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Juxtaposition between two cell types is necessary for dorsal appendage tube formation

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Abstract

The *Drosophila* egg chamber provides an excellent model for studying the link between patterning and morphogenesis. Late in oogenesis, a portion of the flat follicular epithelium remodels to form two tubes; secretion of eggshell proteins into the tube lumens creates the dorsal appendages. Two distinct cell types contribute to dorsal appendage formation: cells expressing the *rhomboid-lacZ* (*rho-lacZ*) marker form the ventral floor of the tube and cells expressing high levels of the transcription factor Broad form a roof over the *rho-lacZ* cells. In mutants that produce defective dorsal appendages (*K10*, *Ras* and ectopic *decapentaplegic*) both cell types are specified and reorganize to occupy their stereotypical locations within the otherwise defective tubes. Although the *rho-lacZ* and Broad cells rearrange to form a tube in wild type and mutant egg chambers, they never intermingle, suggesting that a boundary exists that prevents mixing between these two cell types. Consistent with this hypothesis, the Broad and *rho-lacZ* cells express different levels of the homophilic adhesion molecule Fasciclin 3. Furthermore, in the anterior of the egg, ectopic *rhomboid* is sufficient to induce both cell types, which reorganize appropriately to form an ectopic tube. We propose that signaling across a boundary separating the *rho-lacZ* and Broad cells choreographs the cell shape-changes and rearrangements necessary to transform an initially flat epithelium into a tube.

Keywords: *Drosophila* oogenesis; Broad; *rhomboid*; Tubulogenesis; Boundary; Dorsal appendage

1. Introduction

Epithelial tubes are central components of many animal organs such as the lungs, kidneys and vascular system. These tubes vary in size and structure and are formed by a variety of mechanisms (reviewed by Lubarsky and Krasnow, 2003). One mechanism for forming a simple tube from an epithelial sheet is wrapping. Wrapping occurs when cells constrict apically (or invaginate) to form a curl in the epithelial sheet. The cells on either side of the apically constricted cells meet, establish new cell–cell contacts with one another, and seal off the tube from the rest of the epithelium (reviewed by Lubarsky and Krasnow, 2003). Examples of tubes formed by a wrapping mechanism include the neural tube in vertebrates (Colas and Schoenwolf, 2001) and the transient tube formed by the ventral furrow during gastrulation in *Drosophila* (Costa et al., 1993). While the morphological changes involved with neurulation and gastrulation are well documented in vertebrates and *Drosophila*, the molecular interactions necessary for these morphological changes are not well understood.

As a model for understanding the molecular and cellular interactions necessary to create tubes from simple epithelia, we are studying dorsal appendage formation during egg development in *D. melanogaster*. The egg chamber consists of an oocyte and 15 nurse cells surrounded by a layer of ~650 (Margolis and Spradling, 1995) somatic follicle cells. The follicle cells synthesize the chorionic eggshell, which protects the embryo during external development. In addition, the follicle cells produce specialized chorionic structures including a pore for sperm entry (micropyle) and two dorsal appendages for respiration. Each dorsal appendage arises from a cluster of epithelial follicle cells originally situated anteriorly and on either side of the dorsal

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midline of the oocyte. Midway through egg chamber development, the cells in each primordium reorganize from a flat epithelial sheet to produce a tube, which serves as a mold for secreted chorion destined to become an appendage (reviewed by Spradling, 1993; Waring, 2000).

The dorsal appendage follicle cells are specified by a combination of spatial information provided by both the anterior/posterior and dorsal/ventral axes. Patterning along the dorsal/ventral axis is generated by a signaling cascade involving the RAS/RAF/MAPK pathway (reviewed by Nilson and Schüpbach, 1999). This cascade is activated in the follicle cells when Gurken (GRK), a TGFα-like ligand, signals from the oocyte to the overlying follicle cells via the Epidermal growth factor receptor homologue (EGFR). A second signaling cascade involving three additional EGFR ligands refines the initial GRK signal, leading to two separate dorsal appendage primordia (Wasserman and Freeman, 1998; Peri et al., 1999). Decapentaplegic (DPP), a BMP2/4 homologue, acts in a concentration-dependent manner to position the dorsal appendages along the anterior/posterior axis (Twombly et al., 1996; Deng and Bownes, 1997; Peri and Roth, 2000).

