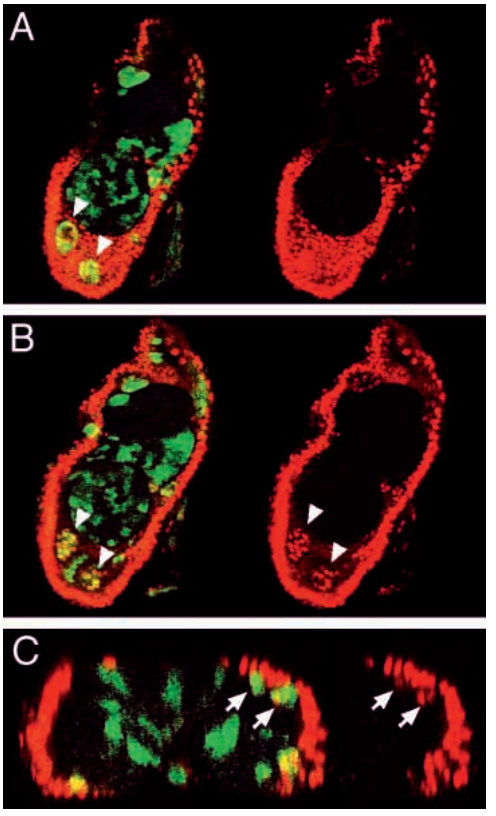

Fig. 5. Dll-expressing clones. (A,B) Optical sections of a leg disc with many Dll-expressing clones (Act>Gal4; UAS-Dll UAS-GFP). Dll-expressing clones are labeled by coexpression of GFP (green). Hth expression (red) is shown separately at right. The plane of sections are slightly oblique so that they pass through the proximal portion of the disc at bottom and through the distal tip in the center, but miss the proximal folds toward the top (see Fig. 1C,D for orientation). Arrowheads indicate two clones of Dll-expressing cells in the proximal fold of the ventral epithelium. The deeper section in B shows that the Dll- and Hth-expressing cells have dropped below the plane of the epithelium, suggesting that they are sorting out from the surrounding cells. Hth expression is only slightly reduced in the clone. (C) Optical cross-section projected from a series of 16 horizontal sections through the disc in A and B (magnification of C is 2× that of A and B). The right half of the Hth channel is shown separately at right. Arrows indicate two Dll-expressing clones. Note that the clones appear to have segregated from the overlying proximal epithelium (red).

defects in distal leg segments (Fig. 9). *hth* adult clonal phenotypes are comparable to those produced by *exd* mutant clones (González-Crespo and Morata, 1995; Rauskolb et al., 1995), as reported previously (Casares and Mann, 1998; Pai et al., 1998).

DISCUSSION

Regulation of Hth expression

The expression patterns of Dll and Hth/Exd reflect an early subdivision of the disc into proximal and distal domains. At early stages of disc development, Dll and Hth/Exd are expressed reciprocal domains, which account for all cells of the disc (Fig. 1; see also González-Crespo et al., 1998). At this stage, Dac is not yet expressed. What is the relationship

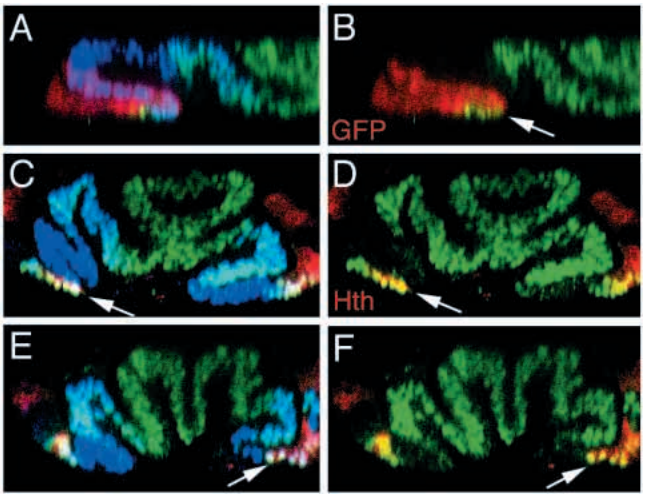

Fig. 6. Hth-expressing clones in the proximal leg. (A,B) Optical cross-section of half a leg disc showing a clone of GFP-expressing cells (Act>Gal4; UAS-GFP) crossing the outer ring of Dll expression. GFP is shown in red, Dll in green and Dac in blue. (B) Same image showing GFP and Dll. (C-F) Optical cross-sections of a disc with several Hth-expressing clones (Act>Gal4; UAS-Hth/UAS-GFP). Hth-expressing clones were labeled by coexpression of GFP (red). (C,D) The Hth-expressing clone at left meets the edge of the Dll ring but does not cross into more distal territory (arrow, overlap of Dll, Dac and Hth labels appears white). (D) Same image showing Hth and Dll, overlap appears yellow). (E,F) The Hth-expressing clone at right meets the edge of the Dll ring but does not cross into more distal territory (arrow).

