Development 126, 3407-3413 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV7720

Proximal to distal cell communication in the *Drosophila* leg provides a basis for an intercalary mechanism of limb patterning

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Accepted 19 May; published on WWW 5 July 1999

SUMMARY

Proximodistal patterning in the *Drosophila* leg is elaborated from the circular arrangement of the proximal domain expressing *escargot* and *homothorax*, and the distal domain expressing *Distal-less* that are allocated during embryogenesis. The distal domain differentiates multiply segmented distal appendages by activating additional genes such as *dachshund*. Secreted signaling molecules Wingless and Decapentaplegic, expressed along the anterior-posterior compartment boundary, are required for activation of *Distal-less* and *dachshund* and repression of *homothorax* in the distal domain. However, whether Wingless and Decapentaplegic are sufficient for the circular pattern of gene expression is not known. Here we show that a proximal gene *escargot* and its activator *homothorax* regulate proximodistal patterning in the distal domain. Clones of cells expressing *escargot* or *homothorax* placed in the distal domain induce intercalary expression of *dachshund* in surrounding cells and reorient planar cell polarity of those cells. *Escargot* and *homothorax*-expressing cells also sort out from other cells in the distal domain. We suggest that inductive cell communication between the proximodistal domains, which is maintained in part by a cell-sorting mechanism, is the cellular basis for an intercalary mechanism of the proximodistal axis patterning of the limb.

Key words: *Drosophila melanogaster*, Leg development, Proximodistal axis, *escargot*, *homothorax*

INTRODUCTION

Patterning in insect legs is organized along anteroposterior (AP), dorsoventral (DV) and proximodistal (PD) axes. In the case of *Drosophila*, AP and DV axes of the leg imaginal discs are established along the embryonic AP and DV axes that are set up based on maternal positional information. The PD axis, on the other hand, is zygotically specified by cellular interactions involving secreted signaling molecules Wingless (Wg) and Decapentaplegic (Dpp) (Cohen, 1990, 1993; Goto and Hayashi, 1997).

PD axis formation in the leg disc becomes first evident when cells expressing either Escargot (Esg) or Distal-less (Dll) are arranged in a circular pattern (Goto and Hayashi, 1997). Dll expression defines the central, distal domain. Esg-expressing cells become the proximal domain that surrounds the distal domain. The Meis family homeodomain protein Homothorax (Hth) is expressed in the proximal domain as well as in the surrounding body wall and regulates nuclear localization of another homeodomain protein, Extradenticle (Exd) (Rieckhof et al., 1997; Pai et al., 1998; Abu-Shaar and Mann, 1998). Exd is active in the nucleus but inactive in the cytoplasm (Mann and Abu-Shaar, 1996). The genetic requirement for Dll, Exd and Hth suggest that the distal domain gives rise to the majority of the adult leg including tarsus, tibia, femur and trochanter (Cohen and Jürgens, 1989; Cambell and Tomlinson, 1998) and that the proximal domain gives rise to the coxa and the ventral thoracic body wall (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995; Wu and Cohen, 1999). Initial PD subdivision in the embryonic leg disc becomes elaborated during larval stages by activation of additional genes such as *dachshund* (*dac;* Mardon et al., 1994) in a circular intermediate domain between the distal and proximal domains. *dac* is required for specification of the intermediate fate (Mardon et al., 1994).

The leg imaginal disc is also divided into a posterior compartment that expresses the secreted molecule Hedgehog (Hh) and an anterior compartment that responds to Hh by expressing Wg and Dpp along the AP compartment boundary (Campbell et al., 1993; Basler and Struhl, 1994). Mutual repression between Wg and Dpp limits Wg expression to the ventral side and Dpp expression to the dorsal side (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). This spatial restriction of Wg and Dpp expression is essential for DV patterning of the leg. In addition, graded activities of Wg and Dpp are required for the expression of *Dll* and *dac* and repression of *hth* in the distal domain (Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). In the proximal domain, target gene activation by Dpp and Wg is inhibited by Hth and Exd, suggesting that the distal and proximal domains have distinct characters to respond to Dpp

and Wg (Gonzalez-Crespo et al., 1998; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999).

Based on the above observations, it was proposed that the circular patterns of gene expression along the PD axis in the distal domain are organized by the gradient of the combined activity of Dpp and Wg (summarized in Fig. 1A; Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998). In the central region where combined activity of Dpp and Wg would be high, *Dll* is activated and *dac* is repressed. An intermediate level of Wg and Dpp activities would allow *dac* expression in the intermediate domain. Gorfinkiel et al. (1997) showed that ectopic expression of *Dll* in the dorsal-proximal region induces *wg*, which is thought to interact with *dpp* to specify a new PD axis. These results suggest that the combination of Wg and Dpp constitute a 'distalizing' signal for the PD axis.

Although these results suggest that the combination of Wg and Dpp activities centered at the distal tip is essential for PD patterning, it is not known whether Wg and Dpp are sufficient to account for all aspects of PD positional information. In fact, the grafting and regeneration experiments using larval cockroach legs (reviewed by French et al., 1976) suggest that the reciprocal communication between distal and proximal parts of a leg segment promotes regeneration of the intermediate part. We thus speculated that a proximal to distal cell communication may also be used in PD patterning of the leg during development, and decided to test this idea by studying the functions of genes expressed in the proximal domain.