Two widely used markers for following dorsal appendage formation are Broad (BR) and *rhomboid* (*rho*). The *broad* gene encodes four zinc-finger transcription factors (DiBello et al., 1991); the Z1 isoform is required for proper dorsal appendage formation (Deng and Bownes, 1997; Tzolovsky et al., 1999). Initially, Broad is expressed in all columnar follicle cells. Midway through oogenesis, Broad expression decreases in most cells with the exception of two clusters of cells located on either side of the dorsal midline; these clusters correspond to the roof cells of the dorsal appendage primordia (Deng and Bownes, 1997; Tzolovsky et al., 1999; Dorman et al., 2004). *rhomboid* encodes a serine type peptidase (Urban et al., 2001; Lee et al., 2001) and is required to establish the two dorsal appendage primordia (Ruohola-Baker et al., 1993). Initially, *rhomboid* is expressed in a saddle pattern of dorsal anterior follicle cells. Then, *rhomboid* expression resolves into two open 'hinges' on either side of the dorsal midline (Ruohola-Baker et al., 1993). Double labeling reveals that these *rhomboid* 'hinges' flank the anterior and dorsal margins of each *broad* cluster (Nakamura and Matsuno, 2003). Thus, the dorsal anterior follicle cells initially co-express both *rhomboid* and *broad* transcripts and subsequently resolve this expression into two distinct populations of dorsal appendage-forming cells.

Recently, we employed a GFP-Moesin fusion protein to image dorsal appendage formation in cultured egg chambers. We also examined fixed tissue to associate the observed cell shape-changes and movements with the molecular markers that define the cells' patterning histories. We used antibodies against E-Cadherin and Broad to visualize roof formation and a *rhomboid* promoter-*lacZ* fusion line (*rho-lacZ*) that mimics the later hinge pattern of *rhomboid* to describe the cell shape changes associated with floor formation (Dorman et al., 2004). Here, we compare the events that occur in wild type with those of several mutants that exhibit defects in the patterning process. Surprisingly, both the *rho-lacZ* and Broad cell types are specified correctly and reorganize appropriately to occupy their normal positions within each abnormal dorsal appendage tube. In addition, ectopic *rhomboid* (in the anterior of the egg chamber) is sufficient to induce both the *rho-lacZ* and Broad cell types, which reorganize appropriately to form an ectopic tube. Although the *rho-lacZ* and Broad cells rearrange in both wild type and mutant egg chambers, they never intermingle. We hypothesize that a boundary exists between these two cell types to prevent mixing between the *rho-lacZ* and Broad cells and to coordinate the two cell populations during their reorganization from a flat epithelium into a tube.

2. Results

Dorsal appendage formation is a complex process involving the coordinated behaviors of *rho-lacZ* and Broad cells. The Broad cells constrict apically to form the roof and the *rho-lacZ* cells elongate to form the floor of the tube (Dorman et al., 2004). Here we show that the Broad cells move in a convergent-extension like manner during tube formation. We identify a new marker for floor cells, Fasciclin 3, which facilitates observation of the *rho-lacZ* cells as they reorganize to close off the ventral midline of the tube. We then take advantage of these new tools to analyze tube formation in a variety of dorsoventral and anterior patterning mutants.

2.1. Broad cells rearrange in a consistent manner during dorsal appendage formation

Broad protein is expressed initially in all cells of the follicular epithelium (Deng and Bownes, 1997). Just prior to dorsal appendage tube formation, Broad protein is down-regulated in anterior follicle cells and concomitantly expressed at a high level in two patches corresponding to the roof cells of the two dorsal appendage primordia (from hereon called the 'Broad cells') (Deng and Bownes, 1997; Tzolovsky et al., 1999; Dorman et al., 2004 and Fig. 1A). The number of Broad cells remains constant during dorsal appendage formation. From stage 10B to late stage 13 we found $\sim 53 \pm 4$ Broad cells in each dorsal appendage primordium ($n = 51$; Deng and Bownes, 1997; French et al., 2003; and our methods). Since neither cell division nor cell death occurs in the follicle cells during these stages (King, 1970; King and Vanoucek, 1960; Nezis et al., 2002), it is likely that the Broad pattern observed at stage 13 (Fig. 1F) represents rearrangements between the cells originally specified at stage 10B (Fig. 1A).

During the first stage of tube formation the Broad cells rearrange along the anterior/posterior axis in a highly

