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 (Basler and Struhl, 1994) (reviewed by Lawrence and Struhl, 1996). One classic example of a compartment boundary is the border that divides *Drosophila* imaginal disks into anterior and posterior compartments (also known as the AP boundary). This boundary is established early in embryogenesis at the anterior of each stripe of *engrailed* expression and is maintained throughout the life of the fly (Garcia-Bellido et al., 1973; Vincent and O'Farrell, 1992). The role of Engrailed in compartment boundary maintenance in the wing imaginal disc was recognized nearly 30 years ago (Morata and Lawrence, 1975). It is now established that this role is dual. On the one hand, Engrailed imparts a specific 'affinity' to posterior cells and thus encourages them to sort out from cells in the A compartment (Blair and Ralston, 1997; Dahmann and Basler, 2000). On the other hand, Engrailed activates the expression of Hedgehog, which signals across the boundary and renders receiving cells immiscible with posterior, *engrailed*-expressing cells (Blair and Ralston, 1997; Rodriguez and Basler, 1997). That Hedgehog signalling is indeed required in anterior cells is demonstrated by the behavior of anterior cells that lack either *cubitus interruptus* (*ci*) or *smoothened* (*smo*), two essential components of the Hedgehog signal transduction pathway. Such clones no longer respect the boundary even if *engrailed* is expressed normally on the other side (Blair and Ralston, 1997; Rodriguez and Basler, 1997). Because Ci is the transcription factor that mediates Hedgehog signaling, it appears that the effect of Hedgehog on boundary maintenance is mediated by the transcriptional activation of one or several genes in anterior cells lining the boundary.

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), $Df(2R)en^{E}$ (Tabata et al., 1992), ci^{94} (Methot and Basler, 2001), ci^{Cell} (Slusarski et al., 1995), $stripe^{DG4}$, $rhomboid^{7M43}$ (Jurgens et al., 1984), $zipper^{1}$ and $hindsight^{E8}$. The wg^{cx4} $Df(2R)en^{E}$ 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 *Bcl*I 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-β-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 proteinase 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% gltuteraldehyde 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 devitelinised 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 regresses (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.

