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Investigation of leading edge formation at the interface of amnioserosa and dorsal ectoderm in the *Drosophila* embryo

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SUMMARY

The leading edge (LE) is a single row of cells in the *Drosophila* embryonic epidermis that marks the boundary between two fields of cells: the amnioserosa and the dorsal ectoderm. LE cells play a crucial role in the morphogenetic process of dorsal closure and eventually form the dorsal midline of the embryo. Mutations that block LE differentiation result in a failure of dorsal closure and embryonic lethality. How LE cells are specified remains unclear. To explore whether LE cells are specified in response to early dorsoventral patterning information or whether they arise secondarily, we have altered the extent of amnioserosa and dorsal ectoderm genetically, and assayed LE cell fate. We did not observe an expansion of LE fate in dorsalized or ventralized mutants. Furthermore, we observed that the LE fate arises as a single row of cells, wherever amnioserosa tissue and dorsal epidermis are physically juxtaposed. Taken together our data indicate that LE formation is a secondary consequence of early zygotic dorsal patterning signals. In particular, proper LE specification requires the function of genes such as *u-shaped* and *hindsight*, which are direct transcriptional targets of the early Decapentaplegic/Screw patterning gradient, to establish a competency zone from which LE arises. We propose that subsequent inductive signaling between amnioserosa and dorsal ectoderm restricts the formation of LE to a single row of cells.

Key words: Dorsal Closure, Amnioserosa, Leading edge, JNK, BMP, *Drosophila*

INTRODUCTION

Morphogenesis refers to the creation of biological structure, or 'morphology', by changing the spatial relationships between cells over time (Slack, 1990). Coupled with cellular growth and fate determination, morphogenetic movements are an integral part of larger developmental programs that direct the final form of tissues and organisms. The biological event of dorsal closure in the fly embryo is a useful example of cell sheet morphogenesis that has been likened to the process of mammalian wound healing, but that has the advantage of being amenable to genetic analysis. Towards an understanding of the regulatory and cellular mechanics that underlie the morphogenetic events of dorsal closure, we have examined how cell types essential for closure come to be specified, namely, a specialized group of cells within the dorsal ectoderm called the leading edge (LE) cells. Genetic analyses have shown that the LE, the dorsalmost row of ectodermal cells, is essential during closure because mutations that compromise LE cell differentiation or function ultimately cause a failure in dorsal closure and eventual embryonic death (Knust, 1997; Noselli, 1998).

During dorsal closure, the lateral epithelia on each side of the embryo undergo coordinated cell shape changes, move dorsally, and eventually meet and adhere at the dorsal midline. Successful completion of closure internalizes the amnioserosa, a transient dorsal covering, and encloses the embryo in a continuous, protective epidermal layer. Two well-known signal transduction pathways, the Jun N-terminal Kinase (JNK) cassette and the Decapentaplegic (Dpp)/Bone Morphogenetic Protein (BMP) pathway, have been shown to cooperate in regulating the initiation and maintenance of epithelial sheet movement associated with dorsal closure (Glise and Noselli, 1997; Hou et al., 1997; Kockel et al., 1997; Ricos et al., 1999; Riesgo-Escovar and Hafen, 1997). Maintaining tight control over the level of JNK signal transduction throughout the entire process of closure is crucial because unregulated signaling activity, whether too high or too low, results in gross disruption of the process.

Although many of the components of the JNK pathway are distributed more or less uniformly throughout the ectoderm, signaling activity is limited to the LE, as revealed by the restricted expression of transcriptional targets such as *dpp* and *puckered* (*puc*; Glise and Noselli, 1997; Kockel et al., 1997; Sluss et al., 1996; Zeitlinger et al., 1997). *puc* encodes a phosphatase that negatively regulates the kinase activity of JNK. This negative feedback provides one mechanism with which to control the level of signaling through the JNK pathway (Martin-Blanco et al., 1998). Another mechanism could involve limited activation of the pathway initially.

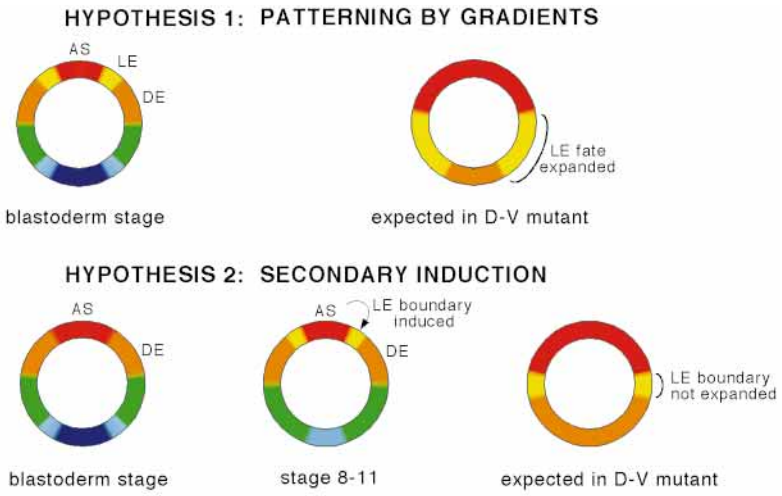

Fig. 1. Two alternative hypotheses could explain the specification of leading edge cells. According to hypothesis 1, patterning of dorsal cell fates in the blastoderm stage embryo is achieved by interpretation of specific threshold levels of BMP activity. High activity is required for amnioserosa tissue (AS), intermediate activity specifies leading edge cells (LE) and low activity patterns the dorsal ectoderm (DE). Dorsalizing mutations that alter the shape or extent of the BMP activity gradient would be expected to expand dorsal cell fates including the LE. Alternatively, hypothesis 2 predicts that LE cells are not part of the blastoderm fate map but arise secondarily, possibly through inductive interactions between differentiating amnioserosa and dorsal ectoderm. According to this hypothesis, dorsalizing mutations would not be expected to expand LE cell fate beyond a single row.

However, the source and identity of upstream signals that trigger and restrict activation of the JNK pathway are currently unknown.