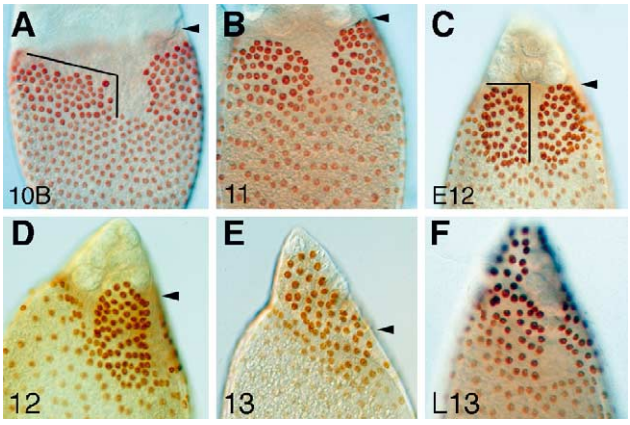

Fig. 1. Broad cells rearrange in a stereotypic manner during dorsal appendage formation. Wild-type egg chambers immunohistochemically stained with anti-Broad antibodies. (A–F) The stage of oogenesis is indicated in the lower left hand corner. In all panels, anterior is up. (A–C) show dorsal views and (D–F) show lateral views. An arrowhead marks the nurse cell/oocyte boundary. S12 NC/oo boundary demarcates the ventral boundary, not the dorsal boundary. (A–F) During stages 10B–13, Broad protein accumulates at a high level in the dorsal appendage primordia and at a lower level in nuclei posterior to the primordia (Tzolovsky et al., 1999). (A–C) The Broad pattern changes from a trapezoid at stage 10B to an oval by stage 12. Brackets illustrate the rows of Broad cells at late stage 10B and early stage 12. (D–F) As the Broad cells move over the nurse cells during stages 12–13, the Broad pattern narrows and lengthens slightly.

consistent manner. Initially, the Broad cells of each primordium are arranged in a trapeziod, with the longest side of the trapezoid parallel to the nurse cell/oocyte boundary. That is, the ~53 Broad cells are present in ~5 rows with ~10 cells/row (Fig. 1A). By early stage 12, the Broad cells reside in an oval-pattern, now elongated along the anterior/posterior axis, forming ~10 rows with ~5 cells/row (Fig. 1C). Thus, prior to moving over the nurse cells the Broad pattern changes from a trapezoid with its longest side parallel to the dorsal/ventral axis, to an oval oriented along the anterior/posterior axis. As the Broad cells move over the nurse cells during stages 12–13 (Figs. 1D,E, 2G,H), the oval pattern narrows and lengthens slightly, suggesting that some minor rearrangements continue among the Broad cells during these stages. Similar cellular rearrangements that shorten and simultaneously lengthen two perpendicular axes occur during convergent extension in vertebrates and during germband extension in *D. melanogaster* (Keller et al., 2000; Irvine and Wieschaus, 1994).

2.2. rho-lacZ *expression defines a unique subset of dorsal appendage follicle cells*

Cells expressing the *rho-lacZ* reporter gene undergo directed elongation to form the floor of the dorsal appendage tube (Dorman et al., 2004). In strains carrying this marker, β-galactosidase localizes to the cytoplasm and as such is an excellent tool to assess the morphology of these cells. Below we review how the *rho-lacZ* cells change shape and rearrange relative to the Broad cells and nurse cells.

Prior to dorsal appendage tube formation the *rho-lacZ* and Broad cells lie in a flat epithelium (Fig. 2A) and the *rho-lacZ* cells lie adjacent to the anterior and dorsal margins of each cluster of Broad-expressing cells (Fig. 2E). During stage 11, the *rho-lacZ* cells slip under the Broad cells, where they remain until the end of oogenesis (Fig. 2B–D,F–H). The *rho-lacZ*-expressing floor cells undergo dramatic cell shape changes during and subsequent to moving under the Broad cells. During stages 11–12 they elongate along their apical/basal axes and narrow their circumferences (Fig. 2F,G). During stage 13 they reverse this process and shorten their apical/basal axes and widen their circumferences (Fig. 2H).

While the *rho-lacZ* cells elongate they also reorganize to seal off the ventral midline of each dorsal appendage tube. Prior to tube formation, the *rho-lacZ* cells are arranged in two perpendicular rows resembling an open hinge (Fig. 2E). As the *rho-lacZ* cells elongate and slip under the Broad cells they reorganize from a hinge to a fan pattern (Fig. 2E–G). This reorganization occurs as the anterior row of *rho-lacZ* cells 'swings' posterior, thereby allowing a pairwise association between the apicolateral membranes of *rho-lacZ* cells originally located in the dorsal and medial sides of the hinge; this fusion results in closing off the ventral portion of the tube. During this process, the *rho-lacZ* cells relinquish contact with their neighbors (the future operculum cells), thereby detaching from the sheet of follicle cells, so that the tube may extend anteriorly over the nurse cells (data not shown).

The Broad cells rearrange at the same time that the *rho-lacZ* cells change shape and reorganize to form the floor of the tube. While the *rho-lacZ* cells convert the hinge into a fan, the Broad cells reorganize from a trapezoid to an oval pattern (Fig. 2F,G). During this tube formation, the *rho-lacZ* and Broad cells maintain epithelial contacts; both cell types continuously express α-spectrin (Fig. 2A–D). Thus, a combination of cell shape-changes and cell rearrangements contribute to sculpting a tube from an initially flat epithelium.

