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# 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 proximodistal 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*$^{01092}$, *tkv*$^{7}$, *tkv*$^{8}$, In(2L)*tkv*$^{Sz-1}$, *punt*$^{1046}$, *punt*$^{135}$, Dp(2;2)DTD48 (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; Staehling-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 (Cappel), 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., 1994), mouse anti-β-gal (Boehlinger Mannheim), rat anti-*D*-α-catenin 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 (*Dll*$^{P}$-β-gal) starts slightly later and becomes localized in the leg primordia. By stage 15, leg disc cells expressing *Dll*$^{P}$-β-gal are encircled by cells expressing Escargot (Esg; Whiteley et al., 1992) in nearly non-overlapping pattern (Fig. 1C). The pattern of *Dll*$^{P}$-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 *Dll*$^{P}$-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 Snail (Sna; not shown) that are required for the commitment to the wing cell fate (Fuse et al., 1996). The domain of *Dll*-304-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, *Dll*-304-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. *Dll*-304-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 *Dll*-304-β-gal mRNA was first detected in the top layer (data not shown), it is likely that the cells expressing *Dll*-304-β-gal had migrated from the surface layer. In stage 12 when some of the *Dll*-304-β-gal-expressing cells started their dorsal migration, the limb primordia 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 *Dll*$^{P}$-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 *Dll*-304-*lacZ* transgene to detect *dpp* mRNA and *Dll*-304-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 *Dll*-304-β-gal was detectable in the lateral region of the embryo (Fig. 3A). In late stage 11, *Dll*-304-