Understanding how LE cells become distinct from other dorsal ectodermal cells may provide additional clues to explain the restriction of JNK pathway activity. Perhaps the LE is intrinsically different from the remainder of the dorsal ectoderm at an early stage in embryogenesis, making LE cells uniquely capable to promote signaling later in development. To explore potential mechanisms by which LE cells are specified, we considered two alternative hypotheses (Fig. 1). In one model, LE cells are fated early in embryogenesis directly in response to dorsoventral (DV) patterning information. By example, dorsal cell fates are thought to be determined by a gradient of signaling activity mediated by the combined action of two BMP family molecules, *dpp* and *screw* (*scw*), herein referred to collectively as the BMP signaling gradient (Ferguson and Anderson, 1992a; Neul and Ferguson, 1998; Nguyen et al., 1998; Wharton et al., 1993). That is, individual dorsal cells directly read the level of BMP signaling to adopt a specific fate, such as amnioserosa, which forms in response to peak levels of signaling, while dorsal ectoderm forms in response to low levels (Ferguson and Anderson, 1992a; Irish and Gelbart, 1987; Wharton et al., 1993). According to this model, LE cell fate is established through a direct readout of an intermediate threshold level of BMP signaling activity. Altering the shape and extent of the BMP signaling gradient in the blastoderm embryo would be expected to alter the number of cells that adopt the LE cell fate, as has been clearly demonstrated for amnioserosa and dorsal cell fates (Jazwinska et al., 1999; Ray et al., 1991; Wharton et al., 1993).

According to an alternative model, LE cells are specified as a secondary consequence of BMP signaling. We imagine the BMP activity gradient may be interpreted as few fairly broad tissue territories, from which additional cellular diversity arises by subsequent signaling and cellular interactions. Among these secondary interactions, inductive signaling at the boundary between amnioserosa and dorsal ectoderm could determine LE cell fate. To test these possibilities, we altered the fate map of the early embryo genetically, and assayed dorsal cell fates using several markers for the amnioserosa, the leading edge and the dorsal ectoderm. In this report, we detail results that support a secondary inductive model for LE specification, which requires the function of target genes downstream of BMP signaling and the juxtaposition of amnioserosa tissue with the dorsal ectoderm.

MATERIALS AND METHODS

Fly stocks

The wild-type stock used was Oregon R. *puc*^{E69} is a P-element enhancer trap insertion into the *puckered* locus, that expresses the *lacZ* gene marking differentiated leading edge cells (Ring and Martinez Arias, 1993). The following mutants were used in this study: *dl*^{1}, *Tl*^{ts}=*Tl*^{r444} /*Tl*^{9QRE}, *cact*^{A2}, *cact*^{HE}, *Df(2L)r10* (*cactus* null), *dpp*^{hr4}, *dpp*^{hr27}, *dpp*^{hr92}, *scw*^{l1}, *tld*^{9B}, *sog*^{S6}, *brk*^{M68}, *hnt*^{E8} and *ush*^{2}. Embryos with extra copies of *dpp*$^{+}$ were generated using an insertional duplication, *Dp(2;2)DTD48* (Wharton et al., 1993). Male flies transheterozygous for the *dpp*$^{+}$ duplication and *puc*^{E69} were crossed to females from the duplication stock (*DTD48/CyO*). The progeny from this cross will contain embryos with two, three and four copies of *dpp*$^{+}$ in the ratio 1:2:1. All alleles are described in the *Drosophila* database (http://flybase.bio.indiana.edu/). For maternal effect mutations, homozygous mutant females were crossed to *puc*^{E69}-bearing males. To test zygotic mutations on the X chromosome (*sog, hnt, brk*), *puc*^{E69} was contributed paternally, but also in the presence of a marked X chromosome. For zygotic mutations on chromosome 2 (*dpp, scw, ush*), transheterozygous males with *puc*^{E69} were crossed to heterozygous females. To test zygotic mutations on chromosome 3, *tld* was recombined onto the *puc*^{E69} chromosome, then crossed to *puc*$^{+}$ heterozygotes. Balancer chromosomes marked with *lacZ* were used to identify the relevant mutant genotypes (http://flybase.bio.indiana.edu/). For experiments using temperature-sensitive mutant alleles, crosses and egg collections were performed at 18°, 25° or 29°C. All other crosses and collections were performed at 25°C. Embryonic stages are defined according to (Campos-Ortega and Hartenstein, 1997).

Immunodetection, histochemistry, X-gal staining and in situ hybridization

The following antibodies were used: rat anti-β-gal at 1:500 (Spana and Doe, 1996), rabbit anti-β-gal at 1:1000 (Cappel Laboratories), guinea pig anti-Kruppel (573) at 1:300 (a generous gift of Dave Kosman), mouse anti-Fasciclin III (IG10) at 1:40 (Patel et al., 1987), mouse anti-hindsight (1G9) at 1:5 (Yip et al., 1997) and rabbit anti-scribble at 1:500 (Bilder and Perrimon, 2000). Immunohistochemistry and immunofluorescence were performed as described (Patel, 1994). The appropriate fluorochrome- or enzyme-conjugated secondary

antibodies were used at recommended dilutions (Jackson ImmunoResearch). Embryos stained by immunohistochemical methods were dehydrated and mounted in methyl salicylate (Patel, 1994). For X-gal staining, embryos were collected, dechorionated in a 1:1 bleach:water solution for 3 minutes, and fixed for 10 minutes in a 1:1 mixture of heptane:fixative (4% methanol-free formaldehyde in phosphate-buffered saline solution with added 0.1% Triton-X-100 (PBST)). After fixation, all liquid was removed from embryos and they were washed extensively in PBST. Embryos were then incubated briefly in staining solution without X-gal substrate (Ausubel et al., 1994) for 5 minutes, followed by incubation in staining solution plus 0.2% X-gal (from 10% stock solution in DMSO) for several hours at 37°C. After staining, embryos were washed, devitellinized in a 1:1 methanol:heptane mixture, rehydrated and mounted in 70% glycerol. In situ hybridization of embryos was carried out as described (Stronach et al., 1996) with digoxigenin-labeled RNA probes (Boehringer Mannheim) corresponding to *dpp*-coding sequences.

Microscopy, image acquisition and processing

Images of stained embryos were captured with the SPOT™ digital camera (Diagnostic Instruments) using differential interference contrast optics on a Zeiss Axiophot microscope. Fluorescent images of embryos were captured using the Leica TCS NT confocal microscope system and subsequently assembled using Adobe Photoshop software.