2.3. *The* rho-lacZ *cells express higher levels of FAS3*

Throughout the elaborate shape-changes and movements that drive tube formation, the Broad and *rho-lacZ* cells never mingle. These two cell types may be maintained separately from one another during this process by differential adhesion. While investigating this hypothesis, we found that the *rho-lacZ* cells express higher levels of the homophilic adhesion molecule Fasciclin 3 (Fas3). FAS3 is an integral membrane glycoprotein that functions in synaptic target recognition during embryonic development (Kose et al., 1997). Using an antibody to FAS3 (Patel et al., 1987), we observed that FAS3 accumulates in all of the columnar follicle cells from stage 10 onwards, with a subset

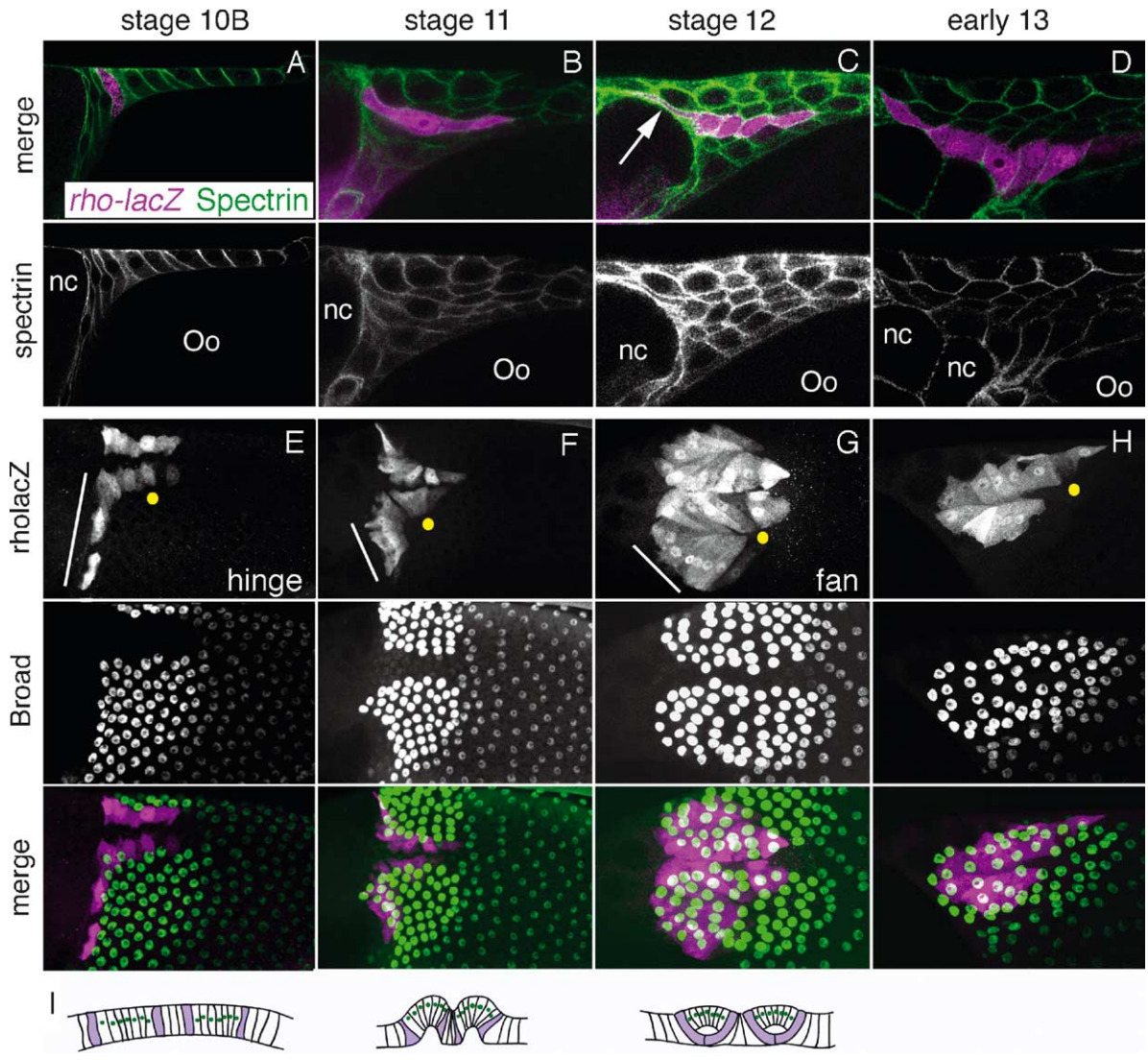

Fig. 2. *rho-lacZ* cells undergo dramatic cell shape-changes during dorsal appendage formation. (A–H) Wild-type egg chambers stained with anti-β-galactosidase antibodies to reveal the cytoplasm of the *rho-lacZ* cells. (A–D) Anti-α-spectrin staining reveals lateral membranes and (E–H) anti-Broad staining defines the roof-forming cells. Stage indicated above each column. (A–D,H) are lateral views and (E–G) are dorsal views, anterior always left. (A–D) Lateral views show the spatial relationship between the *rho-lacZ* cells and other cells in the egg chamber. (A) The *rho-lacZ* cells initially reside in a flat epithelium, with basal up and apical facing down, toward the oocyte. (B–D) They then slip under the Broad cells, where they remain until the end of oogenesis. (C, arrow) The *rho-lacZ* cells follow the contours of the nurse cells as they move over these cells. (E–H) Each dorsal appendage is composed of both *rho-lacZ* and high-Broad cells. Yellow dots indicate apices of *rho-lacZ* cells. (E) The *rho-lacZ* cells are initially adjacent to the