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Hedgehog, but not Odd skipped, induces segmental grooves in the Drosophila epidermis

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The formation of segmental grooves during mid embryogenesis in the Drosophila epidermis depends on the specification of a single row of groove cells posteriorly adjacent to cells that express the Hedgehog signal. However, the mechanism of groove formation and the role of the parasegmental organizer, which consists of adjacent rows of hedgehog- and wingless-expressing cells, are not well understood. We report that although groove cells originate from a population of Odd skipped-expressing cells, this pair-rule transcription factor is not required for their specification. We further find that Hedgehog is sufficient to specify groove fate in cells of different origin as late as stage 10, suggesting that Hedgehog induces groove cell fate rather than maintaining a pre-established state. Wingless activity is continuously required in the posterior part of parasegments to antagonize segmental groove formation. Our data support an instructive role for the Wingless/Hedgehog organizer in cellular patterning.

KEY WORDS: Drosophila, Hedgehog, Wingless, Odd skipped, Patterning, Segmental grooves

INTRODUCTION

Early in development, a cascade of segmentation genes subdivides the Drosophila embryo into parasegmental units along the anterior-posterior axis. Patterning within individual parasegments is controlled by wingless (wg) and hedgehog (hh), which encode secreted signals that emanate from adjacent cell rows flanking the parasegment boundary (Baker, 1987; Lee et al., 1992; Mohler and Vani, 1992). Following their initial activation, Wg and Hh maintain each other’s expression in a positive-feedback loop (DiNardo et al., 1988; Heemskerk et al., 1991; Martinez Arias et al., 1988) and establish organizing centers that control segment polarity. However, the mechanism by which this organizer controls cell behavior and morphology is not well understood.

It has been suggested that Hh induces cell fates posterior to its source in the dorsal epidermis (Heemskerk and DiNardo, 1994). Cells receiving high levels of the Hh signal adopt a smooth cuticle fate and initiate expression of the transcription factor Stripe (Sr), which controls the differentiation of epidermal muscle attachment sites (Frommer et al., 1996). This response is mediated by direct interaction of the transcription factor Cubitus interruptus (Ci) with the sr promoter (Piepenburg et al., 2000). Anterior to the Hh source, sr expression is repressed by the transcription factor Pangolin (Pan), which mediates Wg signaling activity and binds directly to the sr promoter.

hh is also required for the formation of segmental grooves that form posterior to the Hh source in the dorsal and lateral epidermis (Larsen et al., 2003). During stage 12, these cells undergo a series of cell shape changes involving apical constriction and apical-basal elongation that result in segmentally repeated furrows in the epidermis (Larsen et al., 2003; Mulinari et al., 2008).

Recently, the specification of segmental grooves has been used as a model to revise the role of Hh and Wg in epidermal patterning (Vincent et al., 2008). The authors identified the pair-rule gene odd skipped (odd) (Coulter et al., 1990; Coulter and Wieschaus, 1988) as a determinant of groove fate and used the observation that odd expression is initiated normally in hh mutants, but fades prior to groove formation, to suggest that groove fate might be established prior to Hh requirement. Thus, Hh might merely maintain a pre-established cell fate rather than specifying it. The authors further suggested that Wg might not have a direct role in counteracting Hh during groove fate specification.

A prerequisite for this hypothesis is that odd plays a role in groove specification. We show here that odd has no essential role in segmental groove formation. We find that hh, but not odd, is sufficient to induce segmental groove fate in cells of different origin and that Wg signaling is required as late as stage 10 in the posterior part of each parasegment to antagonize Hh activity. Our data reinforce the view that Hh and Wg pattern the dorsal epidermis by inducing cell fates, rather than by stabilizing pre-existing cellular identities.

MATERIALS AND METHODS

Fly strains

UAS-odd (Hao et al., 2003); UAS-hh, UAS-en, UAS-ciP16 and wgcx4 Df(2)enE were gifts of J. P. Vincent (MRC, London, UK). All other strains used are described in FlyBase (www.flybase.org). Experiments were performed at 26°C.

