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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-ci^{VP16}* and *wg^{cx4} Df(2)en^{E}* 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.

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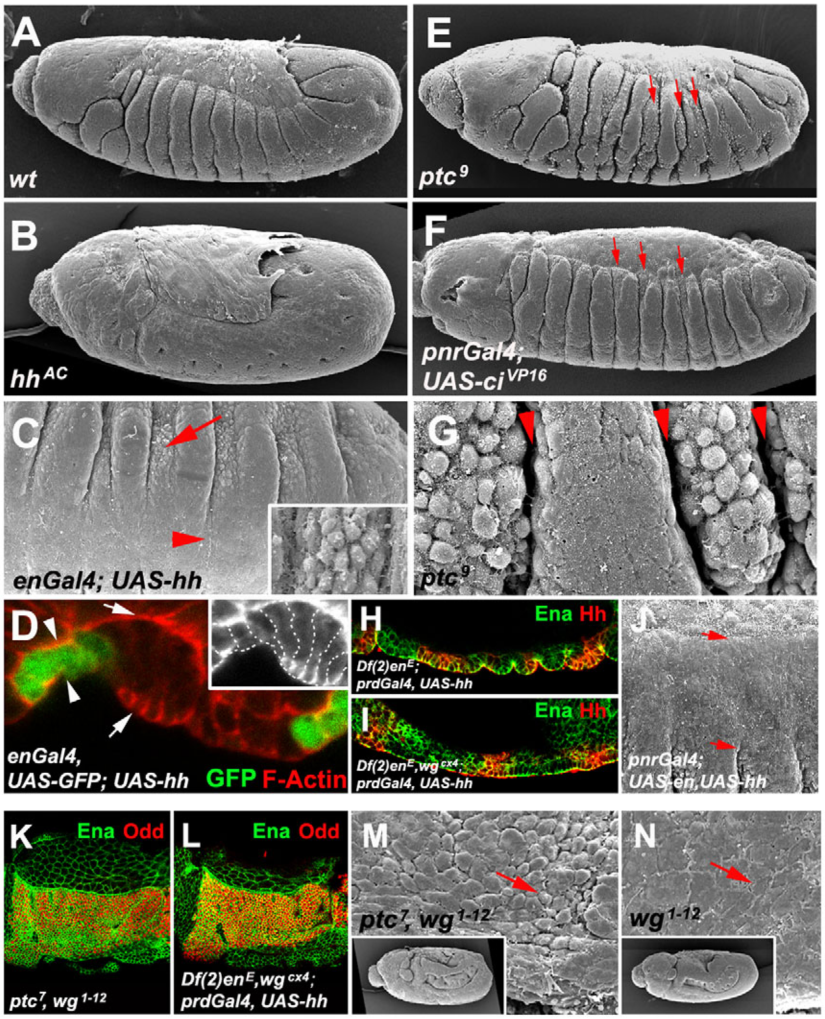

Fig. 1. *hedgehog*, but not *engrailed*, is required for segmental groove formation. (**A**) Segmental grooves in wild-type *Drosophila* embryo. (**B**) Segmental grooves fail to form in *hh* mutants. (**C**) Lateral cells in the anterior part of each segment constrict when *hh* is overexpressed (arrow), but ventral cells do not (arrowhead). Inset is a high-magnification view of the apical constriction. (**D**) Cells posterior to the *en* expression domain (green) elongate their apical-basal axis in response to *hh* expression. The apical and basal ends of cells are indicated in elongating (arrows) and non-elongating (arrowheads) cells. In the inset, cell boundaries are outlined for comparison. (**E**) In *ptc* mutants, several rows of anterior cells in each segment constrict (arrows). See also high magnification in G. (**F**) Dorsal cells at the anterior of each segment constrict (arrows) in response to expression of activated ci^{VP16} using *pnr-Gal4*. (**G**) In *ptc* mutants, grooves form only at the anterior and posterior edges of areas with constricting cells (arrowheads). (**H**,**I**) Expression of *hh* rescues groove formation in *en, inv* (H), but not in *en, inv, wg* (I), mutants. (**J**) *en* represses segmental groove formation cell-autonomously (between arrows). (**K**,**L**) *ptc*, *wg* mutants (K) and *en, inv, wg; prd-Gal4>UAS-hh* embryos (L). Cells throughout the epidermis accumulate the groove markers Odd and Enabled (Ena), but no grooves form. (**M**,**N**) Grooves do not form in *ptc, wg* mutants (M), even though cells throughout the tissue constrict (arrow). Compare with the cell shape in *wg* mutants (arrow in N). Stages shown: 12 (A,B,M,N); 13 (C-L).