anterior and dorsal margins of each cluster of Broad cells. (F–G) Then, the *rho-lacZ* cells slip under the Broad cells and form the ventral floor of the tube. During this process the *rho-lacZ* cells elongate along their apical/basal axes; the anterior row of *rho-lacZ* cells 'swings' posteriorly (indicated by white line) allowing the apicolateral membranes of the cells to fuse and seal the ventral midline of each tube (see text). (H) After tube formation, the *rho-lacZ* cells shorten their apical/basal axes and move over the nurse cells. (I) Diagrams below stages 10B–12 show transverse views highlighting essential features of tube formation. Purple rectangles represent the *rho-lacZ* cells and green dots represent nuclei expressing high levels of Broad. Abbreviations: nc, nurse cell; Oo, oocyte.

of dorsal anterior follicle cells expressing higher levels of FAS3 compared to neighboring cells (Fig. 3A). Lateral views show that FAS3 accumulates in the apico-lateral membrane of all follicle cells, consistent with FAS3 association with septate junctions (Fig. 3B and Woods et al., 1997). In addition to this apico-lateral staining, FAS3 accumulation extends basally along the lateral membrane in a subset of dorsal anterior cells (Fig. 3B). Thus, the higher FAS3 levels observed in dorsal views presumably reflect an increase in FAS3 expression as well as a basal expansion of FAS3 accumulation along the lateral membranes.

Double labeling shows that a subset of cells expressing high levels of FAS3 corresponds to the *rho-lacZ* cells (Fig. 3A,B). Interestingly, FAS3 does not accumulate at a high level in the *rho-lacZ* cell membranes adjacent to the Broad cells (Fig. 3A, inset). This asymmetric accumulation of FAS3 is observed in dorsal views (Fig. 3A) as well as lateral views (Fig. 3B), and may indicate a need to restrict