RESULTS

Markers for differentiated leading edge cells

To follow the differentiation of LE cells, we used an enhancer trap inserted into the *puckered* locus, which responds to JNK signal transduction activity (Glise and Noselli, 1997; Martin-Blanco et al., 1998; Ring and Martinez Arias, 1993). The transgenic insert, puc^{E69}, leads to loss of Puc function, but in the heterozygous state, expresses nuclear localized β-galactosidase (β-gal) in a pattern restricted to the LE cells. These heterozygous animals appear wild type in all assays. β-gal activity is detectable in the LE from the beginning of dorsal closure, stage 13 (~9.5 hours of development), through to the end of closure, when LE cells have formed the dorsal midline at stage 16 (~13 hours of development; Fig. 2). We also followed LE cells by staining for Fasciclin III (Patel et al., 1987), a basolateral membrane protein that is localized asymmetrically in LE cells; specifically, Fasciclin III is absent from the membrane that abuts the amnioserosa directly (see Fig. 4A). This differs from cells of the rest of the dorsal ectoderm that localize Fasciclin III cortically. Finally, in some experiments, we have used *dpp* transcripts to confirm the fate of LE cells. Since *dpp* gene expression is not exclusively restricted to LE cells but, rather, shows a dynamic pattern of transcript accumulation throughout development (Ray et al., 1991), we used *dpp* as a secondary marker in addition to Puc enhancer staining to define LE cell fates.

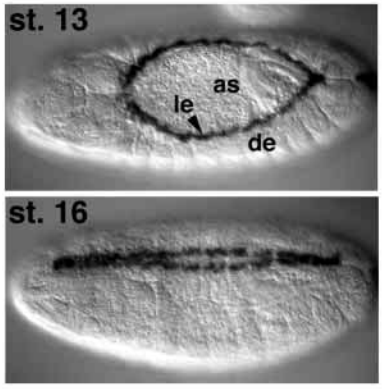

Fig. 2. An enhancer trap in the puckered locus, puc^{E69}, is expressed in leading edge (le) cells of the dorsal ectoderm (de) during dorsal closure. When closure commences at stage 13, the LE appears as a single row of cells forming a ring around the amnioserosa (as), which becomes internalized by stage 16. Closure is complete when the LE cells meet and adhere at the dorsal midline. Panels are dorsolateral views with anterior towards the left.

Dorsalizing mutations fail to expand leading edge beyond a single cell row

In the blastoderm embryo, ventral tissues are specified by a maternal gradient of Dorsal protein activity (Chasan and Anderson, 1993). High levels of Dorsal activity on the ventral side of the embryo direct the formation of mesoderm, while intermediate and low levels direct differentiation of ventral ectoderm. Lack of Dorsal activity on the dorsal side of the embryo allows for the elaboration of a zygotic signaling cascade culminating in a gradient of BMP signaling activity. Maximal levels of BMP signal are required to specify the most dorsally located tissue, the amnioserosa, whereas lower levels of BMP activity direct formation of the dorsal and lateral ectoderm (Ferguson and Anderson, 1992a; Irish and Gelbart, 1987; Wharton et al., 1993). Therefore, mutations in either the maternal Dorsal pathway or the zygotic BMP pathway alter the dorsoventral fate map of the embryo.

To examine whether LE cell fate is altered in response to changes in DV patterning information, we assayed for the presence, position and extent of the LE in dorsalized mutant backgrounds. First, we used a temperature-sensitive mutation to reduce the activity of the maternally required Toll (Tl) receptor, which, under permissive circumstances, signals to promote Dorsal activity. This allowed us to assess the effect of increasing dorsalization (loss of Dorsal activity) by collecting embryos derived from Tl^{ts} mothers at different temperatures. At 18°C, Tl^{ts} activity is slightly impaired causing a reduction in ventral cell fates and concomitant broadening of dorsal pattern elements (Anderson et al., 1985). If an expanded dorsal patterning field can specify a broader domain of LE cell fates, then it should occur under these conditions. In these mutant embryos, Puc-expressing LE cells were present as a single cell row and their position was shifted more ventrally compared with wild type (Fig. 3A,B). As in wild-type embryos, the row of LE cells was located at the interface between amnioserosa and dorsal ectoderm.

When mutant embryos are raised at a nonpermissive temperature, 29°C, Tl^{ts} is unable to signal, resulting in loss of ventrally derived cell fates and further expansion of dorsal fates (Anderson et al., 1985). Indeed, these dorsalized embryos displayed differentiating amnioserosa tissue around the entire central region of the embryo (Fig. 3C). LE and dorsal ectoderm were also formed in these embryos but their axial arrangement was reoriented nearly 90°. Consequently, LE cells were arrayed around the DV circumference of the embryo, rather than in an anterior to posterior orientation. However, they remained in a single row configuration between amnioserosa and dorsal ectoderm cells (Fig. 3C).

A similar phenotype is observed in embryos derived from mothers with a null mutation in the *dorsal* gene (Fig. 4).

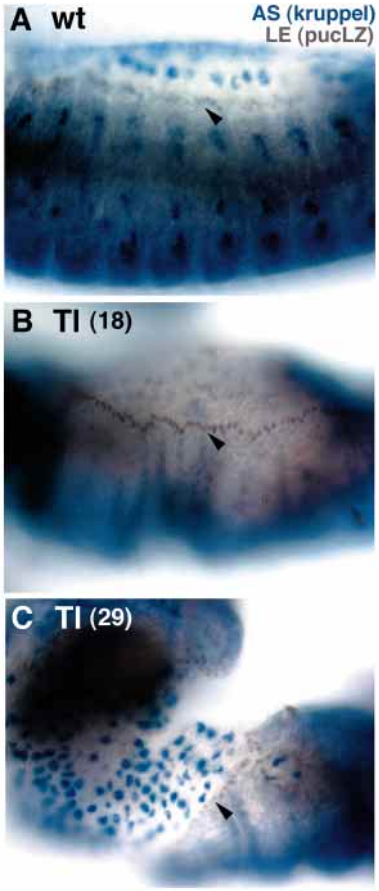

Fig. 3. Examination of amnioserosa and leading edge in dorsalized embryos. Embryos have been double immunolabeled for Kruppel (blue, alkaline phosphatase) to reveal the large amnioserosa cells and β-gal (brown, horseradish peroxidase) to reveal *puc* enhancer expression in the LE (arrowheads). In wild-type embryos (A), a single row of LE cells is detected at the interface between amnioserosa and dorsal ectoderm. At this stage, Kruppel is also detected in segmentally repeated muscle precursors. Weakly dorsalized embryos are derived from mothers bearing a temperature-sensitive *Tl* mutation raised at 18°C (B). LE cells are detected as a single row located more ventrally than in wild type. Further dorsalization of embryos raised at 29°C results in amnioserosa tissue that encompasses the DV circumference of the embryo (C). LE cells are also reoriented circumferentially but remain as a single row of cells at the amnioserosa/ectoderm interface. Lateral views with anterior towards the left.