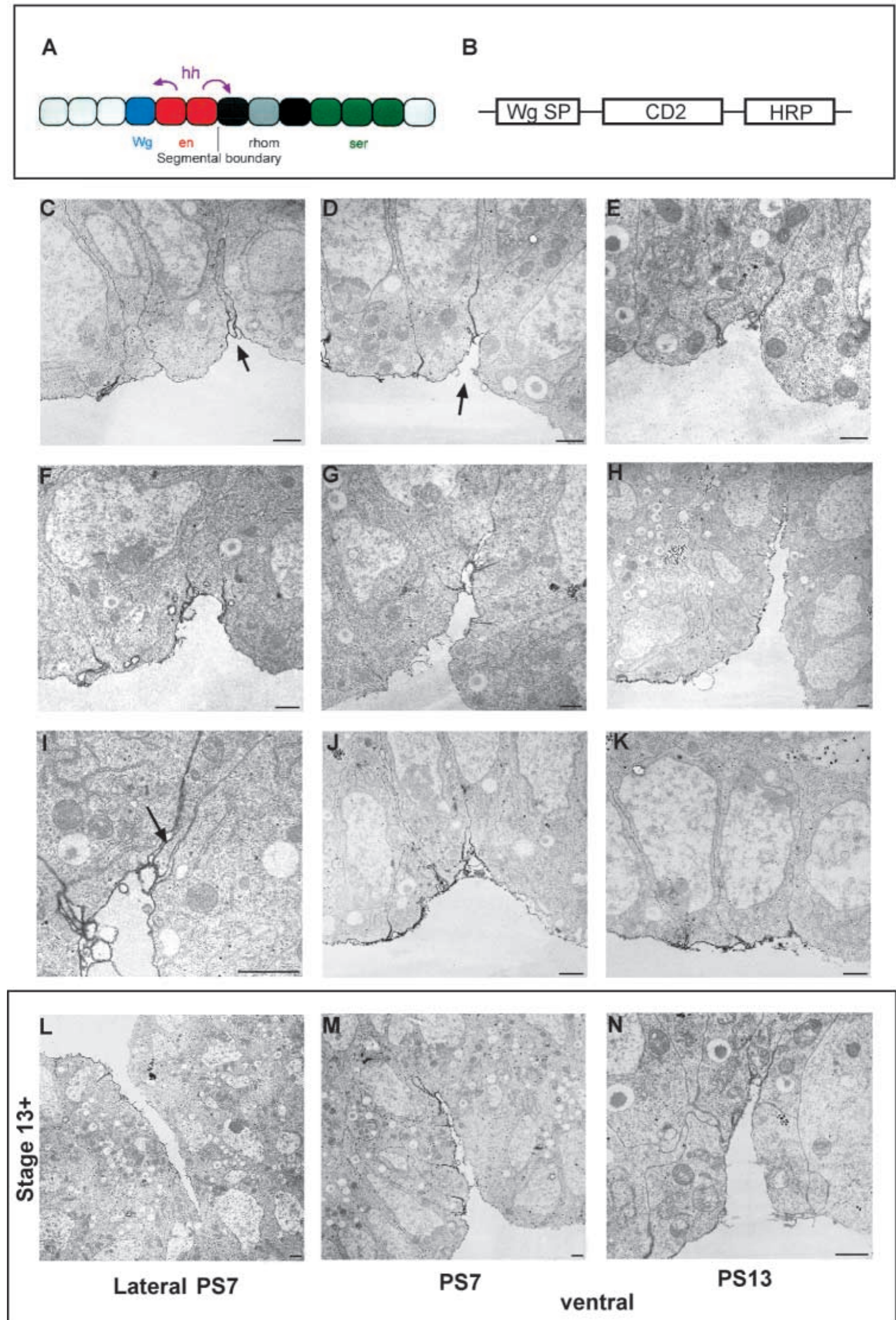

Fig. 1. Morphological changes during segmental boundary formation. (A) Schematic drawing of gene expression patterns in a horizontal section through one segmental unit. The position of the segmental boundary is marked with a vertical bar. (B) Schematic drawing of the fusion protein used to label cell outlines under the EM (under UAS control). It comprises the signal peptide from Wingless, human CD2 (without signal peptide) and HRP. (C-K) TEM images showing the changes in cell morphology as segmental grooves form and regress. Embryos were stained with DAB and sectioned horizontally through the ventral aspect of the embryo. Although some staining appears at the surface of non-expressing cells (maybe as a result of membrane shedding from expressing cells), we were able to confidently identify expressing cells after a bit of practice. An annotated version of this figure highlighting expressing cells is provided at http://dev.biologists.org/supplemental/. (C) Shortly after germ-band retraction is initiated, a small dip (arrow) appears between *engrailed*-expressing and non-expressing cells. (D) Apical contact appears to loosen (arrow). (E) The most posterior *engrailed*-positive cell constricts apically and moves inwards in relation to surrounding cells. (F) This cell finds itself at the bottom of the forming groove and neighbouring cells follow this inward motion. (G) More cells have moved in and the groove is now two to three cell diameters deep. (H) The groove at its deepest reaches at least three cell diameters in depth. (I) At this stage the bottom *engrailed*-expressing cell is bottle shaped and severely constricted apically (arrow). (J) The disappearance of grooves is a very rapid event, which allows the cells to return to their original position. (K) The embryo eventually becomes almost flat ventrally. (L-N) Grooves are much deeper laterally (L) than ventrally (M), and posterior grooves (between abdominal segments 2 and 3; N) are not as deep as anterior ones (between abdominal segments 7 and 8; M). Scale bars: 500 nm.