Immunolocalization and microscopy

Embryos were stained and imaged as previously described (Mulinari et al., 2008). Antibodies used were: mouse anti-En, mouse anti-Ena, mouse anti-Crb and mouse anti-Wg (all from DSHB); rabbit anti-RhoGEF2 (Rogers et al., 2004); rabbit anti-Odd (Ward and Skeath, 2000); guinea-pig anti-Sr (Becker et al., 1997); rabbit anti-Bowl (de Celis et al., 1998); and rabbit anti-Hh (Takei et al., 2004).

RESULTS AND DISCUSSION

Requirement for hedgehog and engrailed in groove formation

It has been reported that segmental groove formation requires the activity of engrailed (en) and hh and that en has a function that is independent of its role in hh activation (Larsen et al., 2003). More recently, it has been found that en is not expressed in groove cells (Vincent et al., 2008), thus creating a non-cell-autonomous requirement for en. To address this issue, we reinvestigated the role of hh and en in segmental groove formation.
We found that, as previously reported (Larsen et al., 2003), segmental grooves do not form in hh mutants (Fig. 1B, compare with 1A). When hh was overexpressed, the four to five cell rows posterior to the Hh source constricted apically, elongated their apical–basal axis and took on a shape characteristic of segmental groove cells (Fig. 1C,D). Very similar cell behavior was observed in patched (ptc) mutants (Fig. 1E,G) or when activated Ci, which mediates hh activity (Larsen et al., 2003), was expressed (Fig. 1F). These observations suggest that Hh can organize segmental groove formation. No cell constrictions were observed in the ventral epidermis (Fig. 1C), indicating that a different mechanism might regulate cell shape there.

To address the proposed hh-independent function of en, we investigated en, invected (inv) double mutants in which hh expression was maintained using prd-Gal4. Segmental grooves were rescued in these mutants, suggesting that en is not required for segmental groove formation independent of its role in hh activation (Fig. 1H). By contrast, we found that en represses groove cell behavior when ectopically expressed together with hh (Fig. 1J and see Fig. S1 in the supplementary material). A previous study that reported a requirement of en in groove formation was based on the analysis of en, inv, wg triple mutants, in which hh expression was maintained but did not rescue groove formation (Larsen et al., 2003). We confirm this result (Fig. 1I), but propose that wg may be required in en mutants to allow the morphological differentiation of grooves (see below).

Groove differentiation requires the presence of non-groove cells

Analysis of ptc mutants, or embryos overexpressing hh, reveals that a broad region of cells posterior to the en expression domain are specified as groove cells. However, groove-like invaginations form only at the edges of these regions (Fig. 1C,G). This is even more obvious in double mutants of ptc and the segment polarity gene sloppy paired 1 (slp1), which is required for maintained wg expression. In slp1, ptc mutants, wg expression fades prematurely (Cadigan et al., 1994) and Hh signaling is constitutively active. This results in a substantial expansion of the number of groove cells (see Fig. S2A,B in the supplementary material). However, furrows differentiate only at the edges of groove cell populations. We propose that the morphological differentiation of segmental grooves can only occur at the interface between groove and non-groove cells.

To test this, we turned to wg, ptc double mutants in which Hh signaling is active throughout the epidermis and all cells take on a groove fates (Fig. 1K). Interestingly, these embryos did not differentiate grooves. A similar observation has been reported in en, inv, wg mutants, in which hh expression is sustained (Fig. 1I), and led to the suggestion that en might be required for groove specification (Larsen et al., 2003).

Analysis of cell behavior in wg, ptc mutants showed, however, that cells throughout the tissue constrict their apices but fail to form invaginating furrows (Fig. 1M, compare with 1N). The failure of wg.
ptc mutants and en, inv, wg; UAS-hh embryos to differentiate grooves might be due to the absence of non-groove cells in the epidermis and the concomitant absence of an interface with groove cells (Fig. 1K,L).