Lacking Dorsal protein, embryos are strongly dorsalized and early *dpp* expression is derepressed ventrally (Ray et al., 1991). Immunofluorescent staining of mutant embryos with various combinations of reagents allowed us to visualize the distributions of amnioserosa, dorsal ectoderm and LE with respect to one another. Although amnioserosa differentiation appeared limited to a central domain of the dorsalized embryos, as observed in embryos from Tl^{ts} females, many embryos displayed a nonuniform distribution of amnioserosa and dorsal ectoderm. Fig. 4 shows several examples of small multicellular islands of one tissue that are interspersed within larger fields of the other cell type (Fig. 4C-F).

This arrangement of tissues allowed several interesting observations regarding the formation of LE cells. First, in most cases, wherever amnioserosa and dorsal ectoderm become juxtaposed, we observed the formation of LE cells by the expression of the *puc* enhancer in a single row (Fig. 4, compare 4A with 4B,C). Second, very small islands consisting of just a few cells are surrounded by LE cells (Fig. 4C,E: amnioserosal islands; 4D,E′,F,F′: ectodermal islands). Third, the ectodermal cells that were adjacent to the amnioserosa showed an asymmetric localization of Fasciclin III, just as in wild-type LE cells (Fig. 4 compare inset in 4A with 4E,E′), lending further support to the conclusion that these were indeed differentiated LE cells. Finally, using *dpp* transcripts as an additional marker of LE, we observed the late pattern of *dpp* in small rings and circumferential stripes around the embryo (Fig. 4G,G′). Most of these stripes, especially those in the central region of the embryo, were only a single cell wide, resembling the pattern we detected with the *puc* enhancer. However, it must be noted that because *dpp* is normally expressed in other tissues besides the LE at later stages, dorsalized embryos displayed additional wider bands of *dpp* expression anterior and posterior. Nevertheless, three markers for LE revealed the differentiation of LE cells in a single row.

These findings were corroborated by observing *puc* enhancer expression in embryos that contain up to four copies of the dpp^+ gene. Increasing the gene dosage of dpp^+ produces a broader domain of maximal signaling which results in expansion of amnioserosa tissue (Wharton et al., 1993). By this criterion, embryos with extra copies of dpp^+ are dorsalized. Under these conditions, LE fate remained one cell wide (not shown). In summary, mutant genotypes that dorsalize the embryo alter the distribution and expand the size of amnioserosa and dorsal ectoderm tissues but do not expand the LE fate beyond a single row nor displace the LE cell row from the amnioserosa-dorsal ectoderm interface.

Leading edge is proportionately lost with increasing ventralization

Ventralizing mutations reduce the domain of *dpp* expression, accompanied by reduction or elimination of dorsal cell fates (Ray et al., 1991). To determine the effect of progressive loss of dorsal patterning activity on LE cell specification, we examined *puc* enhancer trap expression in various ventralized embryos. Simply halving the dosage of Cactus (Cact), an inhibitor of Dorsal activity, can weakly ventralize embryos, owing to an expansion of the Dorsal activity gradient (Roth et al., 1991). As a result, the domain of maximal BMP signaling activity is reduced along with the overall size of the amnioserosa. In embryos heterozygous for a *cact* null mutation, LE cells were observed in a single row; however, the ring of LE cells was positioned slightly more dorsally than in wild type and appeared smaller to account for the reduced area of the amnioserosa tissue (Fig. 5A).

Strong ventralization of embryos derived from *cact* loss-of-function mutant females leads to potent Dorsal activity in all the cells of the blastoderm and therefore, expansion of ventral cell fates at the expense of dorsal fates (Roth et al., 1991). In this genetic background, amnioserosa and dorsal ectoderm are not specified in part because *dpp* expression, which is required for the fate of those tissues, is repressed by Dorsal. As expected, LE cells also appear to be absent. Fig. 5B shows a few β-gal-positive cells in these embryos; however, they are not likely to be LE cells. Double-label immunofluorescence revealed that the β-gal-positive cells were not Fasciclin III-positive ectodermal cells, but were internal to them (not shown). Similar ectopic, ventrally localized *puc* expression has been documented in puc^{E69} homozygous mutant embryos (Glise and Noselli, 1997; Martin-Blanco et al., 1998), suggesting that *puc* expression can be upregulated in cells other than LE under certain conditions. Therefore, we conclude that in the absence of a dorsal patterning activity, LE differentiation is not apparent.

