Segment boundary formation in *Drosophila* embryos

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Summary

In *Drosophila* embryos, segment boundaries form at the posterior edge of each stripe of *engrailed* expression. We have used an HRP-CD2 transgene to follow by transmission electron microscopy the cell shape changes that accompany boundary formation. The first change is a loosening of cell contact at the apical side of cells on either side of the incipient boundary. Then, the *engrailed*-expressing cells flanking the boundary undergo apical constriction, move inwards and adopt a bottle morphology. Eventually, grooves regress, first on the ventral side, then laterally. We noted that groove formation and regression are contemporaneous with germ band retraction and shortening, respectively, suggesting that these rearrangements could also contribute to groove morphology. The cellular changes accompanying groove formation require that Hedgehog signalling be activated, and, as a result, a target of Ci expressed, at the posterior of each boundary (obvious targets like stripe and rhomboid appear not to be involved). In addition, Engrailed must be expressed at the anterior side of each boundary, even if Hedgehog signalling is artificially maintained. Thus, there are distinct genetic requirements on either side of the boundary. In addition, Wingless signalling at the anterior of the domains of *engrailed* (and *hedgehog*) expression represses groove formation and thus ensures that segment boundaries form only at the posterior.

Supplemental data available online

Key words: *Drosophila* embryos, Segmentation, Boundaries, *hedgehog*, *engrailed*, TEM

Introduction

The establishment of boundaries between groups of cells is a general feature of developing animals. Preventing populations of cells to intermingle allows patterning and growth to be controlled in well-defined compartments. Moreover, boundaries are ideally suited to be a source of morphogen that can be controlled in well-defined compartments. Moreover, boundaries are ideally suited to be a source of morphogen that can be controlled in well-defined compartments. Furthermore, the long-standing observation that the boundary is needed for the formation of a number of cell types suggests that it is a signaling center. The cellular changes accompanying groove formation require that Hedgehog signalling be activated, and, as a result, a target of Ci expressed, at the posterior of each boundary (obvious targets like stripe and rhomboid appear not to be involved). In addition, Engrailed must be expressed at the anterior side of each boundary, even if Hedgehog signalling is artificially maintained. Thus, there are distinct genetic requirements on either side of the boundary. In addition, Wingless signalling at the anterior of the domains of *engrailed* (and *hedgehog*) expression represses groove formation and thus ensures that segment boundaries form only at the posterior.

Cell sorting in imaginal discs could depend on a difference in affinity between cells on either side of the boundary (Lawrence, 1993). Differential adhesion models such as that proposed by Steinberg (Steinberg, 1962) state that cells with similar affinity adhere preferentially with each other and sort out from cells of different affinity. Differences in adhesion between two cell populations could result from either a difference in concentration of one type of adhesion molecule or the differential expression of distinct adhesion molecules (Dahmann and Basler, 2000). So far, no specific adhesion molecule has been identified that is required for maintaining the boundary between the anterior and posterior compartment. At the dorsoventral (DV) boundary of imaginal disks, two putative cell adhesion molecules, the single pass transmembrane proteins encoded by tartan and capricious, have been shown to contribute to boundary maintenance (Milan et al., 2001). However, as yet, compartmental expression of tartan and capricious does not fully account for boundary maintenance as loss-of-function clones still respect the boundary. In the vertebrate hindbrain, another class of membrane-associated proteins have been implicated in boundary formation. There, lack of cell mixing across rhombomere boundaries depends on the interaction between...
Eph receptors and their GPI-anchored ligands, the ephrins, which are expressed in a complementary fashion in alternate segments (reviewed by Wilkinson, 2001). Current data suggest that these molecules control cell affinities by activating downstream signalling, which leads to active repulsion between cells in neighbouring rhombomeres.

The *Drosophila* embryo is another system where boundaries can be studied both genetically and morphologically. During early development, the embryonic epidermis becomes divided into a series of repeated patterning units termed parasegments (Lawrence and Struhl, 1996; Martinez-Arias and Lawrence, 1985). Parasegment boundaries are clonal boundaries that form at the anterior edge of each stripe of *engrailed* expression as soon as cellularization is complete (Vincent and O’Farrell, 1992). They are maintained throughout the life of the fly and indeed give rise to compartment boundaries in imaginal disks (Garcia-Bellido et al., 1973). Around stage 11 of embryonic development, another boundary forms at the posterior edge of each *engrailed* stripe. This boundary is easily recognisable as deep grooves in the epithelium and marks the edge of each segment. As a foundation to uncover the cell biological basis of segment boundary formation, we have studied the morphological changes that accompany this process and its genetic requirements.