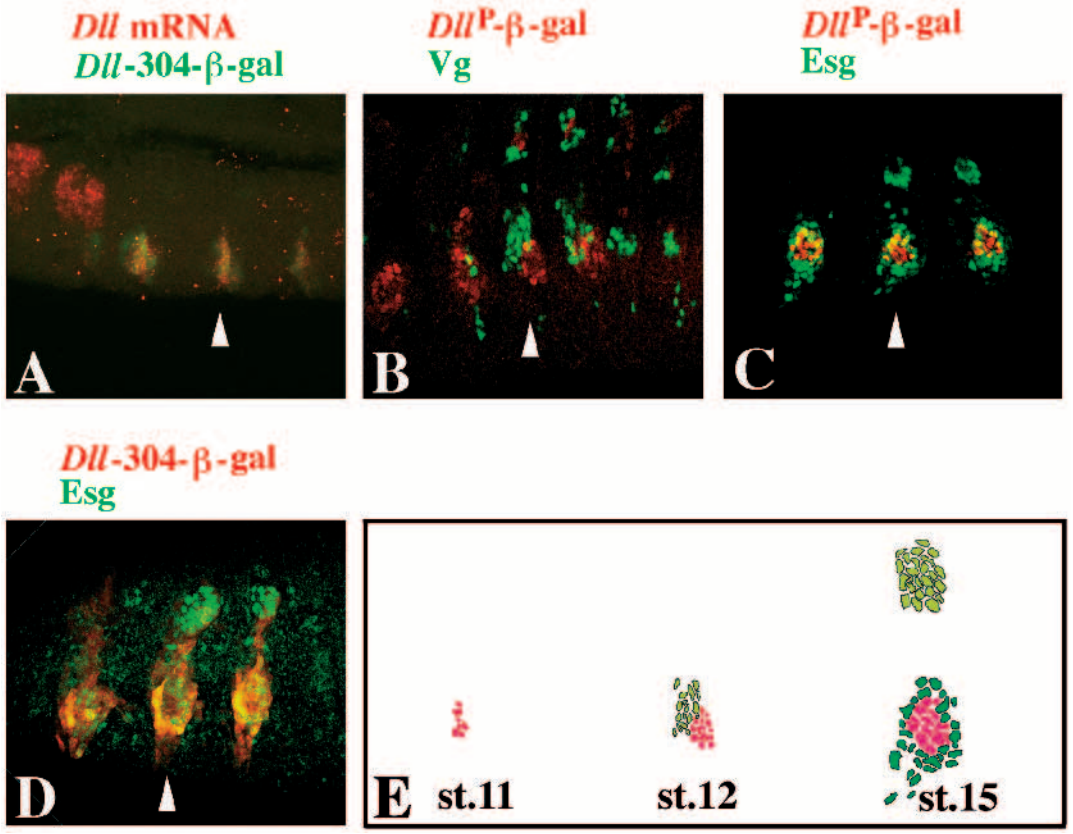


**Fig. 1.** Three positional identities are established in the embryonic limb primordium. Arrowheads indicate the second thoracic segment. Anterior, left; dorsal, up. (A) *Dll* mRNA (red) and *Dll*-304-β-gal (green) are first detected in the cells along the A/P compartment boundary in the early stage 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 $Dll^P$-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 $Dll^P$-β-gal (red). By observation under high magnification, we detected a few cells expressing both of Esg and $Dll^P$-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 *Dll*-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 $Dll^P$-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 $Dll^P$-β-gal is expressed in the distal region (pink).

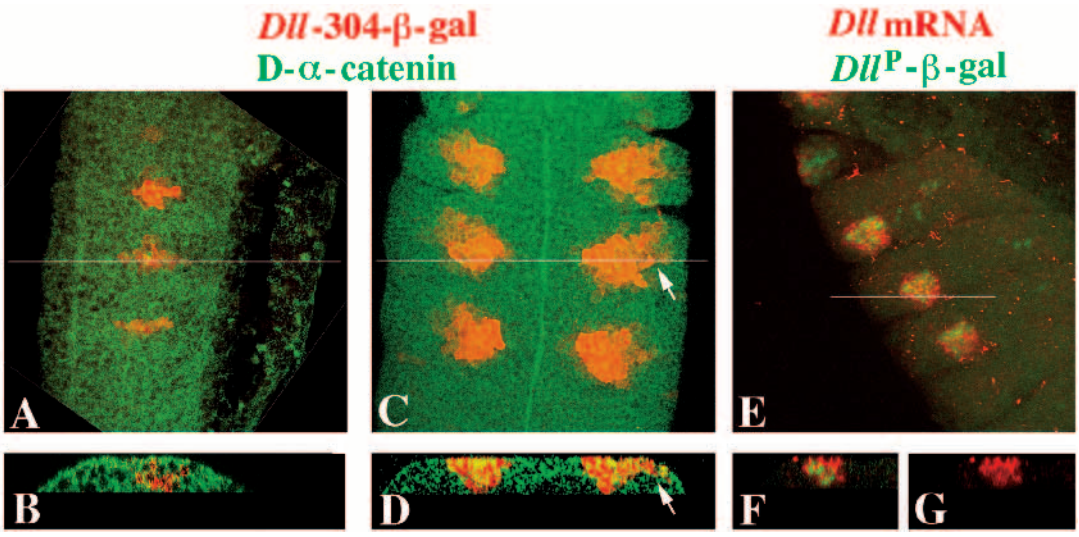


**Fig. 2.** Basal migration of the distal leg cells. (A-D) Embryos were stained to detect expression of *Dll*-304-β-gal (red cytoplasmic stain). *D*-α-catenin staining (green) was used to reveal cell membranes. White lines in A,C,E indicate the plane of sections shown in B,D,F and G. (A) Ventrolateral 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 $Dll^P$-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 $Dll^P$-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. 4J), but the $Dll^P$-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 $Dll^P$-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.

## Dpp and DER set the dorsoventral limit of the limb primordium

The expression of $Dll^P$-β-gal in the near total absence of Dpp receptor activity suggests that the distal part of the leg disc may form independently of Dpp. This idea was confirmed by detection of *Dll* transcripts in *dpp* null mutant embryos. In $dpp^{H46}$ embryos, *Dll* transcripts formed a stripe in each segment that encompassed the dorsal two thirds of the embryo and terminated at the boundary between the dorsal and the ventral ectoderm (Fig. 6A). We also confirmed the previous report that the *Drosophila* EGF receptor (DER) is required to repress *Dll*