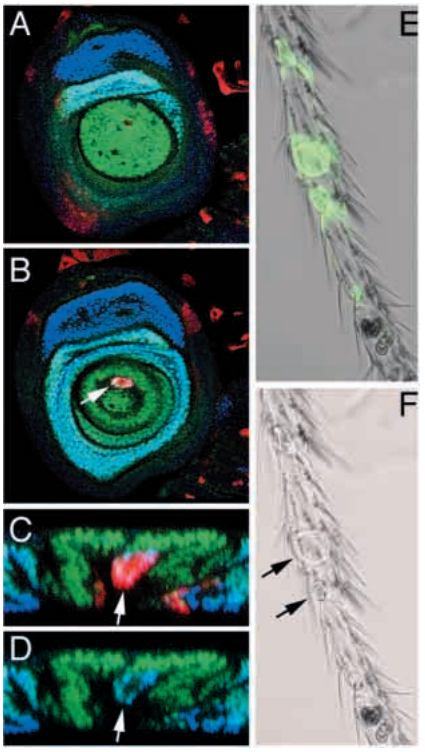

Fig. 7. Hth-expressing clones in the distal leg. (A-D) Hth-expressing clone in the tarsus. the clone was induced in first instar. (A) Section showing the top of the tarsal epithelium; (B) deeper section showing the clone (red, arrow). (C) Cross-section showing that the clone has invaginated and lies below the plane of the epithelium. (D) Same section showing only Dac (blue) and Dll (green, overlap appears light blue). The clone expresses Dac and Dll. Equivalent results were obtained with clones induced in second instar (not shown). (E,F) Tarsal segments of an adult leg carrying GFP and Hth-expressing clones induced in second instar. E shows the overlay of the GFP fluorescence (green) and the bright-field image. The clones form vesicles inside the adult leg (arrows, in F). This is consistent with the invagination of clones observed in the discs (as shown in C,D).

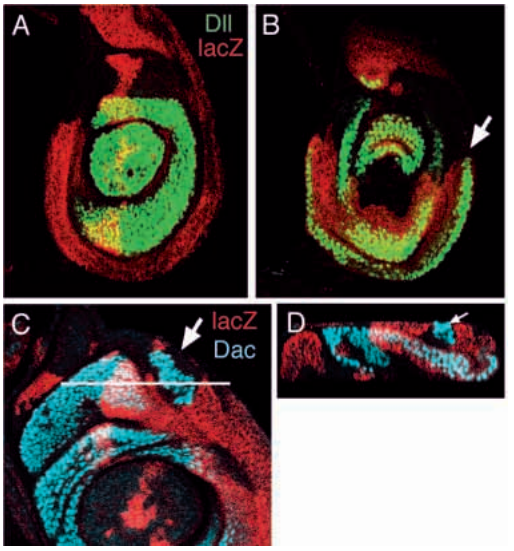

Fig. 8. *homothorax* mutant clones in discs. (A,B) *hth* clones in a disc labeled for Dll protein (green). The clone is marked by the absence of β-gal (red). (A) Optical cross-section through the tarsus and tibia domains of Dll expression. Dll expression is unaffected in the clone (right half of the disc). (B) The proximal ring of Dll is missing in the *hth* mutant cells in a basal optical section (arrow). (C,D) *hth* clones in a disc labeled for Dac protein (blue). Ectopic expression of Dac is seen in a proximal *hth* mutant clone (arrow). (D) Optical cross-section at the position indicated in C shows that the clone is located in the dorsal coxa and ectopically expresses Dac (arrow). Note that more proximally located *hth* mutant cells do not express Dac.

between Dll and Hth/Exd expression in the early disc? The Dll domain is defined by Wg and Dpp signaling (Lecuit and Cohen, 1997). The same signals repress nuclear localization of Exd (González-Crespo et al., 1998) and Hth expression (Fig. 2; Abu-Shaar and Mann, 1998). The reciprocity of Dll and Hth expression suggests a model in which Wg and Dpp act through Dll to repress Hth in the early disc. However, our analysis of marked *Dll* mutant clones shows that this is not the case. Clones of *Dll* mutant cells located in the distal region of the leg do not express Hth (Fig. 3). This contrasts with recent reports by González-Crespo et al. (1998) and Abu-Shaar and Mann (1998) in which evidence is presented for ectopic expression of Exd and Hth in *Dll* mutant clones.