Esg is a Snail (Sna)-type zinc finger transcription factor, and is expressed in all imaginal tissues (Hayashi et al., 1993). It is required for the maintenance of imaginal cell identity in the wing disc (Fuse et al., 1996) and in abdominal histoblasts (Hayashi et al., 1993). A requirement for Esg in leg development was suggested from the analysis of partial loss-of-function mutants of *esg*, which showed a defect in the proximal leg (Hayashi et al. 1993). In the tracheal system, Esg regulates expression of the cell adhesion molecule *D*E-cadherin to promote fusion of tracheal tubes (Tanaka-Matakatsu et al., 1996).

In this study, we investigated the roles of Esg in the patterning of the PD axis in the leg. We first show that Esg is expressed in the proximal domain throughout leg development. Next we show that ectopic expression of Esg and its activator Hth in the

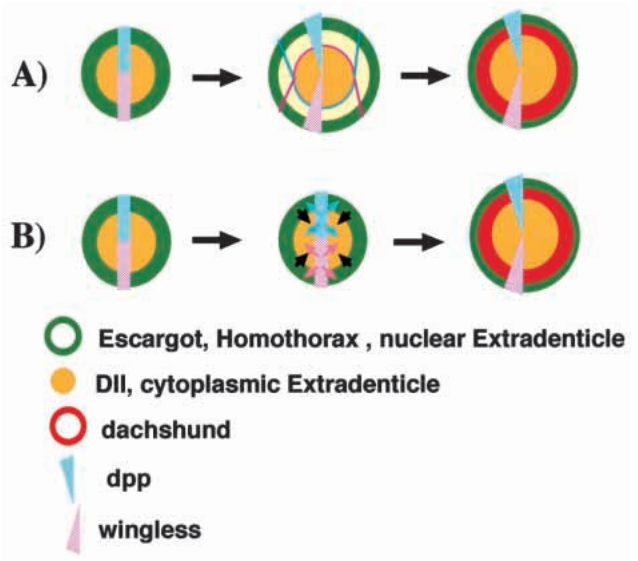

Fig. 1. Two models of the patterning along the proximodistal axis in the leg disc. (A) A model based on graded activities of Wg and Dpp (Lecuit and Cohen, 1997). Wg and Dpp are secreted from the respective ventral and dorsal halves of the cells immediately anterior to the AP compartment boundary. The combined activity of both Wg and Dpp is predicted to form a gradient along the PD axis. Differential response to this gradient leads to circular patterns of Dll and Dac expression. (B) A model that incorporates a proximal to distal signaling (this study). The embryonic leg disc has two circular subdomains along the proximodistal axis, i.e., the *Dll*-expressing distal domain (yellow) and the *esg*-expressing proximal domain (green). Cells in the proximal domain emit a signal (black arrows) to the distal domain, which polarizes planar cell polarity and is necessary for *dac* expression. Wg and Dpp secreted along the anteroposterior compartment boundary (pink and blue arrows, respectively) are also necessary for *dac* expression. The circular arrangement of the proximal domains limits *dac* expression to the intermediate domain.

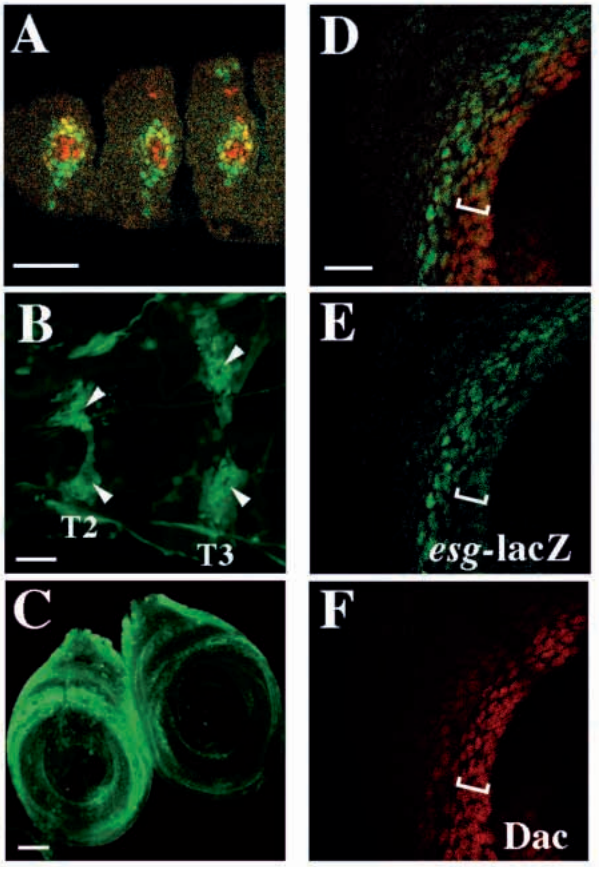

Fig. 2. Esg expression in leg development. (A) Esg (green) and *Dll-lacZ* (red) are expressed in the respective proximal and distal regions in stage-15 embryonic leg discs. (B) A second instar larva bearing both *esg-Gal4* and *UAS-GFP*. Esg expression is excluded from the distal-most regions (arrowheads). (C) Esg is highly expressed in the proximal region of the leg imaginal discs in this late third instar larva. (D-F) A leg disc of an $esg^{P3}lacZ$ larva stained for Dac (red, D,F) and β-galactosidase (green, D,E). A few cells-wide ring (brackets) of the distal most part of *esg-lacZ* expression overlaps with Dac expression in the trochanter. Scale bar: 25 μm.