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

| Class | Signal strength | Genotype | Phenotype‡ | | |
|---|---|---|---|---|---|
| | | | Distal leg | Proximal leg | Wing/haltere |
| strong | very low | $tkv^8$ $tkv^7/In(2L)tkv^{Sz\text{-}1}$* | + | – | – |
| intermediate | intermediate | $In(2L)tkv^{Sz\text{-}1}/tkv^7$† $put^{135}$, $put^{1046}$ | + | + | – |
| wild type | normal | Oregon-R | + | + | + |
| overexpression | high | 32B Gal4/UAS-dpp *Dp(2;2)DTD48 (Dp-dpp)* | + | + | ++ |

*Progeny of a cross between $tkv^7/CyO$ male and $In(2L)tkv^{Sz\text{-}1}/CyO$ female.
†Progeny of a cross between $In(2L)tkv^{Sz\text{-}1}/CyO$ male and $tkv^7/CyO$ female.
‡$Dll^P$-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 *Dll* transcription but is necessary to repress *Dll* transcription in the dorsal part of the embryos, and that DER represses *Dll* 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; Laufer 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 are 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 *Dll*-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 *Dll* 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 *Dll* expression moved ventrally in DER mutants. These results demonstrate that Dpp plays no role in inducing initial *Dll* expression and that the dorsoventral limit of *Dll* expression is defined by repression by Dpp and DER. One likely explanation for the phenotype of the $dpp^{H46}$ 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 *Dll* 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 *Dll* 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 *Dll*-expressing cells and expression of $Dll^{P}$-β-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).

Specification of multiple cell fates by different concentrations of Dpp has been proposed for the patterning of the wing disc (Nellen et al., 1996), but an alternative idea exists. Lecuit et al. (1996) proposed that Dpp determines the pattern of target gene expression in two ways, one by direct activation at a distance by long range diffusion, and the other by activation over a short range, followed by movement of the cells to establish a final pattern. Our results are compatible with either of the ideas. A cell lineage tracing study would distinguish between these two hypotheses.

### 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 *Dll* 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. Cockroach legs can be cut off and grafted to another leg stump. Association of normally nonadjacent 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;

