Specification of the embryonic limb primordium by graded activity of Decapentaplegic

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SUMMARY

Two thoracic limbs of Drosophila, the leg and the wing, originate from a common cluster of cells that include the source of two secreted signaling molecules, Decapentaplegic and Wingless. We show that Wingless, but not Decapentaplegic, is responsible for initial specification of the limb primordia with a distal identity. Limb formation is restricted to the lateral position of the embryo by negative control of the early function of Decapentaplegic and the EGF receptor homolog that determine the global dorsoventral pattern. Late function of Decapentaplegic locally determines two additional cell identities in a dosage dependent manner. Loss of Decapentaplegic activity results in a deletion of the proximal structures of the limb, which is in contrast to the consequence of decapentaplegic mutations in the imaginal disc, which cause a deletion of distal structures. The results indicate that the limb pattern elements are added in a distal to proximal direction in the embryo, which is opposite to what is happening in the growing imaginal disc. We propose that Wingless and Decapentaplegic act sequentially to initiate the proximo-distal axis.

Key words: imaginal disc, wing, leg, cell allocation, wingless, pattern formation, Drosophila

INTRODUCTION

Formation of the limb is one of the key events during the evolution of the animal body plan. The limb grows out from the body wall, which has an anteroposterior (A/P) and a dorsoventral (D/V) axes. While maintaining these two axes, limb patterns are organized along a third axis, i.e., the proximodistal (P/D) axis that is established orthogonally to the first two axes. Recent works on the limb patterning genes suggest that a common mechanism is used in vertebrates and arthropods (Panganiban et al., 1995).

The maintenance of A/P and D/V axes in the limb can be simply explained by the boundary model of Meinhardt (1983), who proposed that the boundaries of distinct cell populations placed along A/P and D/V axes are used to specify a unique point where outgrowth begins. The model predicts that a new limb structure is added in the proximal to distal direction, because the boundary is always placed at the center of the field which is the future distal end. It follows that a loss of cell interaction at the boundary would results in a deletion of the distal structure. Recent studies of pattern formation in the vertebrate limb and the imaginal discs of Drosophila are consistent with this model (Campbell et al., 1993; Riddle et al., 1993; Struhl and Basler, 1993; Basler and Struhl, 1994; Diaz-Benjumea et al., 1994; Parr and McMahon, 1995; Yang and Niswander, 1995). These studies demonstrated that secreted signaling molecules of Hedgehog, TGF-β, and Wnt families mediate cell-cell interactions at the boundaries and that these interactions are essential for locating the point of outgrowth and patterning of the limb. However, most of these studies were focused on growing or regenerating limbs, and crucial information on the earliest event of limb formation is still missing.

Imaginal discs in Drosophila provide a simple model to study limb development (Cohen, 1993). The second thoracic segment of the adult fly contains pairs of wings and legs, each located dorsally and ventrally. Precursors for these two appendages, the wing and the leg imaginal discs, arise in the embryo from a single limb primordium (Wieschaus and Gehring, 1976; Cohen et al., 1993). Unlike imaginal discs in the third instar larva, in which pattern formation involves a great number and diversity of cells and extensive cell proliferation, embryonic imaginal discs contain a small number of cells that hardly divide. For these reasons, embryonic imaginal disc formation would provide information on the simplest form of the limb development, that is, cell allocation from the ectoderm.

The limb primordium located in each thoracic segment is identified by expression of the homeobox gene Distal-less (Dll; Cohen, 1990) at stage 11 of embryogenesis. At this stage, the Wnt family gene wingless (wg) is expressed in a stripe along the A/P compartment boundary and the gene decapentaplegic (dpp) a member of the TGF-β family is expressed in a longitudinal stripe running the length of the lateral trunk region. Dll expression was found at the intersection of the stripes of wg and dpp expression (Cohen et al., 1993). Observations that interaction between wg- and dpp-expressing cells in the imaginal discs promotes the development of the P/D axis (Campbell et al., 1993; Struhl and Basler, 1993; Diaz-Benjumea et al., 1994) suggest that a similar mechanism might be used to specify the embryonic limb.
(Cohen et al., 1993). Indeed, the function of Wg is required for the formation of imaginal discs (Simcox et al., 1989) and expression of Dll (Cohen, 1990; Cohen et al., 1993). However, the role of Dpp in embryonic limb specification has not been experimentally addressed because the early requirement for dpp in dorsoventral patterning makes analyses in later stage difficult.