distal domain induce the intermediate fate in surrounding cells by inducing *dac* expression. We further show that Esg and Hth-expressing cells in the distal domain undergo a change in their adhesive property to sort out from surrounding cells. The proximal to distal inductive communication was unexpected from the model based on the graded activity of Dpp and Wg. Thus we propose an intercalary mechanism that elaborates the PD axis pattern of the leg (Fig. 1B).

MATERIALS AND METHODS

Fly stocks and clonal analyses

esg^{G66B} (Kassis, 1994), *esg*^{P3} (Hayashi et al., 1993), *UAS-esg* (Fuse et al., 1994), *UAS-hth* (Pai et al., 1998), *Dll-lacZ* (*Dll*^{01092}; Goto and Hayashi, 1997), *wg-lacZ* (Kassis et al., 1992), and *omb-lacZ* (Sun et al., 1995) have already been described. *esg*^{NP5130} is a Gal4 enhancer trap line that reproduces larval *esg* expression (S. G, S. H. and NP consortium, unpublished). Flip-mediated mitotic recombination and act5C promoter-Gal4 fusion technique were carried out as described by Xu and Rubin (1993); Ito et al. (1997). Relevant genotypes are: *hsp-70-flp/+; esg*^{G66B} *FRT40A/hs2piM FRT40A* for *esg* mutant analysis, *hsp-70-flp/+; AYGal4 UAS-gfp/UAS-esg, omb-lacZ/hsp-70-flp; AYGal4 UAS-gfp/+; UAS-esg/+* for *esg* misexpression, *hsp-70-flp/+; AYGal4 UAS-gfp/+; UAS-hth/+ or hsp-70-flp/+; AYGal4 UAS-gfp/esg*^{P3}*; UAS-hth/+* for *hth* misexpression. Recombination was induced by a 0.5- to 1-hour heat shock at 37°C applied at 48-72 hours of development.

Immunostaining and histological analyses

Protein expression was detected by a combination of the following antibodies and reagents: rat anti-Esg (Fuse et al., 1994), mouse anti-Dac (Mardon et al., 1994), rat anti-Tsh (Zeng et al., 1993), rabbit anti-Exd (Mann and Abu-Shaar, 1996), rat anti-*D*E-cadherin (Oda et al., 1994), rabbit anti β-galactosidase (Cappel), mouse anti β-galactosidase (40-1a, Developmental study hybridoma bank, University of Iowa), Cy2-and Cy3-labeled secondary antibodies and Streptavidin (Amersham), and biotin-conjugated secondary antibodies (Jackson). Signals were sometimes enhanced by use of the TSA indirect system (NEN). Stained samples were observed with a Zeiss LSM410. Adult legs were mounted in Hoyer's mountant and immediately photographed for GFP images, and then cleared for cuticle analysis.

RESULTS

Expression of *esg* in the leg disc

The first sign of proximodistal axis formation in the leg imaginal disc was a circular arrangement of cells expressing either Esg or Dll during embryogenesis (Goto and Hayashi, 1997; Fig. 2A). As the disc grew in size and evolved circular folds that separated tarsus, tibia, femur, trochanter and coxa, the pattern of *esg* expression was maintained (Fig. 2B,C). At the late stage of the third instar, more Esg protein was detected in the proximal region corresponding to the coxa and trochanter (Fig. 2C). The distal most part of the *esg*-expressing domain partially overlapped with the Dac-expressing domain in the trochanter (Fig. 2D-F). The *esg* expression in the overlapping domain was weaker than that in the more proximal domain where only *esg* was detected. The domain of Esg expression appeared to overlap with the proximal domain