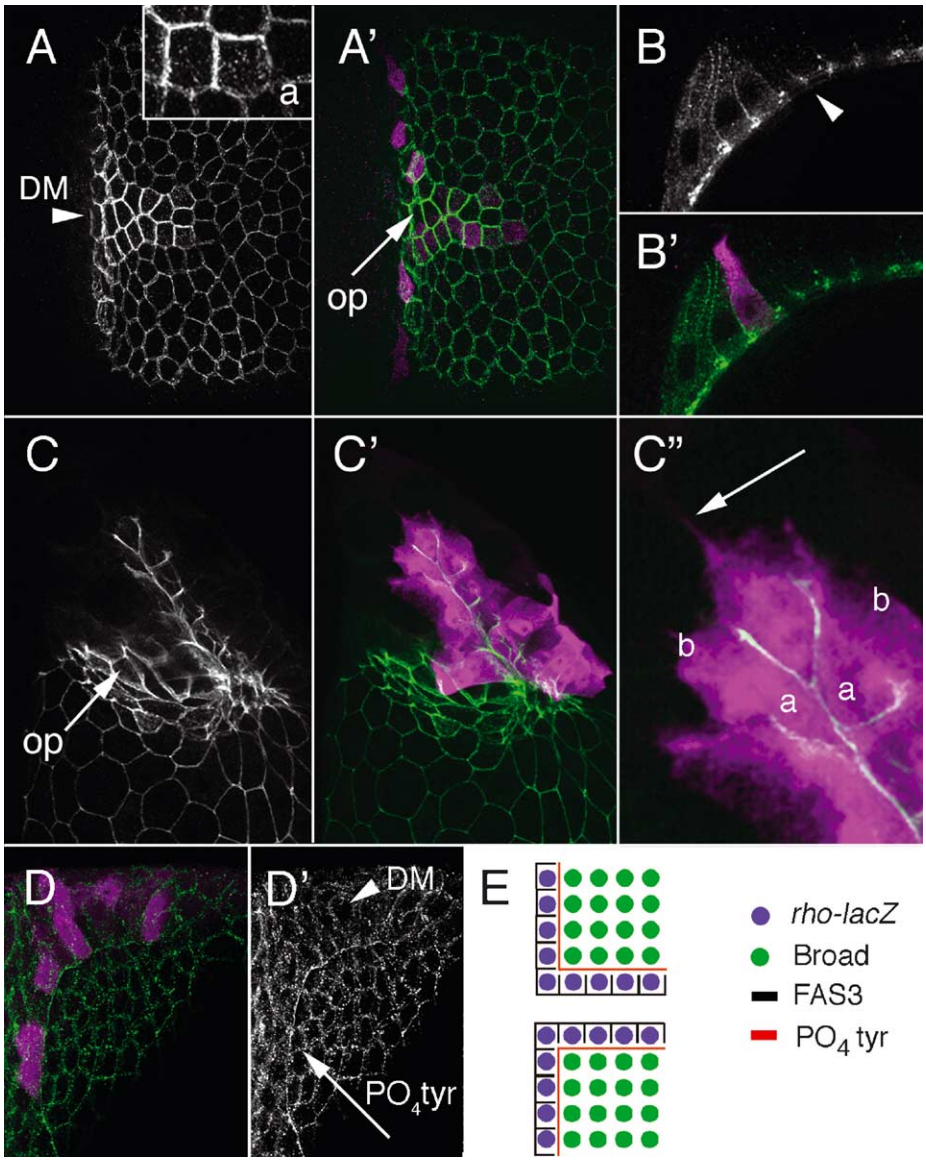

Fig. 3. *rho-lacZ* cells express higher levels of FAS3. Wild-type egg chambers stained with (A–D) anti-β-galactosidase and (A–C) anti-FAS3 or (D) anti-phosphotyrosine antibodies. (A–E) Anterior always to the left, (A,E) dorsal views and (B–D) lateral views. (A,B,D) stage 10B and (C) stage 13 egg chambers. (A–C) FAS3 accumulates in the apicolateral membrane (B, arrowhead) of all follicle cells. A subset of the cells in the dorsal anterior expresses higher levels of FAS3; these cells correspond to the *rho-lacZ* and operculum-forming cells. Note: only a subset of *rho-lacZ* cells express β-gal at this stage, during the period when the marker is turning on. (B) Lateral views show that FAS3 also accumulates along the basolateral membrane of the *rho-lacZ* cells and centripetally migrating cells located anterior to the *rho-lacZ* cells. (C) FAS3 continues to accumulate in the *rho-lacZ* cells as they move anteriorly over the nurse cells. The *rho-lacZ* cells often exhibit protrusions resembling filopodia (C″ arrow). Also, at this stage, the apical FAS3 accumulation marks the ventral midline of the tube. (D) Anti-phosphotyrosine antibodies stain the apicolateral membranes of all the follicle cells over the oocyte. High levels of an epitope recognized by the antibodies accumulate asymmetrically in the membrane(s) between the *rho-lacZ* and Broad cells (D′ white arrow). Since β-galactosidase is just turning on at this stage, the *rho-lacZ* pattern is patchy. (E) Spatial model of dorsal appendage markers at stage 10B. Note that the ventral and posterior limits of the *rho-lacZ* and Broad cells precisely mirror one another. a, apical; b, basal; DM, dorsal midline; op, operculum.

FAS3 function to facilitate the future rearrangements between the roof and floor cells.

FAS3 continues to accumulate at a higher level in the *rho-lacZ* and operculum cells throughout the remainder of oogenesis (Fig. 3C and data not shown). Interestingly, the basal surfaces of the *rho-lacZ* cells often produce protrusions resembling filopodia and lamellopodia, suggesting that the *rho-lacZ* cells are interacting with and migrating along a substrate (Fig. 3C). This substrate appears to be the stretch cells, as the *rho-lacZ* cells contact these cells from stage 11 onwards (see supplementary Figure). Finally, FAS3 activity is not required to prevent mixing between the two dorsal appendage cell types. FAS3 is required, however, to maintain proper tube-lumen size. Homozygous *Fas3* null females lay eggs with normal length dorsal appendages but these appendages have stalks that are wider than normal

(Canton S stalk width = 23.5 ± 4.16 μm; *Fas3*^{null} stalk width = 36.7 ± 4.1 μm). The larger lumens produced by *Fas3* females may result from a subtle increase in the apical surface area of the appendage cells or from sub-cellular defects in apical fusion of floor cells.