In this study, we investigated the role of Dpp in the patterning of embryonic limb primordia. We first show that the proximodistal patterning of the embryonic limb occurs early in stage 11, before segregation of the wing and the leg disc. We next show that Dpp plays a role distinct from that of Wg. While Wg is essential for induction of the limb primordia, specifying the most distal limb identity, Dpp is not required for the induction itself. The early function of Dpp is to restrict limb formation to the lateral side of the embryo. Later on, graded action of Dpp expressed at the dorsal edge of the limb primordium specifies more proximal cell identities. Loss of only the proximal structures upon reduction of Dpp signaling was unexpected from the boundary model (Meinhardt, 1983) and the intersection model (Cohen et al., 1993). Thus, we propose an alternative model for the P/D axis formation.

MATERIALS AND METHODS

Fly stocks

Stocks used for staining were P(w+) Dll 304 B-35 (Cohen et al., 1993), Dll(n1092), tkv, skv, lin(2L)skv; Is1, punt (1046), punt (15), Dp(2;2)DdT48 (Dp-dpp), and Oregon R. Information on the stocks used in this study can be obtained from FLYBASE (http://morgan.harvard.edu/). dpp was ectopically expressed as described previously (Brand and Perrimon, 1993; Stacheling- Hampton and Hoffmann, 1994).

Histochemical methods

In situ hybridization was performed with digoxigenin-labeled Dll RNA probe and detected with the standard procedure (Tautz and Pfeifle, 1989). For double staining with antibody and RNA probe, the standard procedure was modified (S. Goto and S. Hayashi, manuscript in preparation). In brief, fixed embryos were stained for β-gal with a combination of rabbit anti-β-gal (Cappell), biotin-labelled anti-rabbit IgG (Jackson), and FITC-avidin DH (Vector). After the final wash, the embryos were fixed again and hybridized using the standard procedure (Tautz and Pfeifle, 1989). The signal was developed with a fluorescent substrate (HNNP/Fast Red) as described by Kagiyama et al. (1993). The dpp (Padgett et al., 1987), Dll (Panganiban et al., 1994) and the tkv (Okano et al., 1994) probes were described previously. Other antibodies used for immunostaining are as follows: rabbit anti-Vg (Williams et al., 1991), rabbit anti-Sna (Rolf Reuter, personal communication), rat anti-Esg (Fuse et al., 1993), mouse anti-DD (Boehlinger Mannheim), rat anti-D-α-catennin monoclonal antibody DCAT1 (Oda et al., 1993), Cy3-conjugated anti-rabbit and anti-mouse IgGs (Chemicon). The stained embryos were observed using a confocal microscope (LSM410, Carl Zeiss) with a combination of He/Ne 543 nm laser and LP 590 nm emission filter to detect the in situ signal. The FITC signal was detected with a combination of an Ar 488/514 laser and a BP 510-525 nm emission filter. A FT488/543 Dichroic mirror was used.