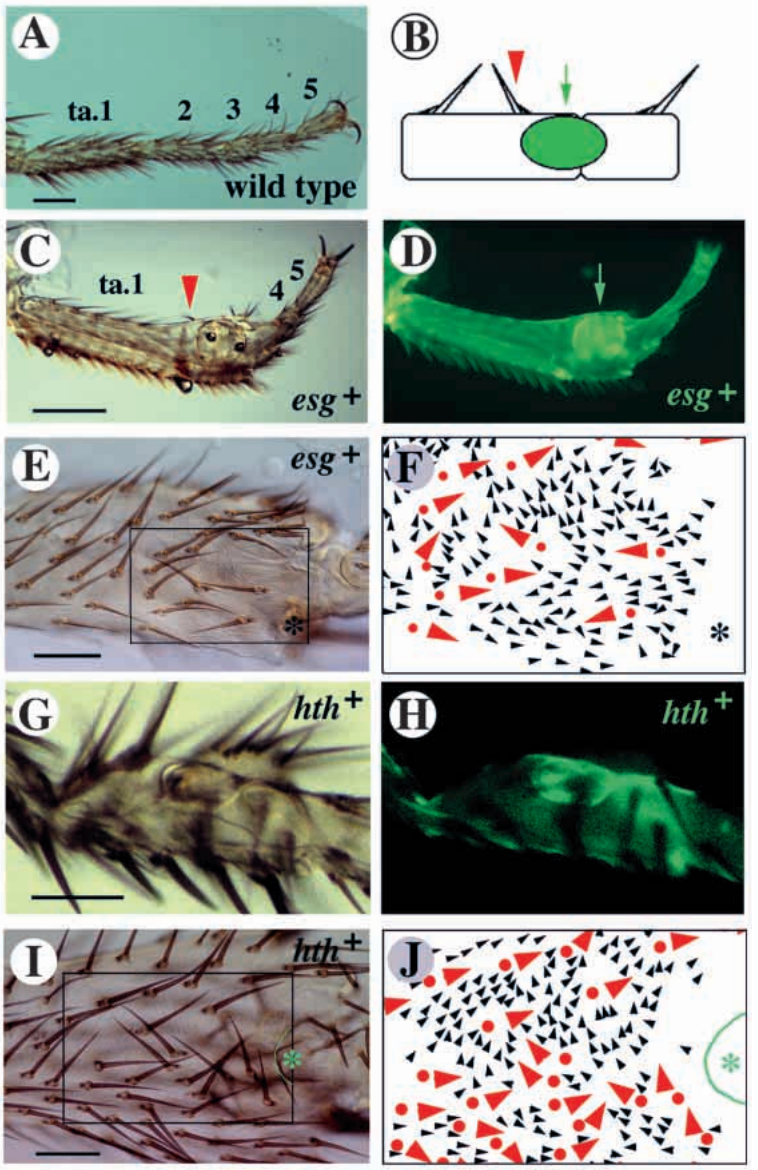

Fig. 3. *esg* organizes proximodistal cell polarity and growth in the adult leg. In all panels proximal is to the left. (A) The distal region of the wild-type leg. The tarsus (ta) has five segments, ta.1 to ta.5, with all associated bristles oriented from proximal to distal. The bract is associated proximally to the base of the bristle. (B-F) The legs in which *esg* was ectopically induced. An *esg*$^{+}$ clone marked with GFP (green) invaginated to form a vesicle (green arrow) in a cell-autonomous manner (B,D). The bristles (red arrowheads) located proximally to the *esg* clones are oriented from distal to proximal (B,C,E,F). The associated bracts are located distally to the reversed bristles. (F) A schematic view of the rectangular area marked in E. The orientation of the bristles (red arrowheads) and the hairs (small black arrowheads) is reversed near the *esg*$^{+}$ clone. The bracts are shown as red dots. Asterisks in E and F indicate the sites where the *esg*-expressing clone invaginated. (G-J) Legs in which *hth* was ectopically induced. (G, H) *hth*$^{+}$ clone marked by GFP (green) invaginated to form a vesicle in a cell-autonomous manner. (I) Epidermis of ta.1 flanking Hth expressing clone (asterisk) showing a reversal of bristle polarity. (J) A schematic view of the rectangular area marked in I. The orientation of bristles (red arrowheads) and hairs (small black arrowheads) is reversed near the *hth*$^{+}$ clone (indicated by the green line). Scale bar: A,C, 100 μm; E,G,I, 50 μm.

defined by expression of *homothorax* and *teashirt*, and nuclear localization of Extradenticle (Gonzalez-Crespo and Morata, 1996; Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998).

Non-cell-autonomous activity of Esg regulates *dac* expression and planar cell polarity

Dll induces distal leg development when expressed ectopically in the proximal domain (Gorfinkiel et al., 1997). To ask if any of the proximal genes have an organizing activity analogous to that of Dll, we induced *esg* ectopically using the flip-out technique (Ito et al., 1997). In the adult, Esg-positive clones marked by GFP were found as vesicles inside the leg cuticle and were often associated with malformation (Fig. 3B-D). In the region proximal to the clones, the bristles and epidermal hairs, which normally point distally, were often reversed (Fig. 3E,F). These bristles and hairs were genetically wild type, suggesting that the polarizing activity of Esg is non-cell-autonomous.

In the third instar leg disc, *dac* is expressed in a partially overlapping manner with the expression of *Dll* and *esg* in an intermediate ring that corresponds to the proximal tarsus, tibia, femur and trochanter (Mardon et al., 1994). When *esg* expression was induced during the second instar, clones in the distal tarsal region showed compact morphology; and many of them were associated with ectopic *dac* expression in cells within and surrounding the clone (Fig. 4A,B). The ectopic *dac* expression resulted in a local reversion of the proximal-distal order of the gene expression (Fig. 4A), which prefigured the change in the cell polarity in the adult leg (Fig. 3B-F). The *esg*-positive clones in the coxa spread normally and did not show induced *dac* (Fig. 4A, arrowhead).

Ectopic Esg expression does not affect Hh, Dpp or Wg signaling

The non-cell-autonomy of the Esg function could be due to a modulation of known secreted molecules controlling anteroposterior and dorsoventral patterning. However, the expression patterns of the Hh target genes *wg* and *dpp*, and *optomoter-blind (omb;* Brook and Cohen, 1996), a target gene of Dpp, were unaffected by misexpression of Esg (Fig. 4D-F). Moreover, the non-cell-autonomous induction of *dac* by Esg showed no strong bias for orientation (Fig. 4B) and position with respect to the compartment boundary and dorsoventral position. These observations suggest it to be unlikely that the inductive activity of Esg involves modulation of *hh*, *wg* or *dpp*; rather, it is probably mediated by an as yet unidentified signaling mechanism.