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

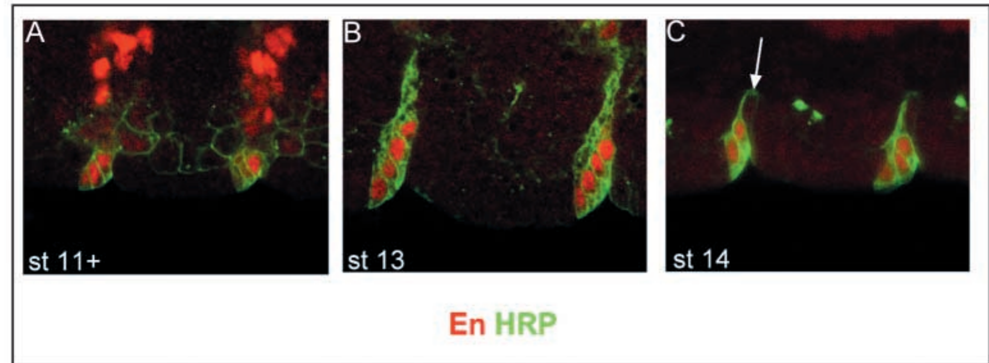

Fig. 2. Loss of *engrailed* expression in the 'bottle cells'. (A-C) Lateral view (focused on the ventral midline) of wild-type embryos stained with anti-Engrailed (red) and anti-HRP (green). (A,B) At stages 12 and 13, Engrailed and HRP immunoreactivity co-localises (although this is not clear at all focal planes). (C) By contrast, at stage 14 the so-called bottle cell downregulates Engrailed expression although it remains labelled with HRP (white arrow). No attempt was made to identify the staining detected inside the embryo, which could be in the mesoderm or the nervous system.

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).

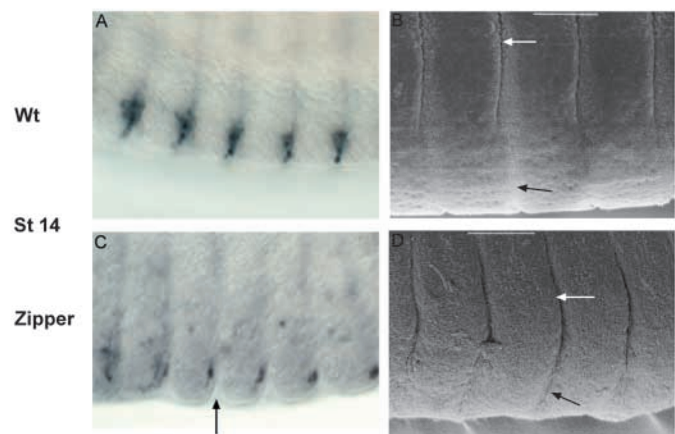

Fig. 3. Persistence of segmental grooves is affected by dorsal closure. (A-D) Wild type and *zipper* mutants, which are defective in dorsal closure, at stage 15 and oriented such that the ventral midline is at the bottom. (A) Wild-type embryo stained with anti-Engrailed (black). (B) Wild-type embryo as seen by scanning electron microscopy (SEM). The ventral epidermis is almost flat (black arrow), whereas shallow grooves are still present laterally (white arrow). (C) Brightfield image of a *zipper* mutant embryo stained with anti-Engrailed (black). Note that grooves persist ventrally (black arrow). (D) Persistent ventral grooves can also be seen by SEM (black arrow). Moreover, lateral grooves appear deeper (white arrow) than in the wild type.