RESULTS

Three distinct cell fates in the embryonic limb primordia

Here we describe the limb development in the second thoracic segment, which is identical to that observed in T3. Limb development in T1 is different from that in T2 and T3 in that the wing disc is not formed. According to the expression of a set of molecular markers, imaginal discs in T2 can be separated into three distinct parts: the wing disc, the proximal leg disc, and the distal leg disc (Fig. 1E, right). In early stage 11 embryos (Campos-Ortega and Hartenstein, 1985), expression of Dll mRNA and β-galactosidase (β-gal) under the control of the Dll early enhancer (Dll-304-β-gal, Vachon et al., 1992) start in a line of cells in the lateral position (Figs 1A, 2A). This expression lies along the anterior-posterior compartment boundary as revealed by double labeling with wg-lacZ and Dll mRNA (data not shown). Expression of β-gal from the Dll enhancer trap allele (DllP-β-gal) starts slightly later and becomes localized in the leg primordia. By stage 15, leg disc cells expressing DllP-β-gal are encircled by cells expressing Escargot (Esg; Whiteley et al., 1992) in nearly non-overlapping pattern (Fig. 1C). The pattern of DllP-β-gal is identical to that of the Dll late enhancer activity (data not shown), which was shown to correspond to the cells that give rise to Keilin’s organ (Cohen, 1993), the distal-most part of the larval limb. Thus the embryonic leg disc consists of two cell populations, the distal part expressing DllP-β-gal and the proximal part expressing Esg. The wing primordial cells are first recognizable in stage 12 as cells expressing Vestigial (Vg; Williams et al., 1991) and moving dorsally (Fig. 1B). After stage 13, the wing primordial cells start to express Esg (Fig. 1C) and Sna1 (Sna; not shown) that are required for the commitment to the wing cell fate (Fuse et al., 1996). The domain of DllP-β-gal expression after stage 12 becomes larger than that of Dll mRNA, because cells that have turned off Dll transcription still retain stable β-gal protein. In stage 15, DllP-β-gal expression overlaps the expression of Esg in both the wing and the leg discs, confirming the previous observation (Cohen et al., 1993; Fig. 1D). These results indicate that Dll expression starts in a common precursor that gives rise to both the wing and the leg discs, and part of the epidermis, but later becomes restricted to the distal part of the leg disc.

Basal migration of distal leg cells

Dll-expressing cells showed a dynamic cell movement in the early stage of limb formation. DllP-β-gal expression starts in stage 11 and it is strongly detected in a group of cells one cell layer beneath the surface layer of the ectoderm (Fig. 2A,B). Since DllP-β-gal mRNA was first detected in the top layer (data not shown), it is likely that the cells expressing DllP-β-gal had migrated from the surface layer. In stage 12 when some of the DllP-β-gal-expressing cells started their dorsal migration, the limb primordium showed a two-tiered shape (Fig. 2C,D). Cells in the basal position remained in the ventral side and the dorsally migrating cells moved in the surface layer. The cells in the basal position will become the distal leg cells, which express the distal leg marker DllP-β-gal (Fig. 2E-G). Thus, the basal movement of the Dll-expressing cells is the earliest sign of the differentiation of the distal leg cells.

Expression of dpp and tkv in the limb primordium

To unambiguously determine the temporal and spatial order of expression of dpp and Dll, we double labelled embryos that have the DllP-lacZ transgene to detect dpp mRNA and DllP-β-gal (Fig. 3). In early stage 11, no significant levels of dpp transcription was detected except for expression in the edge of the dorsal ectoderm, while DllP-β-gal was detectable in the lateral region of the embryo (Fig. 3A). In late stage 11, DllP-β-gal is first detected in a line in the lateral position of the embryo (Fig. 3B). This suggests that Dpp plays a role in the initiation of the basal migration of cells expressing DllP-β-gal. In stage 12, DppP-β-gal is expressed in the distal leg cells as well as in the wing disc, indicating that Dpp is required for the expression of DllP-β-gal in the distal leg cells. In stage 13, DppP-β-gal expression becomes more restricted to the distal leg cells, indicating that Dpp is required for the maintenance of DllP-β-gal expression in the distal leg cells.
Fig. 1. Three positional identities are established in the embryonic limb primordium. Arrowheads indicate the second thoracic segment. Anterior, left; dorsal, up. (A) Dll mRNA (red) and DllP-304-β-gal (green) are first detected in the cells along the A/P compartment boundary in the early stage 11 embryo. (B) By stage 12, Vg expression (green nuclear stain) was detected in the cells leaving dorsally from the leg primordium which was labelled with DllP-β-gal (red nuclear stain). (C) In the stage 15 embryo, Esg (green nuclear stain) is expressed both in the wing primordium (upper cell clusters) and in the proximal part of the leg disc (lower cell clusters) that surround the distal leg cells expressing DllP-β-gal (red). By observation under high magnification, we detected a few cells expressing both of Esg and DllP-β-gal. (D) All of the cells that express Esg (green) coexpress Dll-304-β-gal (red). Note that the wing and the leg discs are connected by Dll-304-β-gal-positive cells that do not express Esg. (E) Summary of the marker expression in the second thoracic imaginal discs. Left: at stage 11, Dll mRNA and DllP-304-β-gal start expression in the limb primordium (pink). Center: at stage 12, Vg expression starts in the wing primordium (light green) that is separating from the leg disc cells labelled with DllP-β-gal (pink). Right. After stage 13, Esg and Sna are expressed in the wing disc (light green). In the leg disc, Esg is expressed in the proximal region (green) and DllP-β-gal is expressed in the distal region (pink).