Intermediate ventralization results from mutations in the

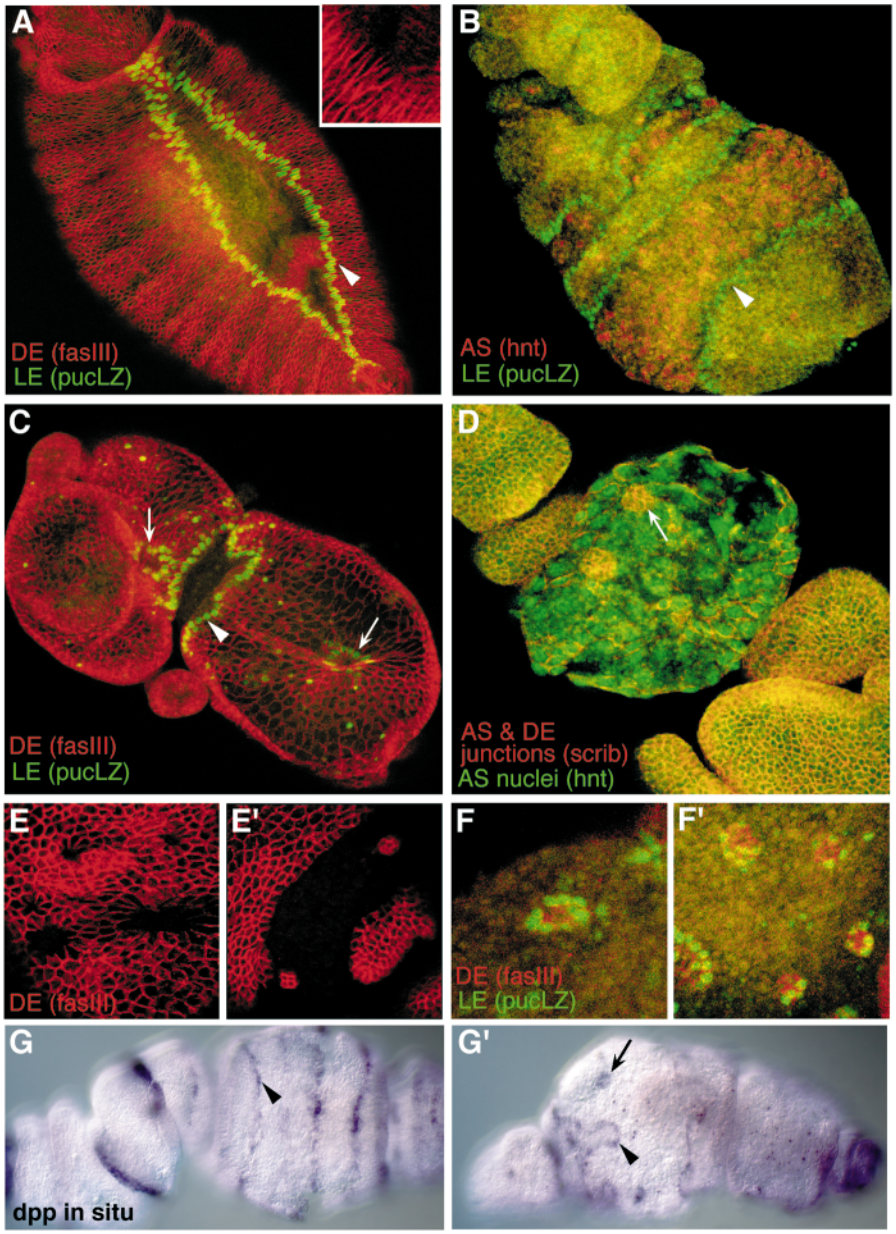

Fig. 4. The leading edge is formed in dorsalized embryos despite disruption of amnioserosa and dorsal ectoderm. Here, dorsal ectoderm (DE), leading edge (LE) and amnioserosa (AS) of dorsalized embryos are examined in close detail with various combinations of antibodies as indicated in the panels. Embryos are from wild-type (A) or *dorsal* mutant mothers (B-G′). Leading edge cells (arrowhead) comprise the first row of ectodermal cells that abut the amnioserosa in wild-type embryos (A). LE cells express β-gal (green) and Fasciclin III (red), which is asymmetrically distributed in these cells (A, inset). Independent of tissue size or position, wherever amnioserosa tissue and dorsal ectoderm are juxtaposed, LE cells are formed. (B) A dorsalized embryo with circumferential single cell wide rings of LE (arrowhead) surrounding amnioserosa tissue. (C) A single row of LE cells at the edge of Fasciclin III-positive ectoderm (arrowhead). In dorsalized embryos, islands of tissue occasionally form (C-F′). (C,E) Unlabeled islands of amnioserosa surrounded by Fasciclin III-positive dorsal ectoderm. Fasciclin III localization is asymmetric in cells adjacent to these islands (E), and these correspond to LE cells that express β-gal (green in C, arrows). (D,E′) Similarly, islands of ectoderm are surrounded by amnioserosa (both tissues labeled in D, only ectoderm labeled in E′ to show asymmetric Fasciclin III). Within a sea of amnioserosa, islands of ectoderm are consistently bordered by β-gal-expressing LE cells (F,F′). Finally, *dpp* RNA is detected in dorsalized embryos by whole-mount in situ hybridization (G,G′). *dpp*, a marker of LE cells, is also observed in rings (arrow) and stripes (arrowheads) consisting of a single row of cells like those seen using the *puc* enhancer. Thus, three LE markers demonstrate the presence of LE cells in dorsalized embryos at the interface between amnioserosa and dorsal ectoderm. Dorsal views with anterior towards the left.

zygotic genes *screw* (*scw*) and *tolloid* (*tld*) because Scw and Tld are necessary to create the peak BMP signal, required for formation of the amnioserosa (Arora et al., 1994; Arora and Nusslein-Volhard, 1992; Ferguson and Anderson, 1992b; Neul and Ferguson, 1998; Nguyen et al., 1998). Consequently, *scw* and *tld* mutant embryos do not differentiate amnioserosa tissue, but they do retain some dorsal epidermal pattern elements. Based on the model whereby dorsal cell fates are specified in direct response to specific threshold levels of BMP signaling, moderate ventralization might be expected to convert cells normally adopting an amnioserosa fate to adopt a more ventral cell fate, the LE cell fate. In *scw* and *tld* mutant backgrounds, we were unable to detect formation of LE (not shown). Similarly, LE cells were not detected in embryos mutant for hypomorphic *dpp* alleles in which amnioserosa fails to form. In all of these cases, either the DV fate map has been shifted too far ventrally to retain the dorsally derived LE fate, or an interaction between amnioserosa and dorsal ectoderm is necessary to specify LE cells within the ectoderm.