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

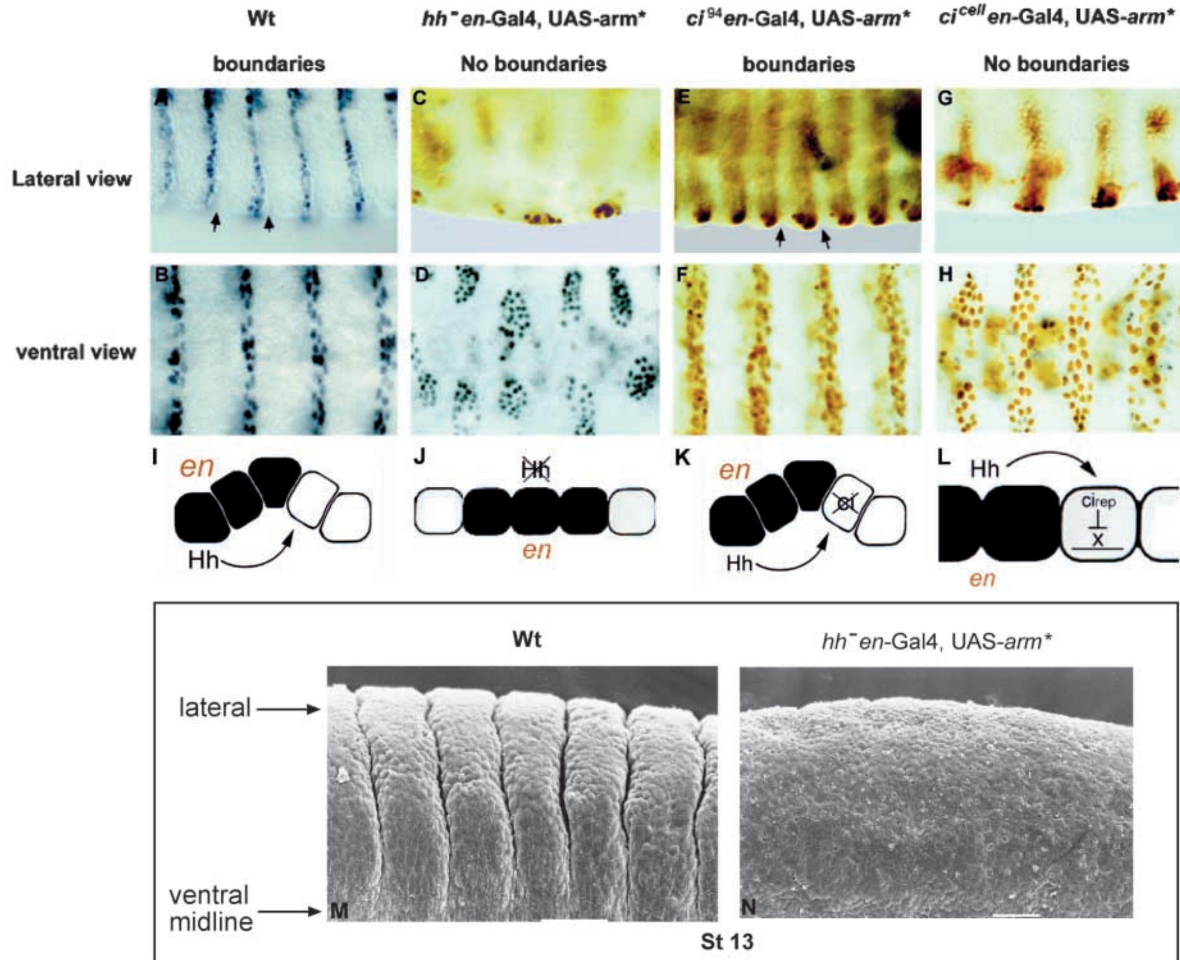

Fig. 4. Boundary formation requires Hedgehog and Ci. (A-H) Stage 13+ embryos stained with anti-Engrailed (black or brown). (A,B) Wild-type embryos. Deep grooves are easily seen (black arrows) in the lateral view in A, while the ventral view shows the normal stripes of Engrailed expression (two to three cell diameters wide). (C,D) Embryos lacking *hedgehog* but continuing to express engrailed (full genotype is shown). No groove form as seen from the lateral view focused on the ventral midline (C) and stripes of *engrailed*-expressing cells are broken up into clumps as seen in the ventral view (D). (E,F) In ci^{94} embryos (with artificially maintained Engrailed), grooves form (black arrows in E) and Engrailed stripes appear normal (F). (G,H) In ci^{Cell} embryos (with artificially maintained Engrailed), grooves do not form (G) and there is moderate disruption of the Engrailed stripes (H). (I-L) Cartoons summarising the results shown in panels above. (M,N) SEM of stage 13 embryos. Compare the wild type in M with a *hedgehog* mutant (with artificially maintained Engrailed) in N.

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: ci^{94}, which lacks all Ci protein (i.e. both the repressor and the activator forms) and ci^{Cell}, which encodes only Ci[75], the repressor form (Methot and Basler, 2001). The result differs for the two alleles. In ci^{94}, segmental grooves and segmental organisation appear normal (Fig. 4E,F) as in the wild type (Fig. 4A,B). By contrast, in ci^{Cell}, 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