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 fate (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,*

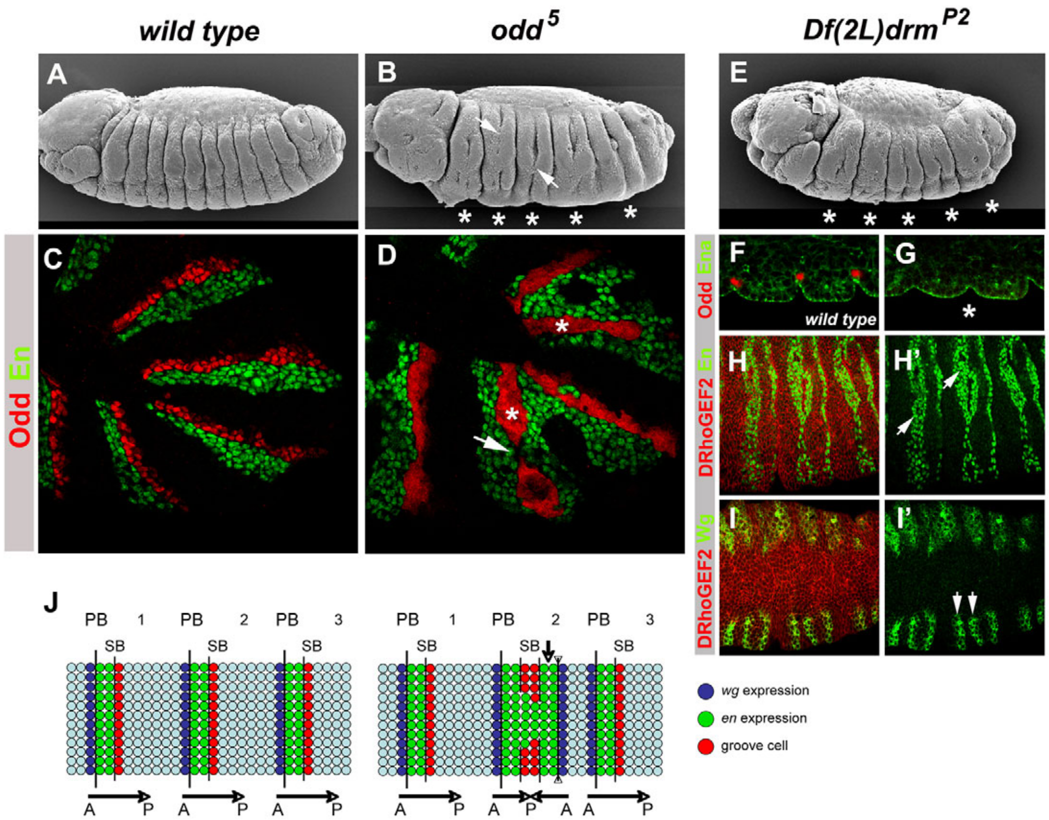

Fig. 2. *odd skipped* is not required for segmental groove formation. (**A**) Segmental grooves in wild-type *Drosophila* embryo. (**B**) In *odd*^{5} mutants, segmental grooves are partially absent (arrows) in parasegments affected by the pair-rule phenotype (asterisks). (**C**) Distribution of En and Odd in the wild type. (**D**) In *odd*^{5} mutants, ectopic En stripes are often partially fused to normal En stripes (arrow). Fused areas correspond to areas in which grooves fail to form. All cells enclosed between En stripes express Odd (asterisks). (**E**) In *Df(2L)drm*^{P2} embryos, the segmental fusions in parasegments affected by the pair-rule phenotype are more severe (asterisks). However, similar to *odd*^{5} mutants, grooves form in areas where the fusion is incomplete. (**F**) In the wild type, groove cells express Odd. (**G**) In *Df(2L)drm*^{P2} embryos, no Odd protein can be detected. Despite this, grooves form in even-numbered (asterisk) and odd-numbered parasegments. (**H**,**H′**) In *Df(2L)drm*^{P2} embryos, ectopic En stripes are often partially fused to normal En stripes (arrows). (**I**,**I′**) Broad En domains are flanked on both sides by Wg expression (arrows). (**J**) Schematic representation of groove patterning defects in *odd* mutants. Left panel, wild type; right panel, *odd* mutant. PB, parasegment boundary. SB, segment boundary. Horizontal arrows denote anterior-posterior polarity. In the even-numbered parasegments of the *odd* mutant, an additional *en* stripe (vertical arrow), which is often partially fused to the normal *en* stripe, forms. Ectopic *wg* expression adjacent to the additional *en* stripe creates an additional parasegment boundary (arrowheads) with reversed polarity. All cells located genetically posterior to *en* are specified as groove cells. Stages shown: 13 (A,B,E-I); 12 (C,D).

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*^{5} mutant embryos, 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*^{5} mutants have been attributed to the hypomorphic nature of the *odd*^{5} allele; however, the molecular lesion in *odd*^{5} is unknown. We therefore determined the nucleotide sequence of *odd*^{5} 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*^{5} 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*^{5} mutants, or that *odd* may be required redundantly, we investigated segmental grooves in *Df(2L)drm*^{P2} 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,I 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)drm*^{P2} mutants (Fig. 2H); however, islands of invaginating 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)drm*^{P2} 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 *odd*^{5} 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).