**Materials and methods**

**Fly stocks**

The following mutant alleles were used: *wg*^CX4^ (Baker, 1987), *hh*^AC^ (Lee et al., 1992), *Df*2Rien^F^ (Tabata et al., 1992), *ci*^M^ (Methot and Basler, 2001), *c^E^*^(Slusarski et al., 1995), *striped*^D^, *rhomboid*^M^ (Jurgens et al., 1984), *zipper* and *hindsight*^P^. The *wg*^CX4^ and *Df*2Rien^F^ recombinant was a kind gift from Peter Lawrence. The following Gal4 drivers and responders were used: *engrailed-Gal4* and UAS-*lacZ* (gift from Andrea Brand, Cambridge, UK), *tubulin-Gal4* (Pignoni and Zipursky, 1997), *buttonhead-Gal4* (gift from Gines Morata, Madrid), *paired-Gal4* (gift from C. Desplan, NYU, USA), UAS-*wingless* (Lawrence et al., 1995), UAS-*arm*^S10^ (Pai et al., 1997), UAS-*engrailed* (Guillen et al., 1995) and UAS-*hedgehog* (Fietz et al., 1995). UAS-CiVP16 was made by inserting DNA encoding the activation domain of HSV VP16 in the *Bcd* site of *ci* located three codons upstream of the stop codon. This C-terminal fusion was then transferred into pUAST. UAS-CD2-HRP was constructed as follows: DNA coding for HRP along with the signal peptide from Wingless was amplified by PCR from UAS-wingless-HRP (Dubois et al., 2001). This was ligated in frame to a PCR fragment encoding most of CD2 (from Lys25 to the C terminus) and then transferred into pUAST.

**Embryo staining and in situ hybridisation**

Standard protocols were used for immunocytochemical staining. Antibodies used were rabbit anti-b-galactosidase (Sigma), mouse anti-Engrailed (4D9) and mouse anti-wingless (4D4) (both from the Developmental Studies Hybridoma Bank), and goat anti-HRP (Sigma). In situ hybridisation was performed as described by Jowett (Jowett, 1997), except that fixed embryos were kept at 100% methanol and no protease K treatment took place. The probe was made from a *hedgehog* cDNA obtained from M. van den Heuvel (Oxford, UK).

**Scanning and transmission electron microscopy**

Visualisation of HRP as well as post-fixation and embedding for TEM was performed as described by Dubois et al. (Dubois et al., 2001) except for the following modifications. The vitelline membrane was permeabilised before fixation by incubating embryos in n-Octane for 3 minutes. Embryos were then washed in 0.1 M sodium cacodylate buffer and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 20 minutes. After fixation embryos were washed in 0.1 M sodium cacodylate (pH 7.2) buffer and then devitellinised by hand in PBS. For SEM, embryos were fixed and processed in the same way as for TEM and then post-fixed in 1% osmium tetroxide in a 0.1 M sodium cacodylate (pH 7.2) buffer. Dehydration was through a graded ethanol series. After dehydration embryos were critical point dried from carbon dioxide and sputter coated with 10 nm gold and viewed in a Jeol 35CF SEM.

**Results**

**Morphogenesis of segmental grooves**

Segmental boundary formation is initiated shortly after germ-band retraction has begun. They are recognisable as periodic indentations in the epidermis that separate cells expressing *engrailed* at the anterior from those expressing *rhomboid* at the posterior (Fig. 1A). To understand the mechanisms involved in boundary formation, we examined the changes in cell morphology before and during boundary formation by transmission electron microscopy (TEM). To allow identification of cells in electron micrographs, we devised a transgenic membrane marker based on horseradish peroxidase (HRP), which catalyses the production of an electron-dense product from diaminobenzidine (DAB). HRP was fused to the transmembrane protein CD2 so that the marker would outline cells and thus reveal cell shapes (Fig. 1B). This inert fusion protein was expressed under the control of *engrailed-Gal4*, so that the membrane of *engrailed*-expressing cells appears dark under the electron microscope.