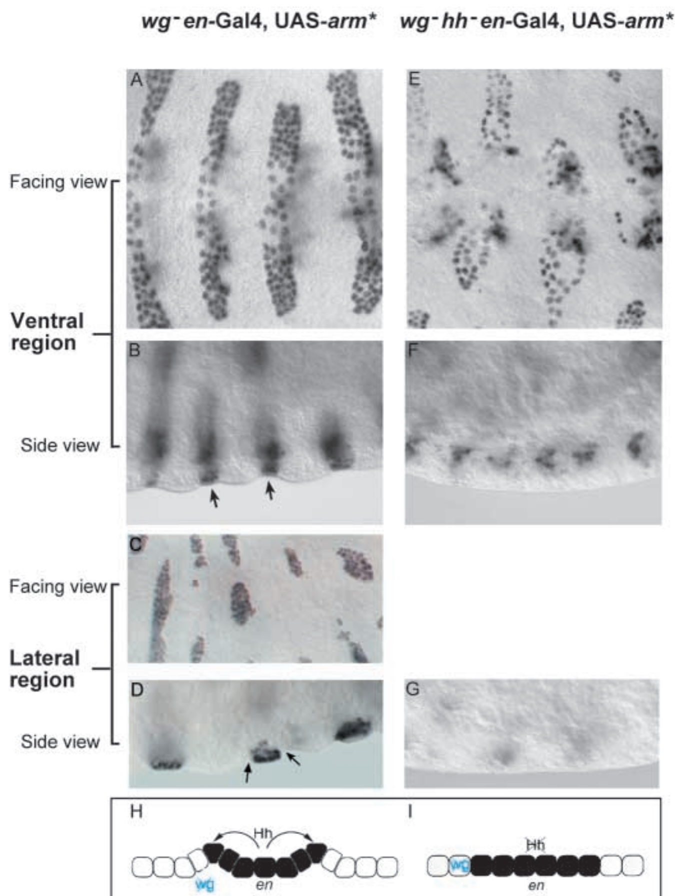

Fig. 5. Wingless signalling inhibits segmental boundary formation. All embryos are at stage 13+ and stained by immunocytochemistry with anti-Engrailed (black). (A-D) Removal of Wingless (while maintaining *engrailed* expression) leads to duplication of segment boundaries. An 'en face' view of the ventral 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.

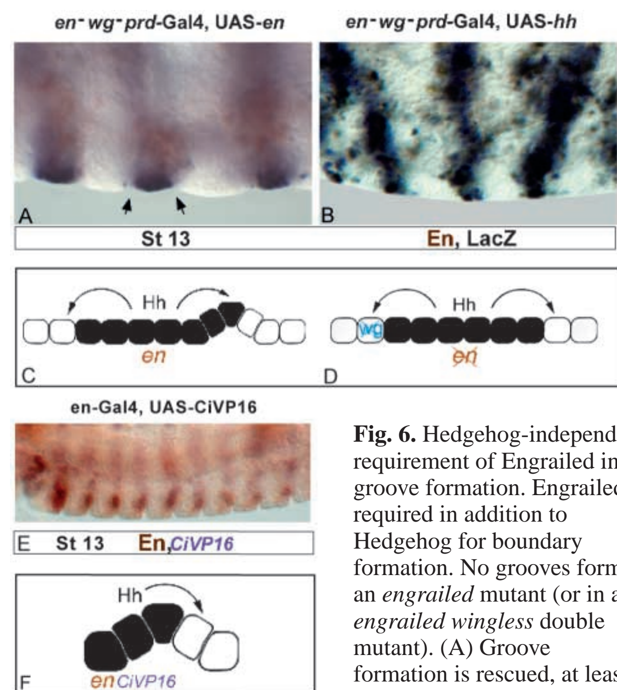

Fig. 6. Hedgehog-independent requirement of Engrailed in groove formation. Engrailed is required in addition to Hedgehog for boundary formation. No grooves form in an *engrailed* mutant (or in an *engrailed wingless* double mutant). (A) Groove formation is rescued, at least in the lateral epidermis (see legend of Fig. 7), by expressing *engrailed* with *paired-gal4*, shown here in the *wingless engrailed* double mutant: grooves form on both sides (arrows) of the expression domain because of the absence of *wingless*. (B) By contrast, no such rescue is seen when Hedgehog is expressed in the same genetic background. (C,D) Diagrams summarising the results in A and B. (E) Stage 13 embryo expressing CiVP16 under the control of *engrailed-Gal4* stained with anti-Engrailed (brown) and a *ci* RNA probe (purple). This embryo is expected to have active Hedgehog signalling on both sides of the presumptive boundaries. Boundary formation is not prevented. This is represented diagrammatically in F.