Esg is required for *dac* expression

We generated esg^{G66B} null mutant clones to assess the requirement of Esg for *dac* expression. esg^{G66B} is a derivative of an enhancer trap and lacks the coding region of *esg* but retains the *lacZ* gene that reproduces the expression pattern of *esg* (Kassis, 1994). *esg* mutant cells are marked by the loss of Myc antigen (Fig. 4G-I) or by the high expression of β-gal produced from the two copies of the *lacZ* gene (Fig. 4J-L). *dac* expression was frequently lost in clones induced at the late second instar larval stage. The partial loss of *dac* expression in large clones may have been due to a non-cell-autonomous rescue by esg^+ cells next to the clones. The clones were sometimes associated with ectopic fold formation (data not shown). Taken together with the gain-of-function analysis, these data suggest that Esg is necessary and sufficient for *dac* induction.

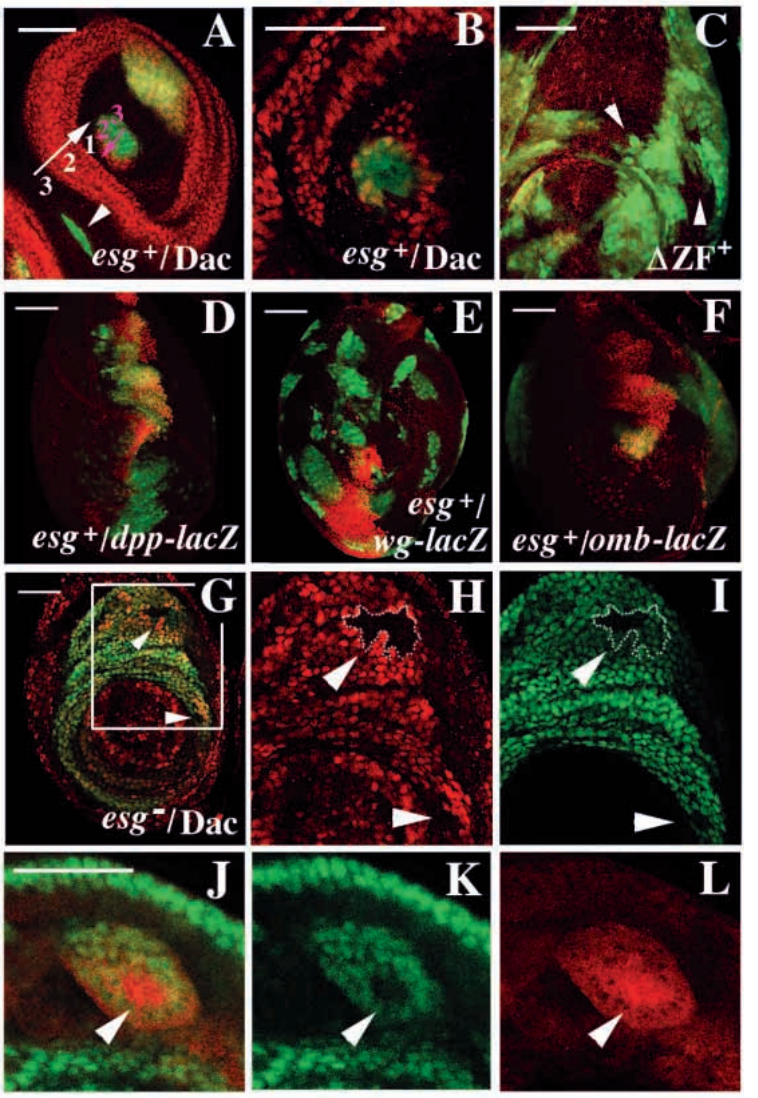

Fig. 4. Non-cell-autonomous effects of *esg* in the distal leg disc. (A,B) esg^+ clone marked by GFP (green) in the distal tarsus induced *dac* expression (red) in the neighboring cells and within the clone. Note a proximal-most clone (in A, arrowhead) that did not induce *dac*. The regions in which only *Dll*, both *Dll* and *dac*, or *esg* is expressed are arbitrarily numbered as 1, 2 and 3, respectively. Proximodistal order of region 1-3 was reversed adjacent to esg^+ clone. (B) Dac was induced in all surrounding cells. (C) Clones expressing non-functional Esg-delta ZF showed a wiggled border (arrowheads). (D,E,F) *dpp-lacZ*, *wg-lacZ*, and *omb-lacZ* expressions (red), respectively, were not affected by ectopic expression of *esg* (green). (G-I) *esg* mutant clones (arrowheads), marked by the lack of nuclear Myc epitope expression (red), lost *dac* expression (green) in all or a part of the clones. (H,I) high-magnification views of the rectangular area marked in G. (J-L) esg^{G66B} mutant clones (arrowheads) positively marked by the strong β-gal expression (red, J,L) lost *dac* expression (green, J,K) at the center of the clone (arrowhead). Scale bars, 25 μm.