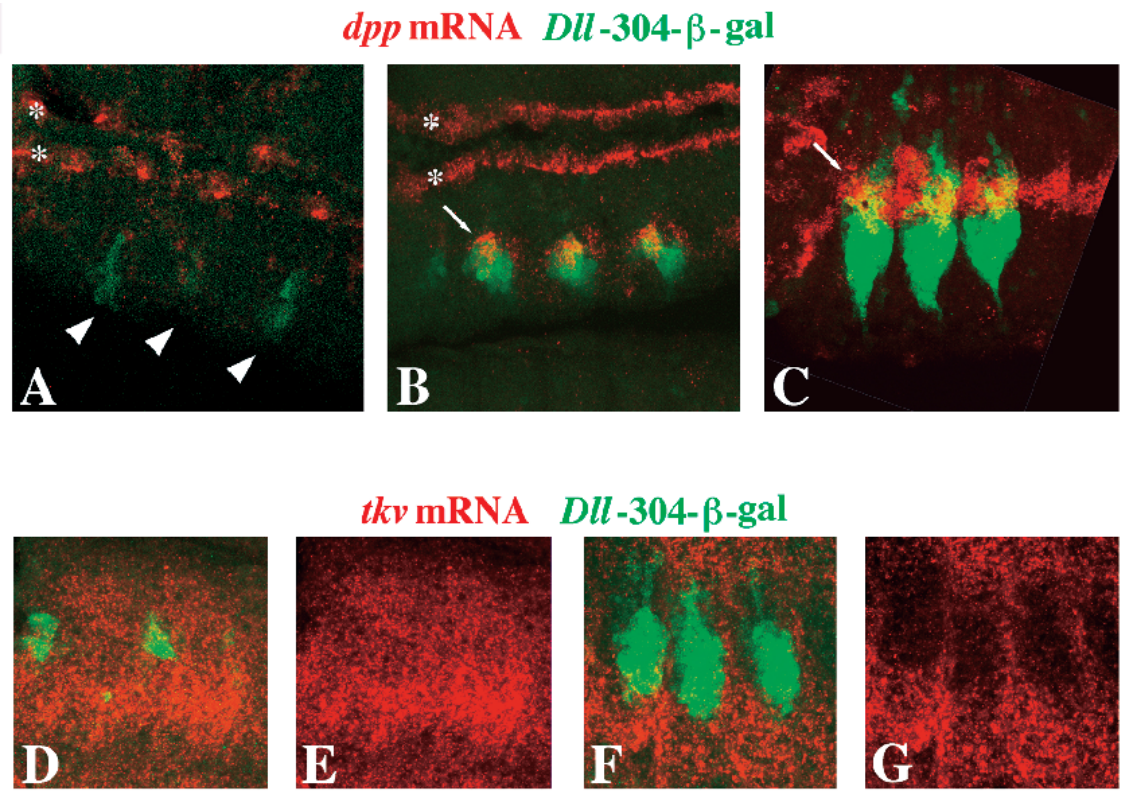


**Fig. 3.** Expression patterns of *dpp* and *tkv* in relation to the expression of *Dll*. (A-C) Expression patterns of *Dll*-304-β-gal (green) and *dpp* mRNA (red). Anterior, left; dorsal, up. (A) An embryo in early stage 11 when *Dll*-304-β-gal expression is first detectable (arrowheads). No significant 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.

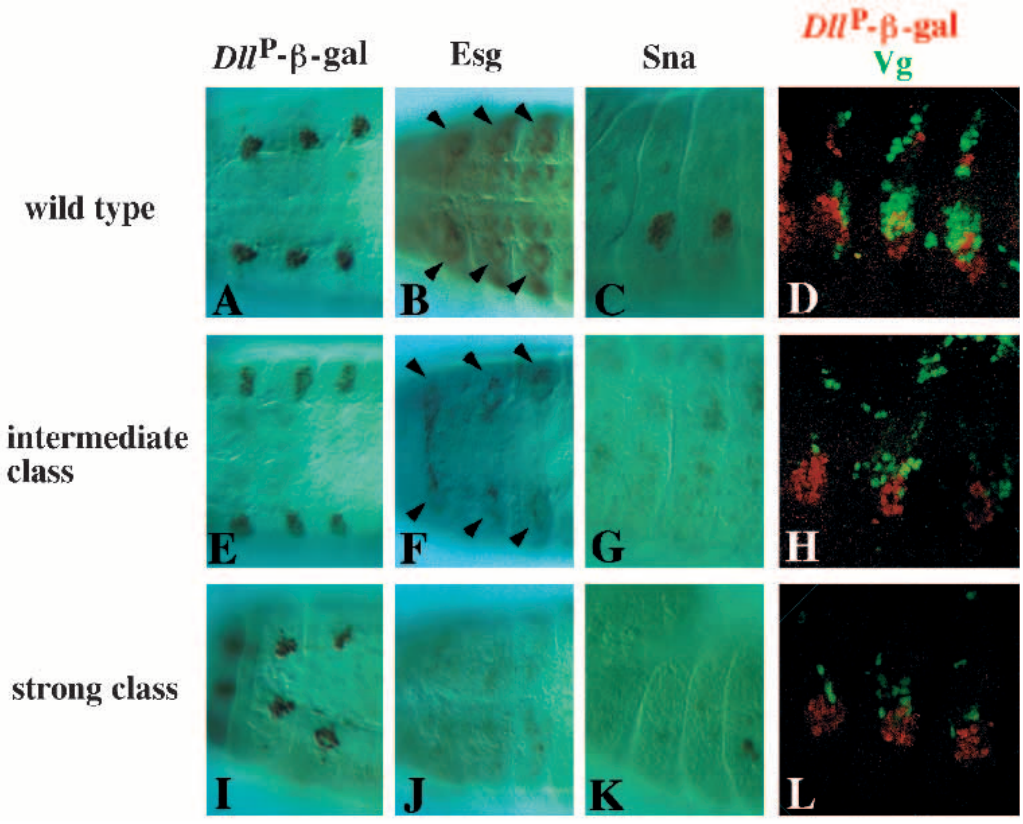


**Fig. 4.** Dpp specifies proximal cell identities in the limb primordium. Embryos were stained for expression of the markers indicated. (A-D) Wild type embryos. (E-H) Intermediate class embryos *(In(2L)tkv$^{Sz\text{-}1}$/tkv$^{7}$)*. (I-L) Strong class embryos *(tkv$^{8}$)*. Expression of *Dll$^{P}$*-β-gal (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.