Fig. 2. Basal migration of the distal leg cells. (A-D) Embryos were stained to detect expression of Dll-304-β-gal (red cytoplasmic stain). D-α-catenin staining (green) was used to reveal cell membranes. White lines in A,C,E indicate the plane of sections shown in B,D,F and G. (A) Ventrolateral view of an early stage 11 embryo. Limb development proceeds from the anterior (top) to the posterior (bottom), so the Dll expression in T3, which appears as a line, commenced more recently than the expression in T1, which appears as a cluster. (B) Transverse section through T2. Cells with strong Dll-304-β-gal expression are found in the subepidermal layer of the ectoderm. (C,D) An embryo in early stage 12. Dll-304-β-gal-expressing cells have increased in number and have formed a cluster that appears two tiered in shape in cross section (D). Some of the Dll-304-β-gal-expressing cells have started their dorsal migration in the top layer of the ectoderm (arrows). (E,F) A stage 12 embryo stained for expression of the distal leg marker DllP-β-gal (green nuclear stain) and Dll mRNA (red cytoplasmic stain). Their expressions coincide in E, but the cross section shows that only the cells in the lower layer express DllP-β-gal (F). In G, only the red channel of the image in F is shown. The same observation was made in all thoracic segments.
β-gal-expressing cells increased in number and formed a cluster. This is the first time when spots of dpp expression were detected in the lateral position. The highest point of dpp expression overlapped with the anterior-dorsal edge of the Dll-304-β-gal-expressing cluster (Fig. 3B). Then the dpp expression gradually expanded in anterior and posterior directions to form a longitudinal stripe running the length of the trunk region (Fig. 3C).

TGF-β like ligands such as Dpp associate with dimers of type I and type II receptor serine/threonine kinases. In Drosophila, two type I receptors, encoded by the genes thick veins (tkv) and saxophone (sax), and a type II receptor, encoded by the gene punt (put), have been identified (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). Since no dpp-related signaling is apparent in the absence of either the Tkv or Put receptor, it was inferred that both receptors act in concert to transduce the dpp signal and that their functions cannot be replaced by the other extant type I and II receptors. We have found that tkv expression in the limb primordia is under dynamic transcriptional control. Cells that had just started to express Dll-304-β-gal also expressed the tkv transcript (Fig. 3D,E). When Dll-304-β-gal-expressing cells increased in number and some of them started their dorsal migration as a wing precursor, tkv transcripts disappeared from the Dll-304-β-gal-expressing cells to form a complementary pattern (Fig. 3F,G, compare with C). It has been shown that tkv transcription changes in a temporally and spatially dynamic pattern, suggesting a requirement to regulate dpp signaling at the level of receptor expression (Affolter et al., 1994; Brummel et al., 1994). Assuming that Tkv protein follows the pattern of the tkv transcript, a very small amount of Tkv protein would be expected to be available after the down regulation of tkv transcription. These observations suggest that when the lateral stripe of dpp expression is established (Fig. 3C), tkv activity is already down regulated in the limb primordium. Thus the dynamic temporal and spatial change in dpp and tkv transcription is likely to limit the period when dpp can maximally affect imaginal disc patterning to a narrow temporal window when a spot of dpp-expressing cells is located at the edge of a Dll-expressing cluster (Fig. 3B).