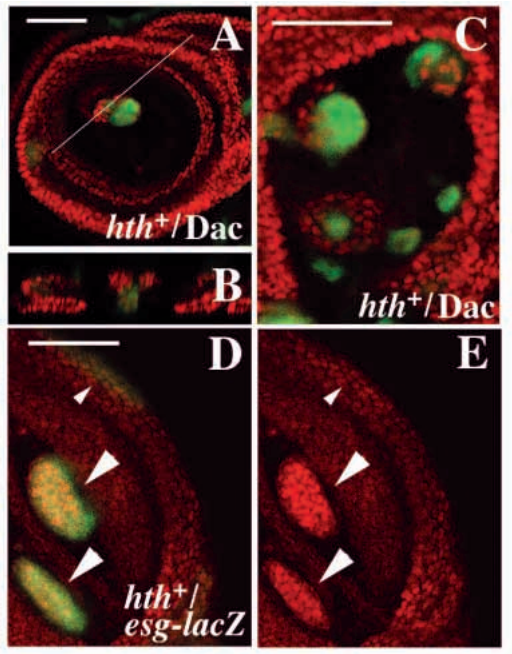

Fig. 5. Non-cell autonomous effects of *hth* in distal leg discs. (A-C) *hth* induced *dac* expression (red) in the neighboring cells and within the clone marked by GFP (green). (B) Transverse optical section of A shows that the *hth* clone invaginated and induced *dac* expression in neighboring cells and within the clone. (C) Dac was sometimes induced in all surrounding cells. (D) *hth* $^{+}$ clones (green) in the distal region induced *esg-lacZ* (red; E) in an cell-autonomous manner (large arrowheads). Proximally located clones did not lead to increased *esg-lacZ* expression (small arrowhead). Scale bars, 25 μm.

Hth activates *esg* and organizes the PD pattern

Proximal cell identity is, at least in part, controlled by the homeodomain protein Hth that regulates nuclear localization of Exd. When expressed ectopically in the tarsal region, Hth caused non-cell-autonomous induction of *dac* expression (Fig. 5A-C) and reversal of bristle and cell polarity (Fig. 3I,J); phenotypes very similar to those caused by Esg. Unlike *esg*-expressing clones, which secrete a smooth cuticle, *hth*-expressing clones in the distal part of the leg sometimes formed thick socketted bristles without bracts, which are characteristics of the bristles in the proximal part of the leg (data not shown). We found that Hth strongly activated a reporter gene under the control of the *esg* enhancer in the distal domain, but did not or only weakly did so in the proximal domain (Fig. 5D,E). This effect was cell-autonomous, suggesting that Hth may directly regulate transcription of *esg*. On the other hand, neither a loss nor a gain of *esg* expression affected the activity of Hth/Exd as assessed by the expression of Hth and nuclear localization of Exd, or by the expression of another proximal gene, *teashirt* (Gonzalez-Crespo and Morata, 1996; data not shown). These results suggest that Esg acts downstream of Hth/Exd to regulate proximodistal patterning.

Hth and Esg regulate cell adhesive properties

The *esg*- or *hth*-expressing clones in the distal region were round in shape with smooth borders and often invaginated basally to form vesicles in the adult legs and in the larval discs (Figs 3D,H, 4-6). In contrast, control clones expressing non-functional *esg*, which lacked the zinc-finger domain, and *esg*-expressing clones located in the coxa and trochanter had ragged borders (Fig. 4A,C). The epithelial-type homophilic cell adhesion molecule *D*E-cadherin (Oda et al., 1994) was expressed throughout the leg discs (Fig. 6A-C) and its apical localization was maintained normally in *esg*-expressing clones (Fig. 6D-F), suggesting that these cells kept their epithelial character. These results of ectopic expression studies, together with the loss of function studies on *hth* (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999; data not shown), indicate that Hth and Esg regulate a cell surface property that distinguishes the proximal and distal domains.

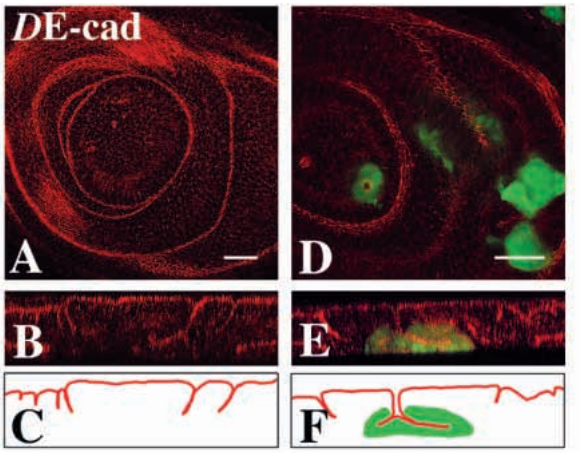

Fig. 6. *D*E-cadherin expression (red) in the wild-type leg disc (A-C) and in *esg*-expressing clones (green in D-F). Transverse sections (B,E) of A and D and their schematic views (C and F), respectively. *D*E-cadherin is expressed in all disc cells (**A**) and is localized at the apical surface, where the staining appears intense at the epithelium folds (B,C). In *esg*-expressing clones, the expression of *D*E-cadherin did not change. Scale bars, 25 μm.