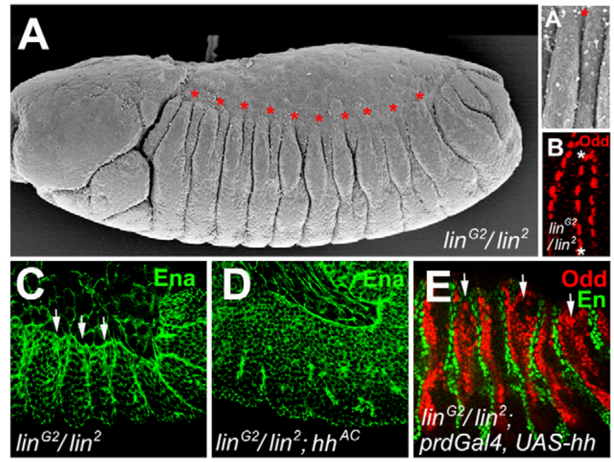

Fig. 3. Hedgehog induces ectopic segmental groove fate in *lines* mutants. (**A**,**A′**) Ectopic segmental grooves form dorsolaterally (asterisks) in *lin* mutant *Drosophila* embryos (high magnification in A′). (**B**) Row of ectopic groove cells expressing Odd (between asterisks). (**C**) Ectopic grooves (arrows) form in *lin* mutants. (**D**) Segmental grooves do not form in *lin, hh* mutants, which were identified by ubiquitous Bowl accumulation (not shown). (**E**) Misexpression of *hh* in *lin* mutants expands the groove cell population marked by Odd to the anterior and posterior of En in many segments (arrows). Stages: 13 (A,B,E); 12 (C,D).

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

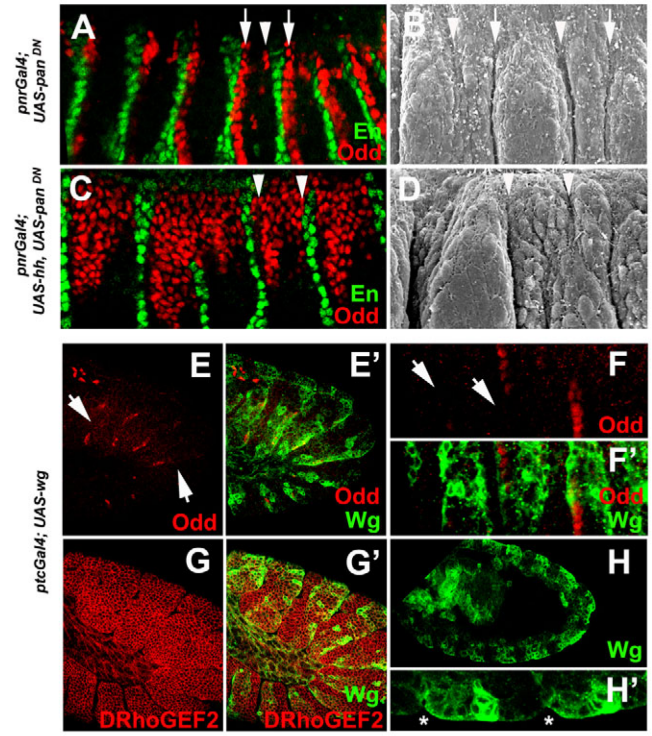

Fig. 4. Wingless antagonizes Hedgehog-induced segmental groove fate at the posterior of each parasegment. (**A**,**B**) *pnr-Gal4>UAS-pan*^{DN} *Drosophila* embryos form ectopic segmental grooves anterior to *en*. Arrows and arrowheads denote wild-type and ectopic grooves, respectively. (**C**,**D**) Co-expression of *pan*^{DN} and *UAS-hh* causes all non-En cells to adopt a groove fate and to assume 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).

specification, we expressed a dominant-negative form of the transcription factor *pan* (*pan*^{DN}), which suppresses Wg signaling. For this, we used *pnr-Gal4*, which initiates expression in the dorsal epidermis at stage 10-11 and thus does not affect early *wg* function. Embryos that express *pan*^{DN} formed a single row of ectopic groove cells anterior to the *en* domain (Fig. 4A,B), confirming our results in *lin* mutants. Strikingly, inactivating Wg signaling and increasing Hh levels at the same time by co-expression of *pan*^{DN} and *hh* resulted in the expansion of groove fate to all cells except those expressing *en* (Fig. 4C,D). These results show that Wg signaling is required after stage 10 to repress groove specification anterior to *en*, thus making the activity of Hh asymmetric. These results also confirm our observations that Hh is sufficient to induce groove fate in cells from different positions along the anterior-posterior axis and suggest that groove fate is not determined before stage 10.

To confirm the ability of *wg* to repress groove fate, we expressed *wg* posterior to *en* in cells that normally take on groove fate. This resulted in the loss of Odd from many cells (Fig. 4E,F), suggesting that *wg* indeed antagonizes *hh* activity. Interestingly, these cells still formed grooves (Fig. 4G). However, these grooves appeared much earlier than segmental grooves (Fig. 4H), suggesting that they are ectopic parasegmental grooves caused by ectopic *wg* expression, as recently suggested (Larsen et al., 2008). Together, our data therefore 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

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