**Dpp specifies proximal cell identities**

We sought to assess the role of the dorsally localized Dpp signal in late stage 11 in patterning the limb primordia by analyzing the marker expression in various dpp signaling mutants (Table 1). In embryos of the intermediate class, expression of the wing disc markers Esg and Sna was lost (Fig. 4G), but the leg disc markers were left intact (Fig. 4E,F). In the strong class of mutants, Esg expression in the proximal leg disc was additionally lost (Fig. 4I), but the DllP-β-gal expression in the distal part of the leg disc was still intact (Fig. 4I). The defect in the wing disc was already apparent at stage 12, because the number of Vg-positive cells migrating away from DllP-β-gal-expressing cells was decreased (Fig. 4H,L). These observations suggest that the reduction in the Dpp signal affected the patterning within the limb primordium before segregation of the dorsal and the ventral discs.

The sequential loss of the wing disc and the proximal leg disc marker expression upon a reduction in the Dpp signal suggests that a high dose of dpp specifies these disc cell types. To test this idea, we expressed dpp ectopically throughout the ectoderm using the Gal4-UAS system (Brand and Perrimon, 1993). In such embryos, the leg disc appeared normal but the cells in the wing disc increased in number (Fig. 5B,D). A similar observation was made in embryos that had 4 copies of the dpp gene (data not shown), suggesting that the phenotype is mainly due to an increase in the peak level of dpp, and not to the ubiquitous expression outside the normal domain of dpp expression. Although ectopic expression of dpp causes cell proliferation in the imaginal disc (Capdevila and Guerrero, 1994; Zecca et al., 1995), this was not the case in the embryo. We found no detectable change in the BrdU incorporation pattern in limb primordia of embryos overexpressing dpp (data not shown). Between the leg and wing discs, there were cells that previously expressed Dll-304-β-gal but were not included in either of the discs (Fig. 1D). These cells might have been recruited to the wing disc upon an increase in the Dpp signal. These phenotypes at the various levels of Dpp signal indicate that the wing disc cells are specified by a high dpp level, and the proximal leg disc cells are specified by an intermediate one.

**Table 1. Phenotypes of Dpp signalling mutants**

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<td>tkv&lt;sup&gt;/2L;tkv&lt;sup&gt;Sc-1&lt;sup&gt;+/+&lt;/sup&gt;&lt;/sup&gt;</td>
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<td>overexpression</td>
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*Progeny of a cross between tkv<sup>/CyO</sup> male and Int<sup>2L;tkv<sup>Sc-1<sup>/CyO</sup></sup> female.
†Progeny of a cross between Int<sup>2L;tkv<sup>Sc-1<sup>/CyO</sup></sup> male and tkv<sup>/CyO</sup> female.
‡DllP-β-gal (distal leg), Esg (proximal leg), and Sna and Esg (wing) were used as markers.
expression in ventral ectoderm (Raz and Shilo, 1993, Fig. 6B). These results demonstrate that Dpp is not required for the initiation of Dil transcription but is necessary to repress Dil transcription in the dorsal part of the embryos, and that DER represses Dil transcription in the ventral ectoderm.

**DISCUSSION**

Studies of growth and patterning in vertebrate limb bud and *Drosophila* imaginal discs have provided a unifying view that cell-cell interactions between distinctly specified cell populations form an organizing center of the P/D axis (Campbell et al., 1993; Basler and Struhl, 1994; Lauf et al., 1994; Niswander et al., 1994; Parr and McMahon, 1995; Yang and Niswander, 1995). Therefore the major question concerning the initial event of the limb formation is how A/P and D/V positional information in the embryo is used to specify distinct founder cell populations of the limb, and how these cells interact to establish the P/D axis. This process inevitably involves interaction between the limb primordial cells and surrounding ectodermal cells, a situation fundamentally different from that in the imaginal disc which autonomously organizes its own pattern independent of its environment. Indeed we showed that gradual reduction of Dpp signaling resulted in gradual loss of limb primordial cells in a proximal to distal direction in the embryo, while distal parts of the adult appendages are deleted first in dpp hypomorphic mutants (Spencer et al., 1982). Our results indicate that the dorsoventral signal Dpp specifies different cell fate that will be arranged along the P/D axis, suggesting the D/V positional information is the major determinant of cell identities in the P/D axis.