DISCUSSION

Proximodistal pattern formation

The finding that *dac* is induced by *esg*-expressing cells in the tarsal region was unexpected from the model (Lecuit and Cohen, 1997) proposing that a combination of Wg and Dpp produces an instructive gradient of a distalizing signal that is highest at the distal tip where *dac* is repressed (Fig. 1A). This model assumes that a response to a certain threshold of a combination of Wg and Dpp directly leads to a circular pattern or that cell proliferation and migration of responding cells lead to a circular arrangement. However, no proof has been given to support the idea that *dac* or other genes respond directly to Wg and Dpp in a circular pattern. Clonal analysis suggested that cells in the leg disc generally migrate in a PD direction (Bryant and Schneiderman, 1969), not along the circumference as the model would predict. We therefore propose a revised model of leg patterning in which the circular arrangement of the proximal cells provides patterning information that regulates the distal gene expression in conjunction with Wg and Dpp (Fig. 1B). The circular arrangement of proximal and distal cells of the leg disc is set up by late embryogenesis (Goto and Hayashi, 1997; Gonzalez-Crespo et al., 1998) by a

mechanism that is not well understood. Once established, the circular pattern is maintained by repression of *hth* in the distal domain by *wg* and *dpp* (Abu-Sharr and Mann, 1998; Wu and Cohen, 1999), and by activation of *esg* by *hth* in the proximal domain (this study). During the transition from the second to third instar, *dac* expression is induced by a combination of a signal from proximal cells, and Wg and Dpp signaling from the AP compartment boundary. The range of each signaling limits *dac* expression to the intermediate domain.

The cell autonomous sorting of Esg- or Hth-expressing cells in the distal domain suggests that Esg and Hth regulate cell adhesive properties in the proximal cells. Similar sorting behavior was reported for cells that lost Dll or Hth (Gorfinkiel, 1997; Wu and Cohen, 1998; Campbell and Tomlinson, 1998). Differential cell affinity controlled by the proximal and distal genes may contribute in part to the separation of the proximal and distal domains into a circular pattern.

Intercalary regeneration

An intercalation mechanism of the proximodistal patterning was suggested (French et al., 1976) from the results of grafting and regeneration experiments utilizing larval cockroach legs. The experiments showed that joining of normally non adjacent positions within a leg segment resulted in intercalary regeneration of the intermediate structures, suggesting that the abutment of distal and proximal positional value can induce an intermediate value. The combination of a graft from a distal level with a proximal level host resulted in a normally oriented regenerate. However, combining a proximal graft with a distal level host produced a regenerate with reversed proximodistal polarity, which is reminiscent of the phenotype caused by ectopically induced Esg in the distal domain. This phenotype was difficult to interpret assuming that the distal tip is the only source of proximodistal positional information. A suggestion that such an intercalary mechanism is also used in normal development came from the work by Schubiger (1974), who showed that the presumptive most proximal (thorax and coxa) and distal (claws) parts were the first to commit to differentiation when premature discs from larvae of 74-80 hour of development were immediately subjected to metamorphosis. Intermediate structures (trochanter, femur, and tibia) were recovered from discs from older larvae. The proximal to distal signaling dependent on Esg and Hth described here may provide a molecular basis for the intercalation mechanism.

Insights into vertebrate development

Proximodistal axis patterning in the vertebrate limb requires interaction between the apical ectodermal ridge (AER) and mesenchyme. *Dlx* (Ferrari et al., 1995), a homolog of *Dll*, and *Slug* (Ros et al., 1997), a homolog of *esg*, are expressed in the AER and mesenchyme, respectively. Nuclear localization of mouse Exd homolog Pbx1 in limb mesenchyme is modulated in a similar manner to Exd in *Drosophila* leg discs (Gonzalez-Crespo et al., 1998). Thus the proximodistal patterning in the vertebrate and insect limb (French et al., 1976) may share a common genetic basis for an intercalary mechanism.

We thank H. Sun, Y. Hiromi, T. Uemura, R. Mann, G. Mardon, D. Andrew, Developmental Study Hybridoma Bank and Bloomington Stock Center for reagents, and Yash Hiromi and Tadashi Uemura for critical comments on the manuscript. This work was supported by grants from the Japan Society for Promotion of Science (Research for the Future, S.H.) and from the Ministry of Education, Sports, and Culture (S.G.).

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.

Basler, K. and Struhl, G. (1994). Compartment boundary and the control of *Drosophila* limb pattern by *hedgehog* protein. *Nature* **368**, 208-214.

Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila leg. *Science* **273**, 1373-1377.

Bryant, P. J. and Schneiderman, H. A. (1969). Cell lineage, growth and determination in the imaginal leg discs of *Drosophila melanogaster. Dev. Biol.* **20**, 263-290.

Campbell, G. and Tomlinson, A. (1998). The roles of the homeobox genes aristaless and Distal-less in patterning the legs and wings of *Drosophila*. *Development* **125**, 4483-4493.

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.

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

Cohen, S. M. and Jürgens, G. (1989). Proximal-distal pattern formation in *Drosophila*: cell autonomous requirement for *Distal-less* gene activity in limb development. *EMBO J.* **8**, 2045-2055.