Cell shape changes during groove formation were studied in horizontal sections through the ventral aspect of the embryo at the level of parasegment 9 (the boundary between abdominal segments 3 and 4). Groove formation begins shortly after initiation of germ band retraction as a slight splaying between HRP-positive and HRP-negative cells (arrow in Fig. 1C). As this slit matures into the boundary, we refer to the cells on either side as ‘groove founder cells’. The groove founder cells further lose contact apically, and a groove forms between them (Fig. 1D). Subsequently, in any one section, the cell at the anterior of the incipient boundary (the one expressing *engrailed*) appears to constrict its apical surface. At the same time, it moves towards the interior of the embryo (Fig. 1E), seemingly pulling neighbouring cells along. As boundary formation proceeds, this cell becomes positioned at the bottom of the groove and begins to adopt a bottle shape (Fig. 1F). The cells neighbouring the groove founder cells follow this inward movement, and also display partial apical constriction. The groove continues to deepen (Fig. 1G), until the bottle cell, which is still HRP positive, ends up three to four cell diameters below the surface of the embryo (Fig. 1H). This cell remains at the bottom of the groove with its apex constricted (arrow Fig. 1I) until late stage 13, coinciding with the onset of dorsal closure. After this stage, in the ventral region, the groove progresses (Fig. 1J) until stage 15, when it has practically disappeared (Fig. 1K). At lateral positions, a similar sequence of events is seen, but with two quantitative differences. Lateral grooves dig deeper into the embryo and regress later than ventral ones (compare Fig. 1L with 1M). In conclusion, groove formation involves specific changes in cell contact between the groove founder cells, apical constriction of the most posterior *engrailed*-expressing cells, and inward migration of cells surrounding the groove.
As indicated above, the most posterior engrailed-expressing cells display a distinctive behaviour during groove formation. So far we have not been able to track the fate of this cell as the grooves disappear. However, we have obtained evidence that it ceases to express Engrailed around the time when grooves are deepest. Embryos expressing HRP-CD2 under the control of engrailed-Gal4 were stained for HRP (green) and Engrailed protein (red) (Fig. 2). As the groove grows deeper, Engrailed and HRP are co-expressed (Fig. 2A, B) as expected. However, at later stages, Engrailed protein is no longer detectable in the bottle cell, whereas HRP membrane stain remains, presumably because HRP is relatively stable (white arrow in Fig. 2C). Thus, during groove formation the most posterior engrailed-expressing cell changes morphology dramatically and, upon completion of this process, stops expressing the Engrailed protein.

We note here that groove formation coincides with germ band retraction as if segments were being compressed, much like an accordion. The first segments to undergo such apparent compression are the most anterior ones and this is where...
grooves are deepest (compare Fig. 1M,N). Another noteworthy temporal correlation is between the disappearance of grooves and dorsal closure, a process whereby the epidermis spreads dorsally to enclose the whole embryo. Thus, it could be that the need for additional surface area during dorsal closure promotes groove regression. To investigate this further, we looked at zipper mutants, which are defective in dorsal closure, albeit with a variable penetrance (Cote et al., 1987). In those zipper mutants that completely fail to undergo dorsal closure, grooves persist longer. For example, ventral grooves can be seen well into stage 15 (staging based on anterior morphology and time of egg laying) (black arrows in Fig. 3C,D), a stage when the ventral surface of wild-type siblings is relatively smooth (Fig. 3A and black arrow in Fig. 3B). Moreover, at lateral positions, grooves appear to be deeper in zipper mutants (white arrow in Fig. 3D) than in wild type (white arrow in Fig. 3B).