cells are often found in small groups (Fig. 5C) surrounded by grooves (arrow in Fig. 5D). Disruption of the integrity of *engrailed* stripes at lateral positions could be due to a failure to maintain parasegment boundaries in the absence of Wingless and to a differential requirement for Wingless along the DV axis. Importantly for the purpose of this paper, grooves forms around all *engrailed*-expressing cells whether they are in stripes or loosely arranged in groups. To confirm that these grooves are indeed due to the action of Hedgehog; the same experiment was repeated in the absence of both *wingless* and *hedgehog* (*wingless*⁻ *engrailed-Gal4 UAS-Arm* hedgehog*⁻). In these embryos, grooves are abolished altogether (Fig. 5F,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

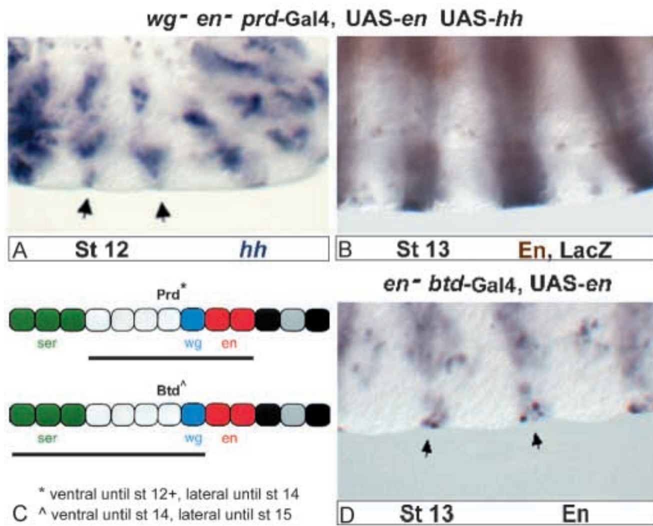

Fig. 7. Continuous requirement of *engrailed* and *hedgehog* in groove formation. When driven by *paired-gal4*, expression of *engrailed* and *hedgehog* rescues segmental grooves only transiently (in an embryo lacking *engrailed* and also *wingless*). (A) Side view of such an embryo at stage 12, focused on the ventral midline. Shallow grooves can be seen (arrows). (B) At stage 13, these grooves are no longer visible. (C) Schematic representation of the domains of *paired* (Prd) and *buttonhead* (Btd) expression. Note that, in the ventral region, expression of *buttonhead* persists longer (up to stage 15) than that of paired (to stage 12+). (D) *buttonhead*-driven *engrailed* expression rescues groove formation (arrows) in an *engrailed* mutant even at stage 13 and beyond. This is not true of *paired-gal4*-driven *engrailed* (not shown).

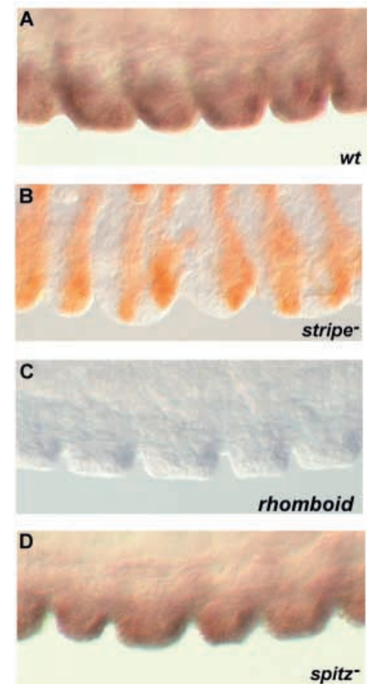

Fig. 8. *stripe*, *rhomboid* and *spitz* are not required for groove formation. Lateral views of stage 14 embryos. (A) Wild type. (B) $stripe^{DG4}$ mutant embryos (here stained with anti-Engrailed) have grooves although they can be irregularly spaced. (C) Normal grooves form in a $rhomboid^{7M43}$ mutant (also stained with anti-Engrailed). (D) $spitz^{1}$ mutant. Again, grooves form although the epidermis can be disorganised

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 *UAS-CiVP16* (which encodes a powerful activated form of Ci; C. A., unpublished) does not prevent boundary formation (Fig. 6E). Thus, activation of Hedgehog signalling on both side of the boundary is compatible with boundary formation.