How can we account for the difference in the results between these reports? In both studies, the clones were induced in second instar larvae using the same allele of *Dll*. In our experiments, clones were marked by the absence of Dll protein and by the absence of a neutral β-gal marker, which permits definitive genotyping of the cells independent of Dll expression. In the other reports, clones were marked only by the absence of Dll. The disc epithelium is highly folded and the proximal Hth-expressing epithelium is very close to the distal Dll-expressing

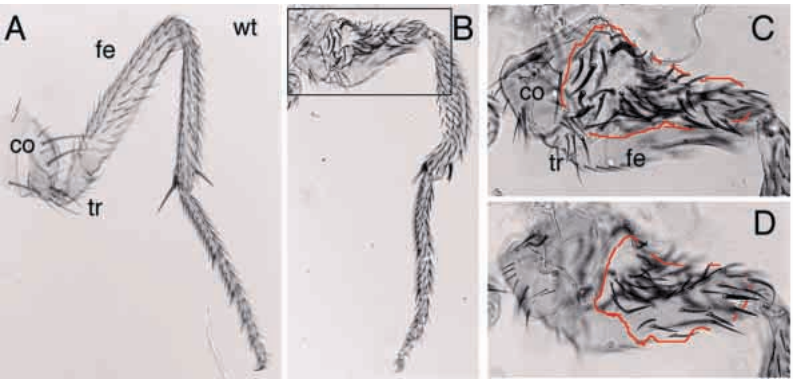

Fig. 9. *homothorax* clonal phenotypes in adult legs. (A) Wild-type second leg; co, coxa; tr, trochanter; fe, femur. (B-D) *Minute*$^{+}$ *homothorax* mutant clone in the second leg. (C,D) Both surfaces at higher magnification. The clone is marked by the absence of a *Dp(f*$^{+}$*)* transgene, so mutant cells have *forked* mutant bristles. Coxa, trochanter and femur segments can be distinguished clearly outside the clone, but are fused in the clone. This phenotype has been described for *exd* mutant clones (González-Crespo and Morata, 1995; Rauskolb et al., 1995).

epithelium (as illustrated in Fig. 1). Unless cells in the clone are definitively genotyped, it is difficult to distinguish a genuine clone from a patch of the overlying Hth-expressing proximal epithelium that has been pushed downward into the plane of the optical section. Serial optical sections of wild-type discs show that this type of distortion of the disc epithelium can occur in damaged discs as well as in discs that are not obviously damaged (data not shown).

How is Hth repressed by Wg and Dpp? Dac is induced by Wg and Dpp toward the end of second instar (Lecuit and Cohen, 1997). Hth expands distally to some extent in Dac mutant discs (Abu-Shaar and Mann, 1998, and data not shown). These observations suggest that Dac contributes to Hth repression. However, our results show that Hth is repressed prior to the onset of Dac expression (Fig. 1E) indicating that Dac cannot be the primary repressor. Whether Wg and Dpp act directly to repress Hth expression or act via another as unidentified repressor remains to be determined.

Sorting out of Dll mutant clones

Our results suggest that cells unable to express Dll are unable to remain integrated into the epithelium of the distal segments. We observed Dll mutant clones in the tarsal segments sorting out from the disc epithelium. If the clone segregates below the disc epithelium the vesicle can be recovered inside the adult leg. If the clone segregates above the disc, the vesicle is likely to be lost when the disc everts. There appears to be less bias against persistence of *Dll* mutant clones in the femur, where Dll expression normally decreases to low levels beginning in early third instar. Most of the distal *Dll* mutant clones that we recovered after clone induction in second instar were in the femur (25/33). *Dll* mutant clones induced in third instar can be recovered at higher frequency and contribute to normal development of femur and tibia structures (Gorfinkiel et al., 1997; Campbell and Tomlinson, 1998).