**odd skipped is not required for groove cell specification**

The pair-rule gene odd is initially expressed in 4- to 5-cell wide stripes in even-numbered parasegments. At early gastrulation, odd expression expands to segmental periodicity and is subsequently refined to a single row of prospective groove cells located posterior to en. Continued expression of odd in these cells requires hh. In odd mutants, grooves are unaffected in odd-numbered parasegments, but partially missing in even-numbered parasegments (Fig. 2B, compare with 2A), and residual grooves coincide with regions in which odd expression is detectable (Fig. 2C,D) (Vincent et al., 2008). These observations have been interpreted as indicating that groove fate might be specified prior to the requirement of Hh and differentiation of the groove. Thus, the later activity of Hh might not induce, but merely maintain, groove cell identity that has been pre-established in the odd-expressing cell population (Vincent et al., 2008). However, this hypothesis is based on the presumption that odd has a function in groove cell specification and this has not been demonstrated.

Residual grooves in odd mutants have been attributed to the hypomorphic nature of the odd allele; however, the molecular lesion in odd is unknown. We therefore determined the nucleotide sequence of odd and found a substitution that mutates codon 84 from CAG to a TAG stop codon. The resulting truncated peptide, which lacks all four putative zinc fingers encoded by wild-type odd, is no longer restricted to the nucleus but uniformly distributed in the cell (Fig. 2D, compare with 2C; see also Fig. S3A-C in the supplementary material). Thus, odd is likely to be a null allele.

To exclude the possibility that groove formation may be rescued by read-through of the stop codon in odd mutants, or that odd may be required redundantly, we investigated segmental grooves in Df(2L)drmP2 mutants, in which odd and its sister genes drumstick...
(drm) and sister of odd and bowl (sob) are entirely deleted (Green et al., 2002). In these embryos, normal grooves formed in odd-numbered parasegments in the complete absence of odd function (Fig. 2E-G).

We next investigated even-numbered parasegments in which grooves are partially missing (Fig. 2B,E). odd encodes a transcriptional repressor that regulates the expression of other segmentation genes in the early embryo. In odd mutants, derepression of the en activator fushi-tarazu in even-numbered parasegments results in the formation of an ectopic en stripe posterior to the normal stripe (DiNardo and O’Farrell, 1987; Mullen and DiNardo, 1995). Simultaneously, wg expands anteriorly and becomes expressed adjacent to the ectopic en-expressing cells (Fig. 2H,1 and see Fig. S3D-G in the supplementary material). This results in the formation of an ectopic parasegment boundary with reversed polarity (Fig. 2J). Thus, the outward-facing edges of both en stripes are genetically anterior and lined by wg-expressing cells that do not form grooves. The inward-facing edges of the normal and ectopic en stripes fuse in some areas, and these corresponded to areas in which grooves were missing, as cells that were genetically posterior to en and could respond to the Hh signal had been replaced by en-expressing cells. The fusion of normal and ectopic en stripes was more severe in Df(2L)drmΔ2 mutants (Fig. 2H); however, islands of invading groove cells could still be observed (Fig. 2E,G), demonstrating that groove fate is specified in the absence of odd, drm and sob function in all parasegments. We conclude that all cells that are genetically posterior to en are specified as groove cells in the absence of odd function and the partial absence of grooves in even-numbered parasegments in odd mutants is a secondary consequence of the pair-rule phenotype of these embryos (Fig. 2J). The slightly more severe pair-rule phenotype seen in Df(2L)drmΔ2 mutants might be due to a contribution from one of the odd sister genes, most likely sob, to pair-rule function, or could be caused by low-level read-through of the stop codon in the oddΔ allele.

Finally, to investigate whether odd is sufficient to trigger cell shape changes, we expressed a UAS-odd transgene either alone or together with hh in the epidermis. No induction of groove cell behavior other than that triggered by hh was observed (see Fig. S4 in the supplementary material). Together, our data show that odd plays no essential role in groove cell specification and that odd paralogs are unlikely to act redundantly in this process.