In the course of other studies (Stephen M. Jackson and CAB, unpublished results) we observed that a subset of columnar follicle cells stain strongly with anti-phosphorylated tyrosine antibodies (Fig. 3D). Like FAS3, the phosphotyrosyl proteins reside in the apical region of all columnar follicle cells. Similar to FAS3 accumulation, the phosphotyrosyl proteins are asymmetrically restricted within the cell (Fig. 3D). Unlike the high FAS3 distribution pattern, however, double labeling shows that antibodies against phosphorylated tyrosine recognize an epitope within or associated with the membranes between the *rho-lacZ* and Broad cells (Fig. 3E). This high level of phosphotyrosine staining could be due to epitopes located in the *rho-lacZ*, the Broad, or both cell types. Nevertheless, within the dorsal appendage-forming cells, FAS3 and phosphotyrosyl proteins segregate to distinct membrane compartments. These results suggest that differential adhesion between the *rho-lacZ* and Broad cells and signaling across the proposed boundary between these two cell types may facilitate the distinct cellular functions of the roof and floor cells and coordinate their diverse activities.

2.4. rho-lacZ and Broad cells rearrange appropriately in patterning mutants

Tube formation involves the coordinated action of a single row of *rho-lacZ* cells coupled to a defined number of Broad cells. How, then, does morphogenesis proceed when the patterning of these cell types is disrupted? Both Broad and *rhomboid* expression patterns are specified via a combination of signals along the anterior/posterior and dorsal/ventral axes (Ruohola-Baker et al., 1993; Deng and Bownes, 1997; Queenan et al., 1997; Peri and Roth, 2000). By stage 10B, a single overlapping expression domain has resolved into two primordia with distinct sub-populations of ~53 Broad cells flanked anteriorly and dorsally by a single row of *rho-lacZ* cells (Fig. 3E). At this stage the ventral and posterior limits of both the *rho-lacZ* and Broad cells precisely mirror one another, suggesting that specification of these two cells types is tightly coordinated. Here we investigate the link between patterning and morphogenesis in a variety of mutants that produce defective dorsal appendages.

2.4.1. Heteroallelic combination of Ras85D^{05703}/Ras85D^{E62K}

Previous researchers demonstrated that Broad and *rhomboid* expression are dramatically altered in *Egfr* signaling mutants, reflecting a loss of dorsal cell fates. A reduction in EGFR signal results in a single domain of Broad cells, instead of two domains (Deng and Bownes, 1997), and a significant reduction in the *rhomboid* expression pattern (Ruohola-Baker et al., 1993). Presumably, these early expression patterns resolve such that the few remaining *rhomboid*-expressing cells do not express Broad and are located anteriorly to the Broad domain. To examine these patterning processes and the subsequent morphogenesis, we took advantage of a heteroallelic combination of *Ras1* alleles (*Ras85D*^{05703}/*Ras85D*^{E62K}) that results in 100% of the eggs having a single dorsal appendage (Schnorr and Berg, 1996).

Both the Broad and *rho-lacZ* cell types are produced in *rho-lacZ.8.3*/+; *Ras85D*^{05703}/*Ras85D*^{E62K} egg chambers (Fig. 4A,B). One large Broad domain straddles the dorsal midline, rather than two dorsolateral domains as found in wild type. As expected, the *rho-lacZ* cells that normally flank the dorsal side of each Broad domain are absent (Fig. 4A) whereas the *rho-lacZ* cells that normally flank the anterior margin of each Broad domain are present. These cells lie in a *continuous* row immediately anterior and adjacent to the Broad cells. The ventral limits of both the *rho-lacZ* and Broad cells precisely mirror one another as in wild type.

Despite the loss of the dorsal midline in *Ras85D*^{05703}/*Ras85D*^{E62K} egg chambers, the Broad and *rho-lacZ* cells undergo their characteristic cell shape-changes and rearrangements to produce a single dorsal appendage. The Broad cells rearrange to form the dorsal roof of the tube and the *rho-lacZ* cells associate in a pairwise fashion to form the ventral midline of the tube. This result demonstrates that the *rho-lacZ* 'hinge' pattern per se is not essential to produce a tube. It also suggests that some gradient of information must be present to direct the lateral-most Broad and *rho-lacZ* cells toward the dorsal mid-line.