Direct modulators of the BMP signaling gradient do not affect leading edge specification

To target the region of the BMP signaling gradient where we imagine LE cell fate might arise, we examined LE differentiation further in zygotic mutant backgrounds where the shape of the BMP activity gradient is directly altered (Fig. 6). Both *brinker* (*brk*) and *short gastrulation* (*sog*) modulate BMP signaling activity such that the intermediate portion of the signaling gradient is enlarged, as observed by the expansion of molecular markers and pattern elements in the dorsal ectoderm (Ashe et al., 2000; Francois et al., 1994; Jazwinska et al., 1999). In other words, loss of either *brk* or *sog* activities results in a lateral shift in the embryonic fate map. With respect to amnioserosa differentiation in particular, *brk* mutants are

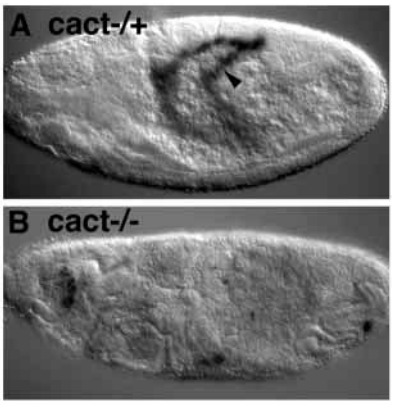

Fig. 5. The leading edge is proportionately lost with increasing ventralization. By enzymatic detection of β-gal from the *puc* enhancer trap, we observe that LE cells are present as a single row (arrowhead) in weakly ventralized embryos derived from mothers heterozygous for a *cactus* deficiency (A). LE is absent, as is amnioserosa, in severely ventralized embryos derived from mothers homozygous for strong hypomorphic *cactus* alleles (B). A few β-gal-positive cells are evident in these embryos, but they are not likely to be LE cells (see text). The embryo in A is oriented dorsal upwards and anterior towards the left.

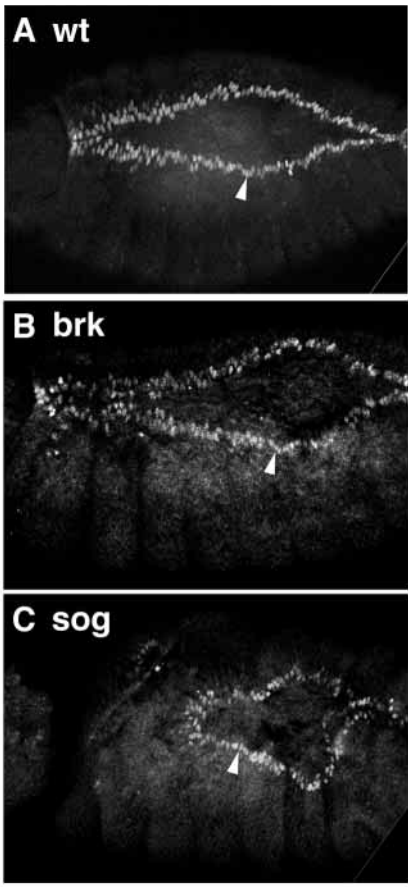

Fig. 6. Leading edge is not expanded in mutants that modulate the shape of the BMP activity gradient. Wild-type (A), *brk* mutant (B) and *sog* mutant (C) embryos were independently labeled with anti-β-gal antibodies to reveal the cells of the LE (arrowheads). Despite significant cell fate changes in the mutant embryos, LE cells are observed in a single row at the interface between amnioserosa and dorsal ectoderm, indicating that LE differentiation is fundamentally normal. In the anterior of each embryo, LE cells interdigitate, causing the appearance of multiple rows. This phenomenon correlates with the dramatic cell movements of dorsal closure and is not specific to the mutant genotypes. All panels are dorsolateral views with anterior towards the left.

relatively normal (Jazwinska et al., 1999). In contrast, *sog* mutants have fewer amnioserosa cells because Sog is essential for achieving the maximum level of BMP signaling required for patterning the amnioserosa (Ashe and Levine, 1999). If LE was specified in response to a discrete intermediate threshold level of BMP activity, mutants such as these might be expected to expand the LE domain.

Interestingly, in null *brk* mutant embryos, we observed that LE specification was normal; Puc-expressing cells were detected in a single row at the edge of the dorsal ectoderm surrounding a normal sized amnioserosa (Fig. 6B). Despite significant changes in the embryonic fate map of *sog* mutant embryos, LE cell specification appeared fundamentally normal (Fig. 6C). The extent of various dorsal tissues are specifically changed in *brk* and *sog* mutant embryos, but both genotypes give rise to embryos with amnioserosa and dorsal ectoderm tissues, and LE was always detected between those tissues.

Leading edge markers are differentially expressed in U-shaped mutants

Taken together, our results raise the possibility that amnioserosa may be required for LE formation. To address the function of amnioserosa for LE specification, we examined *puc* enhancer expression in several mutants of the U-shaped class, including *u-shaped* (*ush*) and *hindsight* (*hnt*). Incidentally, the dorsal expression domains of these genes are directly regulated by DV patterning signals (Ashe et al., 2000; Yip et al., 1997). In these mutant embryos, the amnioserosa tissue is fated normally and begins to differentiate up to stage 11, but then degenerates prematurely (Frank and Rushlow, 1996; Lamka and Lipshitz, 1999). In both *ush* and *hnt* mutants, programmed cell death takes place over the course of a few hours, with elimination of amnioserosa cells by stage 13 – the time when dorsal closure would normally commence. Unexpectedly, we observed different patterns of expression with the *puc* enhancer in the two mutants. In *ush* embryos, β-gal-positive cells were not detected (Fig. 7A). In contrast, *hnt* mutant embryos displayed Puc-positive LE cells at the edge of the dorsal ectoderm, albeit with less uniform expression than normally observed (Fig. 7C). To confirm these observations, we examined the accumulation of *dpp* mRNA in the LE. Similar to *puc* enhancer expression, we observed differential expression of *dpp* in *ush* versus *hnt* mutant embryos. *ush* mutant embryos show a consistent and significant reduction in LE *dpp* expression, although residual *dpp* transcripts are seen (Fig. 7D, arrowhead). *dpp* expression appears relatively normal in *hnt* mutant embryos (Fig. 7E).