**Segment boundary formation requires Hedgehog signalling**

There is circumstantial evidence that both Engrailed and Hedgehog could be involved in segment boundary formation. Boundaries fail to form in engrailed and hedgehog mutant embryos. Moreover, as described in the Introduction, both Engrailed and Hedgehog are implicated in maintenance of the compartment boundary in wing imaginal disks (Rodriguez and Basler, 1997; Blair and Ralston, 1997). Because Engrailed activates hedgehog expression and hedgehog signalling activates wingless expression, which is itself needed for continued engrailed expression, expression of hedgehog and engrailed are interdependent during embryogenesis (di Nardo et al., 1988; Martinez-Arias et al., 1988; Lee et al., 1992), thus complicating the genetic analysis. To investigate the specific contribution of each gene on boundary formation, we devised genetic combinations that allowed expression of one without the other. To maintain continued engrailed expression in a hedgehog null mutant, an activated form of Armadillo (Arm*, armS10) (Pai et al., 1997) was expressed under the control of engrailed-Gal4, thus artificially maintaining wingless signalling in the engrailed domain and rendering engrailed expression independent of Wingless. No segmental groove form in such embryos (Fig. 4C). The surface of the epidermis appears smooth at the time when deep grooves can be seen in wild type siblings (Fig. 4A). As expected, engrailed expression is sustained in these embryos, however segmental organisation is disrupted (Fig. 4D). Engrailed-positive cells are no longer confined to sharply delineated stripes as in the wild type (Fig. 4B), but are randomly positioned in small clumps of cells throughout the epidermis. We conclude that Hedgehog signalling is required for segment boundary formation and also for maintenance of segmental organisation.

Canonical signalling by Hedgehog is mediated by the transcription factor encoded by ci (Aza-Blanc et al., 1997; Methot and Basler, 2001). In the absence of Hedgehog, full-length Ci is constitutively processed to a repressor form, Ci[75]. In the presence of Hedgehog, Ci[75] is no longer produced and full-length Ci[155] can activate target genes. To test whether the role of Hedgehog signalling in boundary formation requires ci, as is the case in the wing disk, we looked
at groove formation in ci mutant embryos. As above, engrailed expression was artificially maintained (with engrailed-Gal4 UAS-arm*). Two alleles of ci were used: ci94, which lacks all Ci protein (i.e. both the repressor and the activator forms) and ciCell, which encodes only Ci[75], the repressor form (Methot and Basler, 2001). The result differs for the two alleles. In ci94, segmental grooves and segmental organisation appear normal (Fig. 4E,F) as in the wild type (Fig. 4A,B). By contrast, in ciCell, grooves are lacking (Fig. 4G) and the domain of engrailed expression (artificially maintained) is disorganised (Fig. 4H) much as in a hedgehog mutant. This suggests that a target of Ci is required for boundary formation and that, in the absence of signalling, expression of this target is repressed by Ci[75].

**Wingless signalling inhibits segmental boundary formation**

Hedgehog signals to cells located both at the posterior and the anterior of the engrailed-expressing compartment. Yet, segment boundaries only form at the posterior. What could be the reason for this asymmetry? One obvious possibility is that Wingless, which is active at the anterior of each engrailed stripe, could prevent boundary formation there. Indeed, such a regulatory mechanism ensures that rhomboid is only expressed at the posterior of each stripe of hedgehog expression – rhomboid expression is activated by Hedgehog signalling and repressed by Wingless signalling (Alexandre et al., 1999). To assess the role of Wingless signalling on segmental grooves, we looked at wingless mutants in which engrailed (and hedgehog) expression was artificially sustained with the engrailed-Gal4 UAS Arm* system. In the ventral region, engrailed expression is maintained in defined stripes (Fig. 5A) and grooves form on both sides (Fig. 5B) suggesting that, indeed, Wingless signalling normally prevents Hedgehog from activating groove formation at the anterior. More laterally, the segmental organisation is disrupted and engrailed-expressing
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Fig. 5. Wingless signalling inhibits segmental boundary formation. All embryos are at stage 13+ and stained by immunocytochemistry with anti-Engrailed (black). (A-D) Removal of Wingless (while maintaining engrailed expression) leads to duplication of segment boundaries. An ‘en face’ view of the ventral area (A) shows that engrailed stripes are sharply delineated on both sides. In a side view of the ventral region (B), one can see grooves on both sides of engrailed stripe (e.g. black arrows). In the lateral region, an ‘en face’ view (C) shows that Engrailed stripes are broken up into clumps. (D) Grooves are generated around the islands of engrailed-positive cells as seen in a side view. (E-G) In a double mutant (wingless–hedgehog), no groove forms. (E) Engrailed stripes are disrupted throughout (en face view of the ventral region as in A). (F) Ventral grooves are no longer generated, as seen in a side view as in B. (G) Likewise no groove can be recognised laterally in a side view similar to that in D. (H,I) Schematic drawings summarising the results shown in A-G.

Furthermore the stripes of engrailed expression are disrupted ventrally (Fig. 5E) as well as laterally.