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).

This provides additional evidence against the possible requirement of EGFR signalling, although further analysis of EGFR mutants is needed before a definite conclusion can be reached.

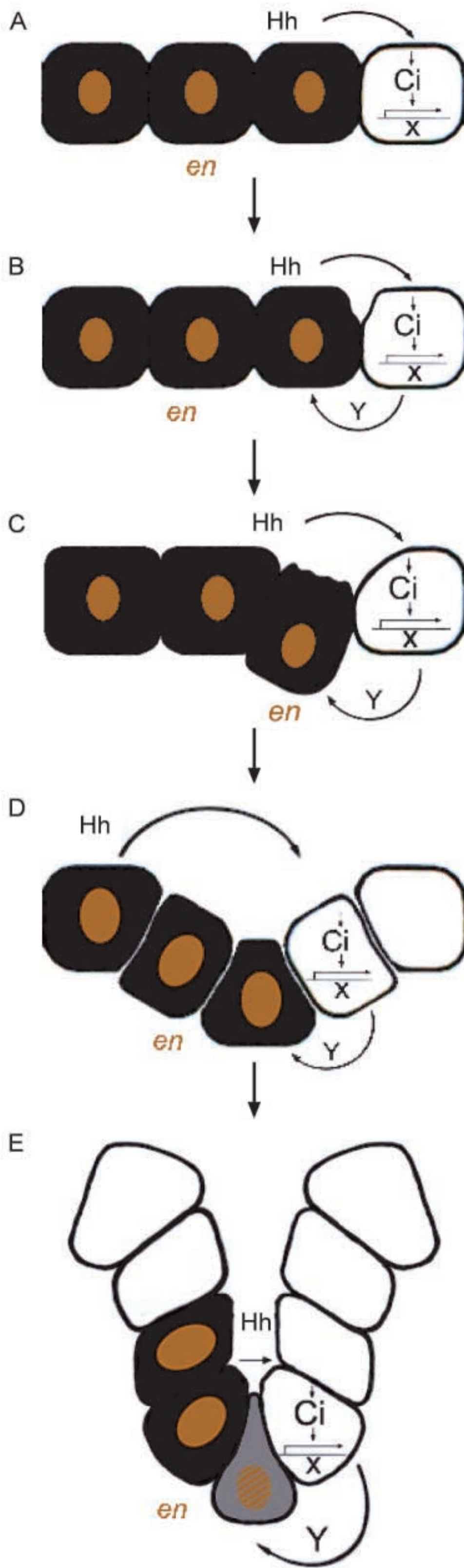

Fig. 9. Morphological changes accompanying segmental groove formation. Schematic representation of the changes in cell morphology and genetic interactions before and during boundary formation. Here, drawings are oriented such that the apical side of the epithelium is upwards, according to convention. (A) Groove formation is initiated by Hedgehog signalling in cells adjoining the most posterior *engrailed*-expressing cells. Signalling by Hedgehog prevents repression by Ci[75], leading to the expression of gene(s) *x*. (B) The groove founder cells loose contact on their apical side and an unknown signal (Y) feeds back on the *engrailed*-expressing cell. (C) The most posterior *engrailed*-expressing cell constricts its apical surface and moves inwards. (D) It comes to lie at the bottom of the forming groove while continuing to constrict its apical surface. (E) As the groove reaches its deepest point, the most posterior *engrailed*-expressing cell acquires a bottle shape. At the same time, it turns off *engrailed* expression.

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 germband shortening in groove formation, we looked at *hindsight* mutants, which are deficient in germband 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 germband 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 germband 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^{94}), we conclude that the activator form of Ci is not required for segment boundary formation. However, no boundary forms in ci^{Cell} mutant embryos indicating that the presence of Ci[75] (the repressor) prevents boundary formation. We suggest therefore that boundary formation requires the expression of a gene (*x*) that is repressed by Ci[75] but does not require Ci[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[75]. Hedgehog signaling would remove Ci[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^{Cell} but is still present in the complete absence of Ci, in ci^{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 (Sanson et 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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