**Allocation of the limb primordium**

It has been shown that Dil-expressing cells are located along the wg stripe and that wg is necessary for induction of the limb (Cohen et al., 1993), but the mechanism that restrict the limb formation to the lateral region was not known. We have shown that Dil expression persists and expands dorsally in the absence of Dpp, a result contrary to the model of Cohen et al. (1993). In contrast, the ventral limit of Dil expression moved ventrally in DER mutants. These results demonstrate that Dpp plays no role in inducing initial Dil expression and that the dorsoventral limit of Dil expression is defined by repression of Dpp and DER. One likely explanation for the phenotype of the *dpp* mutant is that the dorsal ectoderm loses its identity and permits the expansion of the ventrolateral fate. A similar phenotype for the embryo mutant for *shnurri*, which encodes an essential downstream component of dpp signaling (Grieder et al., 1995), supports this idea. It is not clear whether the inhibition of Dil transcription by Dpp is direct or through inhibition of Wg expression. DER plays a central role in the patterning of the ventral ectoderm (Raz and Shilo, 1993), and secretion of the Spitz ligand is thought to be the key step in DER activation (Schweitzer et al., 1995). We thus propose that the domain of Dil expression is defined by a combination of three secreted molecules. Wg provides an activating cue, and Dpp and Spitz provide inhibitory cues from the dorsal and ventral side, respectively (Fig. 7A).

**Limb patterning by graded dpp activity**

The first sign of the specification of distal leg cells is the basal movement of Dil-expressing cells and expression of Dil-$\beta$-gal. The distal leg specification depends on Wg, and occurs before the onset of dpp expression within the limb primordium, and is also observed in the absence of the zygotic tkv activity. We therefore propose that the distal leg fate is the default state of the limb primordia without Dpp activity. We have presented evidence suggesting that the Dpp signal in the limb primordia can be maximally transmitted only during a short period during stage 11 when transcription of dpp and tkv overlap. In this period, cells expressing dpp are located in the anterior-dorsal edge of the limb primordium (Fig. 2B). This sets the stage when a localized source of the dpp signal specifies cell fates in a single field of the limb primordium. One explanation is that Dpp forms a gradient with a peak at the dorsal edge of the limb primordium, inducing the wing primordia in the dorsal position and the proximal leg in the medial position in a concentration dependent manner (Fig. 7B). Indeed, Vg-expressing wing disc cells arise in the dorsal side of the limb primordium (Fig. 1B), and the future distal leg cells are located in the ventral side of the limb primordium (Fig. 2E). This model assumes that the wing is the most proximal part of the limb, an idea consistent with the argument that, evolutionarily, the wing originates as the proximal part of the primordial limb (Kukalová-Peck, 1982) and also the embryological observation in *Dacus*, that the wing disc segregates out from the dorsal edge of the leg disc (Anderson, 1963). We propose that the primary P/D axis of the limb is established along the D/V axis (Fig. 7B).

**P/D axis formation**

We have shown that specification of the distal leg cells depends on Wg and that proximal leg specification additionally requires Dpp. The distal leg cells form Keilin’s organ, the distal-most structure of the embryonic limb. In Dil mutants, the leg disc consists only of the proximal leg cells that express Esg (S. H., unpublished) and these cells will form the body wall and a part of the coxa, as was shown in transplantation experiments (Cohen et al., 1993). It is thus possible that the two cell populations in the embryonic leg disc have positional value of extreme proximal and extreme distal parts, respectively. This idea is consistent with the result reported by Schubiger (1974), who showed that immature leg discs forced to undergo metamorphosis differentiate only extreme proximal and distal structures. Elaboration of the leg pattern may involve intercalation of intermediate values as previously suggested from the study of cockroach limb development. Cockroaches can be cut off and grafted to another leg stump. Association of normally non-adjacent P/D positional values results in localized growth and intercalary regeneration of the intermediate structures (French et al., 1976). A similar sequence of marker expression in normal cockroach development has been described (Norbeck and Denburg, 1991).