Role of Engrailed in groove formation

So far our results demonstrate the requirement of hedgehog in segment boundary formation but they do not exclude the possibility that engrailed might also be required. By analogy with the experiments above, where engrailed expression was artificially maintained in a hedgehog mutant, we added back hedgehog expression in an engrailed mutant to specifically test the requirement of Engrailed. To drive hedgehog expression, we used paired-Gal4, a driver whose posterior limit of expression correlates roughly with the position of wild-type segment boundaries (wingless–engrailed–paired-Gal4 UAS-hedgehog). As shown in Fig. 6B, exogenous expression of Hedgehog does not rescue segmental grooves in the absence of engrailed, function, and such embryos exhibit a flat surface. As positive control, we asked whether grooves are rescued by adding exogenous engrailed (thereby also inducing hedgehog expression) using the same driver in the otherwise same genetic background (wingless–engrailed–paired-Gal4 UAS-engrailed) and indeed they are (Fig. 6A).

Thus co-expression of hedgehog and engrailed is required for grooves to form.

Engrailed could contribute to segment boundary formation
by regulating the expression of one or several effector genes. A minimalist view is that the only relevant target of Engrailed in this respect is Ci. Engrailed is known to repress ci expression (Eaton and Kornberg, 1990) and this ensures that no Hedgehog signalling takes place where engrailed is expressed. Conceivably, the juxtaposition of cells undergoing Hedgehog signalling (HH ON) with cells that are unable to activate the pathway (HH OFF) could be sufficient to cause segment boundary formation. However, artificial activation of Hedgehog signalling in the Engrailed domain, using engrailed-Gal4 and buttonhead expression (Eaton and Kornberg, 1990) and this ensures that no Hedgehog requirement of EGFR signalling, although further analysis of EGFR mutants is needed before a definite conclusion can be reached.

Continuous requirement of Engrailed and Hedgehog in groove maintenance

We noticed that, on the ventral surface of the embryos described above (wingless− engrailed− paired-Gal4 UAS-engrailed; Fig. 6A), groove formation is initiated normally and maintained until stage 12 (Fig. 7A). Such grooves then disappear prematurely, before stage 13 (Fig. 7B). At lateral positions, in the same embryos, boundaries are maintained until at least stage 14 (Fig. 6A). The reason for this spatial difference could be due to the expression of paired-Gal4, which starts to decay around late stage 12 ventrally (Fig. 7C) while laterally, it is maintained until at least stage 14. Thus, the presence of grooves in this genetic background (wingless− engrailed− paired-Gal4 UAS-engrailed), correlates temporally and spatially with the expression of engrailed and hedgehog. This suggests that these two genes could be continuously required throughout the lifetime of the groove. To test this possibility, we performed an experiment analogous to that above, but with buttonhead-Gal4, which is expressed in the ventral epidermis beyond stage 14 (engrailed− buttonhead-Gal4 UAS-engrailed). Ventral grooves are concomitantly detectable until stage 14 in such embryos (Fig. 7D). This confirms the suggestion that continuous expression of engrailed and hedgehog is required for groove maintenance.

Neither Stripe nor the EGFR pathway appears to be required for boundary formation

Our results suggest that segment boundary formation requires the activation of specific genes in cells on both sides of the boundary. One important challenge for the future is to identify such target genes. No obvious relevant targets of Engrailed have been reported so far. However, there are candidate targets of Hedgehog signalling that could be involved in boundary formation. In particular, expression of both rhomboid and stripe are activated by Hedgehog signalling and repressed by Wingless signalling (Alexandre et al., 1999; Piepenburg et al., 2000), as expected from a ‘boundary-forming gene’. However stripe null mutants exhibit normal grooves (Fig. 8B) when compared with wild-type embryos (Fig. 8A), although the spacing of engrailed stripes is a little irregular. Likewise, rhomboid mutants also make segmental grooves (Fig. 8C). As Rhomboid is limiting for the activation of Spitz, which itself activates the EGFR (Guichard et al., 1999; Lee et al., 2001), we also looked at spitz mutants. They too form normal grooves (Fig. 8D).
Discussion
In this paper we have characterised the boundary that delineates individual segments during Drosophila embryogenesis. We described the morphological changes that accompany groove formation and identified two key genetic requirements for this process. These are the presence of Engrailed at the anterior of the boundary and the activation of Hedgehog signaling at the posterior. In the absence of either, grooves do not form and, in addition, the segmental organization of the germ band is disrupted.