In mature discs, we were unable to find any Dll mutant clones that interrupt the outer ring of Dll expression (Fig. 4). This is also true for discs where Dll mutant cells were given a growth advantage (Minute$^+$, data not shown). In contrast, we did observe Dll mutant clones that interrupt the outer ring of Dll expression at a reasonably high frequency in early to mid third instar discs (2/10 clones; data not shown). This suggests that Dll mutant cells must sort out soon after Dll expression is turned on in the ring. Clones that sort distally are likely to be found in the femur, whereas those that sort proximally are likely to contribute to the coxa and body wall (some of these clones may also sort out from the epithelium, see also Campbell and Tomlinson, 1998). All of the *Dll* mutant clones found proximal to the outer ring express Hth, suggesting that repression of Hth must be alleviated in those clones that sort out proximally to the ring. As indicated above, Hth expression in these clones cannot be attributed directly to the lack of Dll expression, but may be an indirect consequence of their altered position relative to the source of Wg and Dpp.

Hth activity defines a functional boundary between proximal and distal regions of the leg

Our results suggest that Hth functions to keep the proximal and distal domains of the leg separated. When Hth is removed, proximal and distal segments fuse together, suggesting that the segment primordia are unable to maintain a clear separation in the absence of Hth function. This correlates with loss of Dll expression in the outer ring and with ectopic proximal expression of Dac (Fig. 8). Ectopic expression of Dac is seen in only some Hth mutant cells suggesting that Hth does not directly repress Dac. Dac is normally activated in response to low levels of Wg and Dpp. If Hth reduces sensitivity to Wg and Dpp, proximal clones of *hth* mutant cells might respond as though they were located higher up the activity gradient and therefore induce Dac. This suggestion gains additional support from the observation that Hth-expressing clones in the femur repress Dac (data not shown) and that Hth-expressing clones in the tarsal segments express Dac (Fig. 7). Dac is normally repressed in the distal tarsal segments by the combined action of Wg and Dpp (Lecuit and Cohen, 1997), so reducing sensitivity of distal cells to Wg and Dpp by ectopic Hth would alleviate repression of Dac. Our results suggest that Hth limits the size of the Dac domain by reducing sensitivity of proximal cells to low levels of Wg and Dpp. Thus Wg and Dpp define the size of the Hth domain which in turn delimits the territory in which Wg and Dpp activate Dac and other target genes. Similar conclusions have recently been reported by González-Crespo et al. (1998) and Abu-Shaar and Mann (1998).

How does Hth activity help to keep proximal and distal regions separate in the leg disc? The outer ring of Dll expression forms at the interface between proximal and distal domains of the disc. Cells in the ring express Hth, Dac and Dll. The distal limit of the Hth coincides with the distal edge of the ring. Clones of cells expressing Hth appear to be unable to leave the ring on the distal side. When Hth-expressing clones occur in the distal domain, they sort out of the disc epithelium. Clones of cells mutant for Dll appear to be excluded from the ring and, in some cases, appear to grow along the proximal border of the ring. Previous studies have shown that clones of cells mutant for Dll induced in first instar are limited to contributing to the body wall and coxa (Cohen and Jürgens, 1989). This is true even if the mutant cells are given a growth advantage. The boundary for Dll mutant cells appear to coincide with the position at which the proximal ring of Dll expression will arise later in development. Taken together these observations suggest that cells in the Dll ring have special characteristics that allow them to interface with both proximal and distal cells. We observed that *hth* mutant clones lose expression of Dll where they cross the outer ring, suggesting that Hth activity is required for Dll expression in the ring. The disturbance of the interface between of proximal and distal leg segments caused by *hth* mutant clones may reflect the loss of the Dll ring.

In conclusion, Hth and Dll expression appear to define alternative fates in the second instar disc. Under normal circumstances, there does not appear to be a cell lineage restriction between these populations (i.e. no compartment boundary). Our results suggest that cells can cross between these territories if they are able to switch between Hth and Dll expression. This situation appears to be analogous to the DV subdivision of the leg disc. DV subdivision is stable at the level of gene expression in a cell population, but is not a clonal lineage restriction boundary (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). Similarly, the separation of proximal and distal cell populations requires Hth function. Our results suggest that cells at the interface between these two territories are

specialized to allow integration of otherwise immiscible populations of cells.

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