Our model on the role of Wg and Dpp in embryonic limb specification may be used to explain the consequence of ectopic expression of Wg in the leg disc (Campbell et al., 1993;
Fig. 3. Expression patterns of *dpp* and *tkv* in relation to the expression of *Dll*.

(A-C) Expression patterns of *Dll-304-β-gal* (green) and *dpp* mRNA (red). Anterior, left; dorsal, up. (A) An embryo in early stage 11 when *Dll-304-β-gal* expression is first detectable (arrowheads). No significant level of *dpp* transcript is detectable except at the edges of the dorsal ectoderm (asterisks). (B) In the late stage 11 embryo, spots of *dpp* expression (arrow) appear in the anterior-dorsal edge of a cluster of cells expressing *Dll-304-β-gal*. (C) At stage 12, the *dpp* expression expands to form a longitudinal stripe that overlaps the dorsal edge of *Dll-304-β-gal*-expressing cell cluster.

(D-G) Expression of *tkv* mRNA (red) and *Dll-304-β-gal* (green). (D) In this embryo, which is slightly younger than the one shown in B, *Dll-304-β-gal* expression overlaps with that of *tkv*. (E) Red channel of D. (F) In stage 12 embryos *Dll-304-β-gal* and *tkv* are expressed in a complementary pattern. (G) Red channel of F.

Fig. 4. Dpp specifies proximal cell identities in the limb primordium. Embryos were stained for expression of the markers indicated. (A-D) Wild type embryos. (E-H) Intermediate class embryos (*Int(2)LtkvSc-8/tkv7*). (I-L) Strong class embryos (*tkv8*). Expression of *DllP-β-gal* (A,E,I) and Esg (B,F,J) was detected at stage 16. Sna expression was detected at stage 15 (C,G,K). Vg expression was detected at stage 12 (D,H,L). A,B,E,F,I and J are ventral views. Other panels show the lateral view. Arrowheads indicate the proximal leg discs visualized with anti-Esg antibody.

**dpp mRNA**  **Dll-304-β-gal**

**tkv mRNA**  **Dll-304-β-gal**
Struhl and Basler, 1993). In the normal leg disc, the center of the P/D axis is located at the point where strong expressions of Wg and Dpp are juxtaposed. Placement of Wg-expressing cells near the stripe of Dpp-expressing cells caused formation of a supernumerary axis. Such a situation would bring together distal and proximal positional values, each specified by Wg and Dpp to close apposition. This apposition would lead to intercalary growth and filling of the positional values to form the second axis. We think this interaction between the proximal and the distal cells, combined with cell interactions at the compartment boundaries are the driving force of the limb patterning. A study at the cellular resolution as reported in this work is necessary to test this idea in imaginal discs.

The imaginal discs occasionally change their fates when they are forced to regenerate. For example, wing tissue is observed in leg discs cultured for a long time in vivo (Hadorn, 1978). This ‘transdetermination’ can be induced by the ectopic expression of Wg. Wg induced leg duplication is often associated with ectopic wing tissue (Maves and Schubiger, 1995). Given the ability of a high level of Dpp to induce wing disc cells in embryos, this ‘transdetermination’ phenomenon may be better understood as ‘redetermination’ of the limb.

Vertebrate limb patterning involves a similar set of signaling molecules as in the *Drosophila* limb. Analogous to *Drosophila*, BMP-4 (Dpp homolog) and Dlx (Dll homolog) are expressed in the apical ectodermal ridge (AER), the distal most structure in the vertebrate limb, and Wnt-5a (Wg homolog) is expressed near the Shh (Hh) expressing region (Dealy et al., 1993; Francis et al., 1994; Ferrari et al., 1995). Like cockroach leg, grafting experiments on the amphibian leg shows that a similar mechanism may be used in the P/D axis formation in vertebrates (French et al., 1976). It will be interesting to investigate whether the mechanism used in the *Drosophila* limb specification is also used in the initial event of the vertebrate limb formation.

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