Why boundaries and grooves?
The primary function of boundaries must be to ensure that distinct populations of cells can be patterned separately during development. This is evident from the classic clonal analysis of Drosophila appendages. Because segment boundaries form after most embryonic mitoses have occurred, clonal analysis is of limited use to demonstrate the separation of cells between different segments in the embryo. Nevertheless, in the absence of visible boundary grooves i.e. in the absence of Hedgehog, engrailed-expressing cells are no longer confined to well-demarcated stripes suggesting that segment boundaries are needed to maintain the segmental organization of the epidermis. Therefore, segment boundaries, like compartment boundaries in imaginal discs keep distinct cell populations separate. However, unlike the compartment boundary in disks, segment boundaries are associated with a groove, which could be functionally significant. For example, it is conceivable that grooves contribute to muscle attachment by bringing the appropriate epidermal cells (epidermal muscle attachment) EMA cells (Becker et al., 1997; Frommer et al., 1996) in close proximity to the mesoderm, thus helping muscle recognise its epidermal target.

Groove morphogenesis
Our morphological analysis reveals that groove formation involves apical constriction within the most posterior engrailed-expressing cells and the eventual acquisition of a bottle cell morphology (Fig. 9). Such changes in cell shape are encountered during many morphogenetic events. For example, invagination of the Drosophila mesoderm is characterised by apical constriction (Kam et al., 1991; Leptin and Roth, 1994; Oda and Tsukita, 2001). Likewise, a large reduction of the apical surface of eye imaginal disks cells is seen in the morphogenetic furrow (Wolff and Ready, 1991). In sea urchins, bottle cells have been shown to be required for invagination of the ectoderm (Kimberly and Hardin, 1998). In vertebrates, classic examples include the formation of the neural tube in...
chick (Schoenwolf and Franks, 1984), and of the blastopore lip in amphibians (Hardin and Keller, 1988). Thus, local changes in cell shape may be an important component of the mechanics of groove formation, although in the case of segmental grooves, specific ablation would be required to demonstrate the importance of the bottle cells.

Segmental grooves, when they are deepest, include three or four cells on either side of the bottle cells. It is therefore conceivable that additional forces contribute to groove formation. One possibility is that muscles could pull epithelial cells towards the interior of the embryo. However, grooves still form in stripe mutants, which lack muscle attachment sites (Becker et al., 1997; Frommer et al., 1996). We can therefore exclude a role of muscles in groove formation. Although local changes occur at incipient segment boundaries, a large-scale epithelial rearrangement called germ band shortening takes place and could contribute to groove formation. For example, compression of the germ band by the amnioserosa could conceivably lead to buckling of the epithelium at weak points. Indeed, it has been proposed that convergence of cells toward the vegetal pole in sea urchin embryos creates compression that causes the vegetal plate to buckle (Ettensohn, 1985). To assess the role of germ band shortening in groove formation, we looked at hind sight mutants, which are deficient in germ band retraction (Yip et al., 1997). We found that such embryos do form grooves (data not shown). However, as some degree of germ band shortening still occurs in these mutants, it could be that modest compression of the germ band is sufficient to cause groove formation. Alternatively, as suggested by Shock and Perrimon (Schock and Perrimon, 2002), groove formation could facilitate, but not be absolutely required for, germ-band retraction. A definitive assessment of the role of germ band shortening awaits the isolation of mutations that completely prevents it.

Although germ band shortening leads to a reduction of the exposed surface area of the epidermis, dorsal closure has the opposite effect and this is accompanied by groove regression. In this case, evidence for a causal relation is better because, as we found, groove regression does not occur in mutants such as zipper, which are defective in dorsal closure. This suggests that the surface area needed for dorsal closure could be supplied by cells that are buried in segmental grooves at stages 12-13. More importantly, it shows that manipulating the total surface area of the germ band does impact on grooves, indicating that general morphological changes, in addition to local cell shape changes, could be important in groove formation or maintenance.

In conclusion, we found that cells undergo specific morphological changes at incipient boundaries, especially those cells that line the anterior side of the boundary (the most posterior engrailed-expressing cells). At the same time, it may be that global rearrangements within the epithelium also contribute to groove formation.

