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

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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). 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; 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; Penton and Hoffmann, 1996; Theisen et al., 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...
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. 1. Comparison ofDll 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).

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* 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,H) A horizontal 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., 1992), anti-Dll (Vachon et al., 1992; Fangman 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: dppGal4 (Morimura et al., 1996), UAS-Hth12 (Pai et al., 1998), UAS-Tkv* (Lecuit et al., 1996), act>CD2>Gal4 (Zecca et al., 1996), and FRT82 hthC1 (Rieckhof et al., 1997).

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...
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 Diaz-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 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 depress 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 DllSA1 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 DllSA1 clones along the PD axis of the leg differs from the distribution of control clones. Only 20% of DllSA1 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 DllSA1 clones in the tarsal segments is that the clones segregate out of the surrounding wild-type epithelium. Fig. 3C,D shows clones of DllSA1 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 DllSA1 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 Gorfinkeil et al., 1997; Campbell and Tomlinson, 1998), indicating that the DilSAl clones survive until adult stages. Together these observations suggest that DilSAl 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 Dil clones, 27 (21%) touch the outer ring of Dil expression. None of these clones enter the Dil ring. A similar proportion of control clones touch the Dil 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 Dil mutant clones are unable to cross the outer Dil ring. In some cases, the DilSAl 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 DilSAl 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 DilSAl clones were restricted to the coxa (Cohen and Jürgens, 1989). Together these observations suggest that cells must be able to express Dil in order for clones to cross the ring.

We have observed DilSAl 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).

Dil-expressing cells segregate from the Hth domain
Removing Dil expression causes cells to sort out from the distal region of the disc. We asked whether forced Dil expression would cause cells to sort out from the proximal, Hth domain. Clones of Dil-expressing cells were produced using Act>CD2>Gal4 to direct expression of UAS-Dil and UAS-GFP. Dil-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 Dil-expressing clones in the proximal region either sort out into the Dil-expressing domain or are lost from the disc.

Dil-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 Dil-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 Dil-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 Dil 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 Dil expression. The proximal edge of the Dil 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 Dil 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 Dil but causes ectopic expression of Dac in the central domain (Fig. 7D, overlap of Dil 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 Dil and Dac expression. Clones of hth mutant cells induced in second instar do not affect Dil expression in its central domain, (Fig. 8A), but lead to loss of Dil 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 Dil. 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
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...
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. 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.

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
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