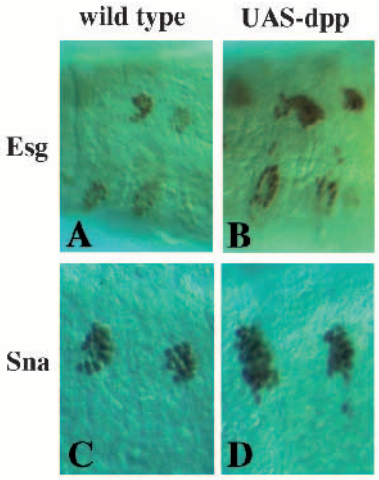


**Fig. 5.** High levels of Dpp induces ectopic wing disc cells. Dpp was ectopically expressed by a combination of UAS-*dpp* and 32B Gal4. (A,C) Wild-type embryos, (B,D) UAS-*dpp* / 32B Gal4 embryos labelled with anti-Esg antibody (A,B) and anti-Sna antibody (C,D). Overexpressed *dpp* caused an increase of a number of wing disc cells, while the number of proximal leg disc cells expressing Esg was unaffected. This increase is already apparent in stage 12 when Vg starts expression (not shown). 32B Gal4 is active throughout the ectoderm after stage 10.

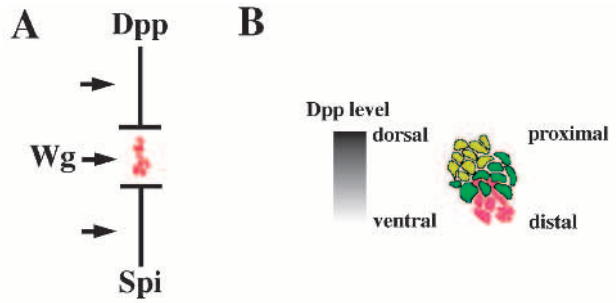


**Fig. 7.** A model for the allocation of the limb primordium and the formation of the P/D axis. (A) At stage 11, a stripe of Wg induces the limb primordium that expresses *Dll* (red). Repression by Dpp and Spitz limits the limb formation only in the lateral position. (B) At late stage 11, a dot of Dpp expression in the dorsal edge of the *Dll*-expressing cell cluster forms a concentration gradient. Cells in the dorsal position exposed to a high level of Dpp become the wing disc cells (light green; Fig. 1B). Cells in the middle position receive intermediate level of Dpp and become the proximal leg (green). Cells in the ventral position receive little Dpp and become distal leg by default (pink; Fig. 2E,F). These cells migrate and rearrange to form thoracic imaginal discs (Fig. 1E, right). Position of the proximal leg disc cells at this stage is assumed based on its sensitivity to intermediate levels of the Dpp signal.

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.

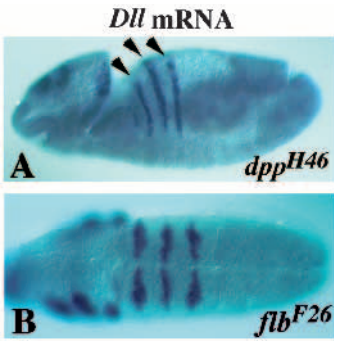


**Fig. 6.** *Dll* transcription is repressed by the D/V signaling molecules Dpp and DER. (A) *Dll* expression in $dpp^{H46}$ embryo (lateral view). Arrowheads indicate expression of *Dll* that extends to the dorsal midline. In the ventral ectoderm, the expression terminates near the border between dorsal epidermis and ventral epidermis. (B) *Dll* expression in $flb^{F26}$ embryo cultured at 25˚C (ventral view). $flb^{F26}$ is a temperature sensitive allele of *DER* (Raz and Shilo, 1993).

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