**Hedgehog induces segmental groove fate**

The identification of odd as a groove cell marker led Vincent et al. to suggest that groove fate might be specified prior to Hh requirement and that Hh may merely maintain groove fate instead of having an inducing role (Vincent et al., 2008). We demonstrate that grooves are specified in the absence of odd function; however, this could be due to an odd-independent, early-acting mechanism present in the cells from which grooves arise.

In order to address whether groove fate is pre-established in the odd-expressing cell population, we asked if groove fate could be induced in cells of a different origin at a later point in time. We used lines (lin) mutants in which late wg expression is altered, which results in the formation of an ectopic segment boundary at the anterior edge of the en domain in the dorsal epidermis. Importantly, the early expression of pair-rule or segment polarity genes is not affected (Hatini et al., 2000).

In lin mutants, ectopic expression of the groove marker odd was initiated at stage 12 in a single row of groove-forming cells anterior to en that are derived from a previously non-odd-expressing cell population that does not contribute to grooves in the wild type (Fig. 3A,B). Ectopic grooves require hh as they were not induced in hh, lin double mutants (Fig. 3D, compare with 3C), and ectopic odd expression was not induced in this background (not shown). An increase in hh levels in lin mutants resulted in the specification of groove fate in all cells except those expressing en (Fig. 3E). These results suggest that hh is sufficient, late in development, to specify groove cell fate in cell populations of different origins and that earlier-acting factors present in the population of odd-expressing cells posterior to en are not required. Very similar results have been reported by Piepenburg et al., who showed that segment border cells form solely in response to the Hh signal that emanates from the en domain (Piepenburg et al., 2000).

Our findings are consistent with the role of Hh in the regulation of cell shape in other systems. Thus, during Drosophila eye development, Hh has been shown to control cell shape in the morphogenetic furrow, and Hh activation in other tissues is sufficient to induce apical constriction and groove formation (Corrigall et al., 2007; Escudero et al., 2007). It is likely that Hh plays a similar role in tissue morphogenesis in other organisms. During neural tube closure in vertebrates, cells undergo similar shape changes involving apical-basal elongation and apical constriction, which is likely to be in response to Hh sources in the notochord and floor plate. Accordingly, knockout of sonic hedgehog is associated with defects in neural tube closure in mice (Jessell, 2000). These observations suggest that Hh might be a principal inducer of cell shape across species.

**The role of wingless in groove formation**

It has previously been established that wg antagonizes the activity of hh in the specification of segment border cells (Piepenburg et al., 2000). However, it is not clear whether wg has a similar role in segmental groove formation, and a late requirement of wg to antagonize Hh-mediated groove specification has been questioned (Vincent et al., 2008). To investigate a direct role of wg in groove...
support the contention that Wg signaling is required to repress Hh-mediated induction of groove fate after stage 10, thus permitting the formation of segmental grooves posterior, but not anterior, to en in the wild type.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/23/3875/DC1

References

Fig. 4. Wingless antagonizes Hedgehog-induced segmental groove fate at the posterior of each parasegment. (A,B) ptc-Gal4>UAS-panen embryos form ectopic segmental grooves anterior to en. Arrows and arrowheads denote wild-type and ectopic grooves, respectively. (C,D) Co-expression of panen and UAS-hh causes all non-En cells to adopt a groove fate and to form a constricted groove-cell-like morphology (between arrowheads). (E,F) ptc-Gal4>UAS-wg embryos. Odd expression is lost in many cells expressing ectopic wg (arrows). (G-H*) Grooves that form anterior to the ptc domain in ptc-Gal4>UAS-wg embryos (G) are likely to be parasegmental grooves, as they already form by stage 10 (asterisks in H*; H* shows high magnification of H). Stages: 13 (A-D,F); 12 (E,G); 10 (H).