Genetic requirements for groove formation

A parallel with the compartment boundary in wing imaginal disks

As described in the Introduction, Engrailed has both a cell autonomous and a non-cell autonomous function in the establishment of the compartment boundary in wing imaginal discs. Although the compartment boundary does not trace its embryonic origin to segment boundaries (see Introduction), there is a striking parallel between the two. As we have shown, for segmental grooves to form, Hedgehog signaling is required in cells at the posterior of the boundary, even if engrailed expression is artificially maintained at the anterior side. Conversely, Hedgehog signaling is not sufficient as exogenous expression of hedgehog in the absence of engrailed does not lead to groove formation.

Two-way signaling across the boundary

As described above, it is the cells that line the anterior side of segment boundaries (the most posterior engrailed-expressing cells) that undergo the most distinctive behaviour during groove formation. This behaviour requires Hedgehog signalling, and yet engrailed-expressing cells are not responsive to this signal. Therefore, their morphological changes must be in response to a signal originating from neighbouring non-engrailed expressing cells. This could be achieved through standard paracrine signaling or by contact-dependent signal mediated by cell surface proteins. Whatever the mechanism, Hedgehog-responsive cells influence the behaviour of adjoining engrailed-expressing cells across the boundary, and crosstalk between the two cells takes place. This is reminiscent of the situation at rhombomere boundaries where cross communication between neighbouring rhombomere cells are required for their formation.

The role of ci

Because, as we have shown, boundaries form in the complete absence of Ci (in ci\textsuperscript{94}), we conclude that the activator form of Ci is not required for segment boundary formation. However, no boundary forms in ci\textsuperscript{Cell} mutant embryos indicating that the presence of Ci\textsuperscript{75} (the repressor) prevents boundary formation. We suggest therefore that boundary formation requires the expression of a gene (x) that is repressed by Ci\textsuperscript{75} but does not require Ci\textsuperscript{155} to be activated. Presumably, an activator of x is constitutively present but, in the absence of Hedgehog, it is prevented from activating x expression by Ci\textsuperscript{75}. Hedgehog signalling would remove Ci\textsuperscript{75} and thus allow activation to occur. Two characterized target genes of Hedgehog (wingless and rhomboid) follow the same mode of regulation. For example, expression of wingless in the embryonic epidermis decays in ci\textsuperscript{Cell} but is still present in the complete absence of Ci, in ci\textsuperscript{94} embryos (Methot and Basler, 2001).

Repression of x expression by Wingless signalling

Although Hedgehog signaling is activated both at the anterior and the posterior of its source, segment boundaries only form at the posterior. One reason for this asymmetry is that Wingless signaling represses boundary formation at the anterior. Indeed, in the absence of Wingless, boundaries are duplicated, as long as expression of Engrailed and Hedgehog is artificially maintained. We conclude that expression of x is repressed by Wingless signalling. Two obvious candidates for x are rhomboid and stripe. Both genes are activated by Hedgehog signaling and repressed by Wingless signaling (Sansoulet al., 1999; Alexandre et al., 1999; Piepenburg et al., 2000) and, indeed, both are expressed in cells that line the segment boundary. To determine if either gene could mediate the role of Hedgehog in boundary formation we looked at the
respective mutants. No effect on grooves could be seen. We conclude that neither rhomboid nor stripe is required for boundary formation although we cannot exclude the possibility that these genes could contribute in a redundant fashion. Overall our genetic analysis suggests that additional targets of Hedgehog must be involved in boundary formation. It will be interesting to find out whether any of these targets will turn out to be implicated in compartment boundary maintenance as well.

The cell-autonomous role of engrailed

Although we have emphasised the role of a Hedgehog target gene in boundary formation, it is clear from our analysis that engrailed also has a cell-autonomous role. We have provided evidence that, even though Engrailed represses ci expression, its role in boundary formation is likely to involve the transcriptional regulation of another target gene (see Fig. 6E). One possibility is that Engrailed could be a repressor of x and that boundaries would form at the interface between x-expressing and non-expressing cells. However, we think that instead, or in addition, Engrailed has a Hedgehog-independent effect on cell affinity and that this could contribute to boundary formation. Of note is the observation that engrailed-expressing cells remain together in small groups even when boundaries are lost for lack of hedgehog. This suggests that engrailed-expressing cells have increased affinity for one another. Thus, Engrailed could specify P specific cell adhesion independently of Hedgehog. Clearly, future progress will require the identification of Engrailed target genes that control such preferential affinity and/or contribute to boundary formation.

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