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

Ferrari, D., Sumoy, L., Gannon, J., Sun, H., Brown, A. M. C., Upholt, W. B. and Kosher, R. A. (1995). The expression pattern of the *Distal-less* homeobox-containing gene *Dlx-5* in the developing chick limb bud suggests its involvement in apical ectodermal ridge activity, pattern formation, and cartilage differentiation. *Mech. Dev.* **52**, 257-264.

French, V., Bryant, P. J. and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. *Science* **193**, 969-981.

Fuse, N., Hirose, S. and Hayashi, S. (1994). Diploidy of *Drosophila* imaginal cells is maintained by a transcriptional repressor encoded by escargot. *Genes Dev.* **8**, 2270-2281.

Fuse, N., Hirose, S. and Hayashi, S. (1996). Determination of wing cell fate by the *escargot* and *snail* genes in *Drosophila*. *Development* **122**, 1059-1067.

Gonzalez-Crespo. S., Abu-Shaar, M., Torres, M., Martinez, A. C., Mann, R. S. and Morata, G. (1998). Antagonism between extradenticle function and Hedgehog signalling in the developing limb. *Nature* **394**, 196-200.

Gonzalez-Crespo, S. and Morata, G. (1995). Control of *Drosophila* adult pattern by extradenticle. *Development* **121**, 2117-2125.

Gonzalez-Crespo. S. and Morata, G. (1996). Genetic evidence for the subdivision of the arthropod limb into coxopodite and telopodite. *Development* **122**, 3921-3928.

Gorfinkiel, N., Morata, G. and Guerrero, I. (1997). The homeobox gene Distal-less induces ventral appendage development in Drosophila. *Genes Dev.* **11**, 2259-2271.

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

Hayashi, S., Hirose, S., Metcalfe, T. and Shirras, A. (1993). Control of imaginal cell development by the *escargot* gene of *Drosophila*. *Development* **118**, 105-115.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. (1997). The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124**, 761-771.

Jiang, J. and Struhl, G. (1996). Complementary and mutually exclusive activities of decapentaplegic and wingless organize axial patterning during Drosophila leg development. *Cell* **86**, 401-409.

Kassis, J. A. (1994). Unusual properties of regulatory DNA from the Drosophila engrailed gene: three 'pairing-sensitive' sites within a 1.6-kb region. *Genetics* **136**, 1025-1038.

Kassis, J. A., Noll, E., VanSickle, E. P., Odenwald, W. F. and Perrimon, N.

(1992). Altering the insertional specificity of a Drosophila transposable element. *Proc. Natl. Acad. Sci. USA* **89**, 1919-1923.

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

Mann, R. S. and Abu-Shaar, M. (1996). Nuclear import of the homeodomain protein extradenticle in response to Wg and Dpp signalling. *Nature* **383**, 630-633.

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

Oda, H., Uemura, T., Harada, Y., Iwai, Y. and Takeichi, M. (1994). A Drosophila homolog of cadherin associated with armadillo and essential for embryonic cell-cell adhesion. *Dev. Biol.* **165**, 716-726.

Pai, C. Y., Kuo, T. S., Jaw, T. J., Kurant, E., Chen, C. T., Bessarab, D. A., Salzberg, A. and Sun, Y. H. (1998). The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, extradenticle, and suppresses eye development in Drosophila. *Genes Dev.* **12**, 435-446.

Penton, A. and Hoffmann, F. M. (1996). Decapentaplegic restricts the domain of wingless during Drosophila limb patterning. *Nature* **382**, 162-164.

Rauskolb, C., Smith, K. M., Peifer, M. and Wieschaus, E. (1995). extradenticle determines segmental identities throughout *Drosophila* development. *Development* **121**, 3663-3673.

Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M. and Mann, R. S. (1997). Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. *Cell* **91**, 171-183.

Ros, M. A., Sefton, M. and Nieto, M. A. (1997). Slug, a zinc finger gene previously implicated in the early patterning of the mesoderm and the neural crest, is also involved in chick limb development. *Development* **124**, 1821-1829.

Schubiger, G. (1974). Acquisition of differentiative competence in the imaginal leg of *Drosophila*. *Wilhelm Roux's Arch. Entwmech. Org.* **174**, 303-311.

Sun, Y. H., Tsai, C. J., Green, M. M., Chao, J. L., Yu, C. T., Jaw, T. J., Yeh, J. Y. and Bolshakov, V. N. (1995). White as a reporter gene to detect transcriptional silencers specifying position-specific gene expression during Drosophila melanogaster eye development. *Genetics* **141**, 1075-1086.

Tanaka-Matakatsu, M., Uemura, T., Oda, H., Takeichi, M. and Hayashi, S. (1996). Cadherin-mediated cell adhesion and cell motility in *Drosophila* trachea regulated by the transcription factor Escargot. *Development* **122**, 3697-3705.

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

Wu, J. and Cohen, S. M. (1999). Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less. *Development* **126** 109-117.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Zeng, W., Andrew, D. J., Mathies, L. D., Horner, M. A. and Scott, M. P. (1993). Ectopic expression and function of the Antp and Scr homeotic genes: the N terminus of the homeodomain is critical to functional specificity. *Development* **118**, 339-352.