In addition to the differential expression of two LE markers in the U-shaped mutants, we observed ectopic expression of LE markers only in *hnt* mutant embryos. β-gal-positive cells were observed in the region of the amnioserosa in *hnt* mutants as early as stage 11 (Fig. 7B), raising the possibility that this could be an example of expanded LE cell fates. We demonstrate that these cells adopt only partial LE cell fate, for the following reasons. These cells do not express the LE marker Fasciclin III, but do express two other LE molecules, albeit aberrantly. *puc*, for example, is expressed precociously in these cells, preceding Fasciclin III expression in the ectoderm (Fig. 7B,C), and *dpp* is rarely but reproducibly expressed (Fig. 7E′, arrow). Additionally, Frank and Rushlow have shown that cells in this region express amnioserosa fate markers such as *race*, through stage 11 (Frank and Rushlow, 1996). Thus, based on the possibility that these cells may co-express LE and amnioserosa markers during stage 11, their identity cannot be unequivocally determined. Our results may indicate that these cells are of mixed fate. The presence of ectopic LE-like cells in *hnt* mutant embryos, coupled with the severe reduction of LE fate markers in *ush* mutants, suggest that the distinction between amnioserosa and LE is a

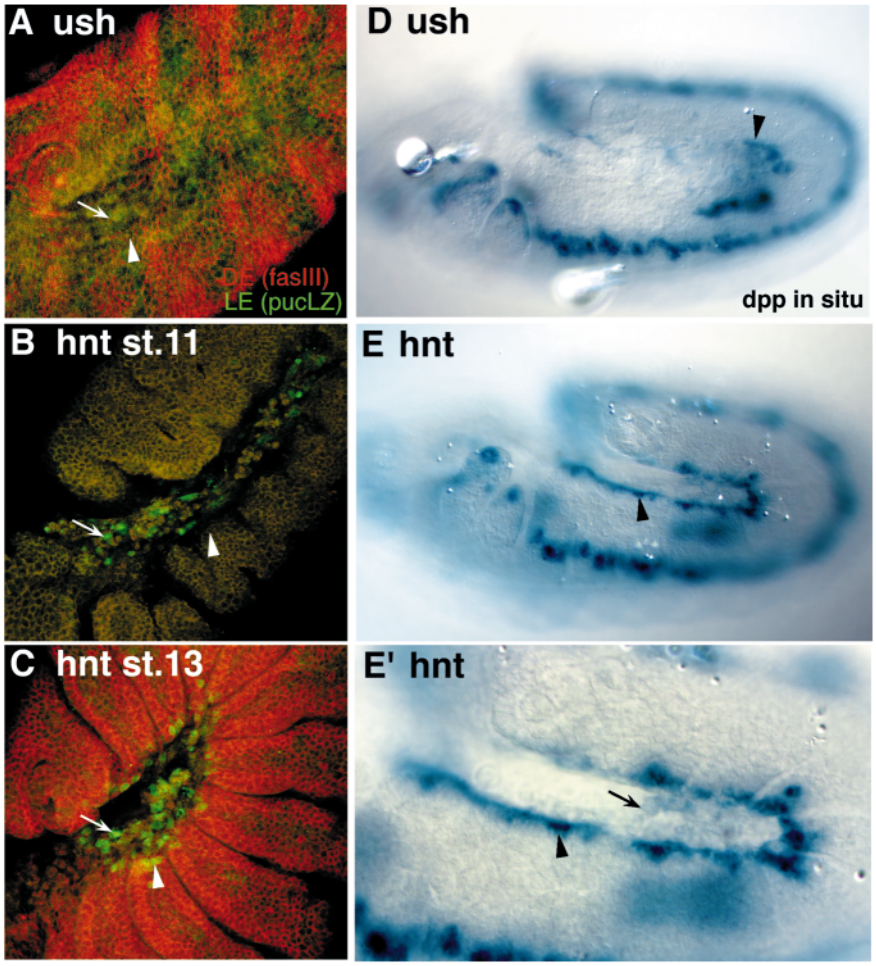

Fig. 7. Leading edge markers are altered in U-shaped mutants in which amnioserosa is prematurely lost. *ush* and *hnt* embryos have been double immunolabeled for Fasciclin III (red) and β-gal (green) to identify dorsal ectoderm and LE, respectively (A-C) or used for whole-mount in situ hybridization to reveal *dpp* transcripts (D-E′). In *ush* mutant embryos (A), β-gal is never expressed at the LE (arrowheads). In contrast, *hnt* mutants exhibit ectopic β-gal expression in the region of the dying amnioserosa (arrows) from stage 11 (B), before Fasciclin III expression levels peak, through stage 13 (C). Additionally by stage 13 (C), β-gal expression is clearly evident at the edge of the ectoderm indicating that LE fates are present in *hnt* mutant embryos, although in a less uniform arrangement compared with wild-type (compare with Fig. 3A). Similar results are shown with *dpp* transcripts in the LE (D-E′). *dpp* expression in the LE is substantially reduced in *ush* mutant embryos (D), although some residual staining is apparent (arrowhead), suggesting that LE specification is compromised. LE expression of *dpp* in *hnt* mutant embryos is relatively normal (E,E′, arrowhead). Higher magnification of the same embryo (E′) reveals some ectopic *dpp* expression in the amnioserosa (arrow), however, these ectopic transcripts are detected in less than 10% of mutant embryos. Lateral views with anterior towards the left.

secondary consequence of Hnt and Ush functions, not a direct result of specific BMP signaling thresholds.

DISCUSSION

As a basis for understanding the complex morphogenetic events of dorsal closure in the fly embryo, we sought to characterize the origin of cell types essential for closure and how they become determined. Examination of the mechanism that underlies LE cell specification during embryogenesis may provide insight as to how they signal and precisely coordinate cell sheet movement later during the closure process. To examine how the LE forms, we asked whether LE cell fate responds to early DV patterning information by first perturbing the BMP gradient genetically and assaying whether the width of the LE stripe is directly responsive to the levels of BMP signal.

Primary versus secondary specification of leading edge cells

Using mutations that influence DV patterning, it is possible to alter the size and distribution of BMP target gene expression patterns, which indicate the extent of amnioserosa and dorsal ectodermal cell fates. If LE fate was specified directly by a particular threshold level of BMP signal, then one would expect LE fate to be perturbed in concert with amnioserosa and dorsal ectoderm fates in DV mutants. Mutations in genes such as *dl*, *Tl*, *brk* and *sog* alter the size of BMP target gene expression domains; however, these mutants failed to alter specification of LE fate. Among these genotypes, *brk* and *sog* specifically modulate the shape of the BMP signaling gradient in a region where LE fate might arise (Jazwinska et al., 1999), yet LE formation in these mutants is fundamentally normal. Furthermore, in dorsalized embryos, LE cells were observed regularly at the boundary between amnioserosa and dorsal ectoderm even when the morphology of these tissues was severely disrupted. Islands of amnioserosa cells within a field of ectoderm were consistently surrounded with a single row of LE cells, independent of the number of amnioserosa cells constituting the island. The converse situation also occurred;

Table 1. Effect of dorsoventral patterning mutations on leading edge formation

Mutant	Class	Leading edge
Tl	Maternal dorsalizing	+
dl	Maternal dorsalizing	+
dpp^{+} (4×)	Zygotic dorsalizing	+
cact	Maternal ventralizing	–
tld	Zygotic ventralizing	–
scw	Zygotic ventralizing	–
dpp^{-}	Zygotic ventralizing	–
brk	Zygotic ‘lateralizing’	+
sog	Zygotic ‘lateralizing’	+
hnt	Zygotic U-shaped	++
ush	Zygotic U-shaped	–

again, a single row of LE cells formed at the boundary between the ectoderm and amnioserosa.

We also analyzed DV mutants to determine whether a decrease in BMP signaling activity converts amnioserosa to LE as predicted by a gradient patterning model. A range of ventralizing mutations (*cact*, *sog*, *scw*, *dpp*) displaying progressive loss of amnioserosa tissue did not give rise to embryos with an expanded domain of LE cells. In fact, LE cells were not detected in the absence of amnioserosa. We found no situation in which an altered BMP gradient was associated with expanded LE fate (Table 1), thus the prediction of a direct gradient response model does not explain LE fate specification.

Notably, DV mutant embryos that perturb the BMP gradient, also perturb the expression domains of target genes including *ush* and *hnt* (Ashe et al., 2000; Yip et al., 1997), without altering LE specification (this report). However, we demonstrate that loss of *ush* and *hnt* function results in specific and distinct perturbations in LE formation. Thus, we favor the interpretation that LE fate specification is not a direct early response to the BMP gradient, but rather is a secondary consequence of the specification of dorsal fates through the action of BMP target genes like *ush* and *hnt*.

Mechanisms for leading edge formation in a single cell row

If LE cells are specified as a secondary consequence of DV patterning gradients, then what additional mechanisms are at work to define LE as a single row of cells? Our data are consistent with several mechanisms. One possibility is that specification of the LE involves the combinatorial action of nested sets of transcriptional regulators, including Hnt dorsally and Ush in a broader domain (Ashe et al., 2000; Cubadda et al., 1997; Fossett et al., 2000; Jazwinska et al., 1999; Yip et al., 1997). Accordingly, loss of Hnt function is predicted to result in a failure to differentiate amnioserosa, coupled with dorsal expansion of more lateral fates, such as the LE. Consistent with this model, *hnt* mutant embryos displayed Puc-positive cells with partial LE character in the region of the dying amnioserosa during stage 11. These results suggest that Hnt may be necessary to distinguish amnioserosa from LE fate at the time of extended germ band stage. This timing is late, relative to the timing of the early BMP threshold response, further supporting the notion that LE specification is a secondary consequence of initial BMP signaling.

Ush may play a role in differentiation of more lateral fates adjacent to the amnioserosa and the Hnt expression domain. Indeed, we show that Ush function is essential for LE development because LE does not form in *ush* mutant embryos. Based on these results, we imagine Ush could define a competency zone from which LE cells arise, or Ush could participate in generating or modulating a signal(s) for communication between the differentiating amnioserosa and dorsal ectoderm. Ush is related to mammalian zinc-finger protein family, Friend of GATA (FOG), which has been shown to participate as a cofactor with GATA transcription factors. Together, these protein complexes regulate cell fate determination multiple times during both mammalian and *Drosophila* development (Cubadda et al., 1997; Fossett et al., 2000; Fox et al., 1999; Haenlin et al., 1997). Interestingly, FOG2, a mammalian homolog of Ush, appears to be required during an inductive signaling event between two distinct tissues in the mouse heart (Tevosian et al., 2000), suggesting that inductive processes in development may commonly use the function of Ush family members. We have not determined whether the function of Ush in LE cell specification is localized to the amnioserosa, the dorsal ectoderm, or both. Experiments to replace Ush function in a tissue-specific manner should address that issue.

Although transcriptional targets of BMP signaling, such as *ush* and *hnt*, among others, define at least three specific threshold responses (Ashe et al., 2000; Jazwinska et al., 1999), the size difference between the nested expression domains of these markers still fails to account for a cell fate defined by a single row of cells. An additional mechanism to explain the spatially restricted stripe of LE cells is through an inductive signaling event. From the analysis of dorsalized mutants, we observed that LE forms as a result of the juxtaposition of amnioserosa tissue with dorsal ectoderm, which may provide spatially limited activation of the JNK pathway. Thus, restricted expression of JNK target genes, such as *puc* and *dpp* may be a direct result of a signal that specifies LE.

Communication between the amnioserosa and the dorsal ectoderm during embryogenesis has been suggested in two cases recently. First, Hnt expression in the amnioserosa is required nonautonomously for proper cell rearrangements in the dorsal ectoderm, associated with retraction of the embryonic germband (Lamka and Lipshitz, 1999). Second, the *raw* gene product appears to be expressed in the amnioserosa, though it influences the activity of the JNK pathway in the ectoderm during dorsal closure (Byars et al., 1999). As amnioserosa and ectoderm develop, they may acquire different cell affinities, which cause them to sort into separate domains or islands (in the case of dorsalized embryos), displaying smooth borders at their interface. A difference in cell adhesion at the boundary may be sufficient to generate signaling for LE specification similar to inductive mechanisms at work at the compartmental boundaries of larval imaginal discs (Dahmann and Basler, 1999; Vincent, 1998). The challenge now will be to identify molecules that may participate in an inductive signal.

Concluding remarks

Our results suggest that a multistep process determines the LE as a single row of cells. We demonstrate that LE does not form directly in response to discrete intermediate levels of BMP signaling activity, but forms secondarily by the action of transcriptional regulators that are themselves BMP target genes. Among these targets, Hnt and Ush define a LE competency zone that is expanded in *hnt* mutants and eliminated in *ush* mutants. We propose that from within the competency zone, LE fate is further refined to a single row by an unknown inductive signal generated by the physical juxtaposition of amnioserosa with dorsal ectoderm. This signal activates the JNK pathway that regulates localized expression of *dpp* and *puc*.

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