Although the overall events of tube formation resemble wild type, the *rho-lacZ* cells often exhibit abnormal cell shapes and spatial arrangements relative to one another. Previously, we showed that *Ras*^{null} clones exhibit cell shape defects (James et al., 2002). Thus, the phenotypes we observe here may result from a reduction in the *Ras* activity necessary for appropriate cell shape changes. Additionally, or alternatively, the unusual cell shapes and arrangements may be due to the abnormally wide dorsal appendage tube produced in this mutant.

2.4.2. fs(1)K10

fs(1)K10 (K10) is required to localize *gurken* mRNA to the dorsal anterior corner of the oocyte. In *K10* mutants, *gurken* mRNA is present in a ring around the anterior circumference of the oocyte (Neuman-Silberberg and Schüpbach, 1993). As a result, the EGFR activation domain expands, producing eggs with enlarged dorsal appendages that are shifted laterally along the egg chamber (Wieschaus et al., 1978). Consistent with the ventral expansion of dorsal-appendage material in *K10* mutants, the expression domains of Broad and *rhomboid* expand ventrally as well (Deng and Bownes, 1997; Ruohola-Baker et al., 1993). Given the huge increase in the number of Broad and *rhomboid* cells in *K10* egg chambers, we asked whether

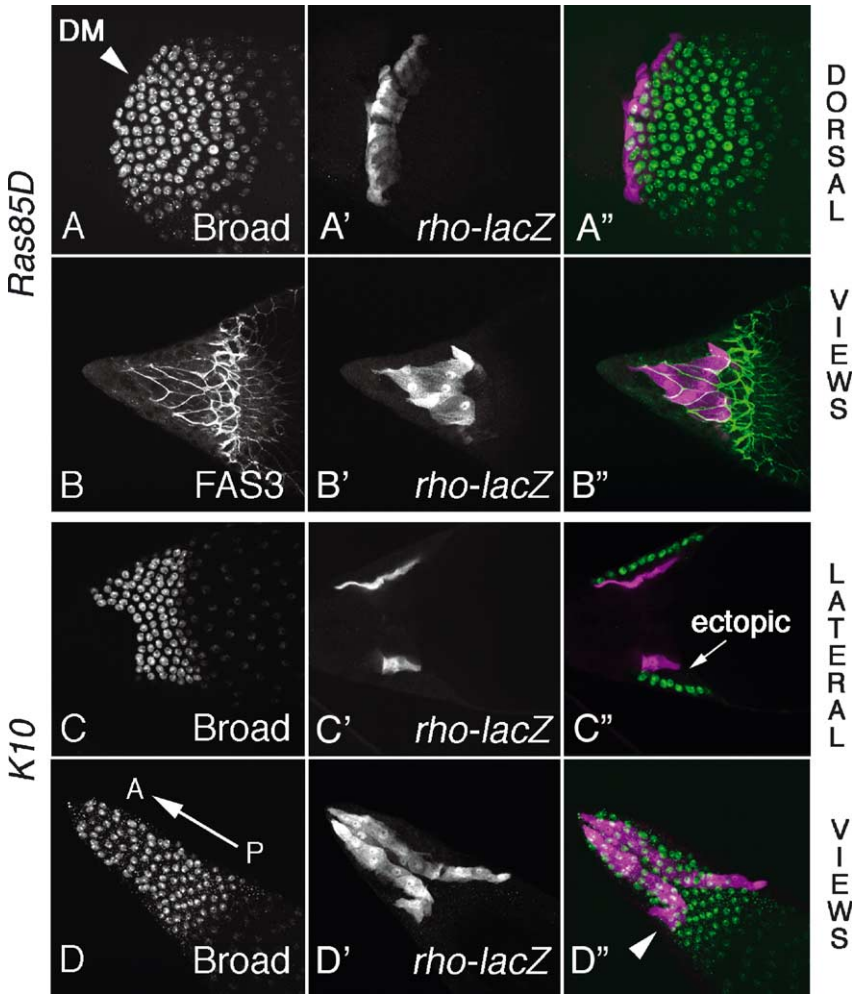

Fig. 4. Dorsal/ventral patterning mutants coordinately regulate *rho-lacZ* and Broad expression. (A,B) Egg chambers from $Ras85D^{05703}/Ras85D^{E62K}$ and (C,D) *fs(1)K10* females. (A–D) Anterior left, (A,B) dorsal views (C,D) lateral views of stage (A) 11, (B,C) 12 and (D) 13 egg chambers. (A,B) The dorsal midline (DM) is missing in egg chambers from $Ras85D^{05703}/Ras85D^{E62K}$ females. (A) Broad accumulates in nuclei of a single, large, dorsal-anterior domain; a single row of *rho-lacZ* cells arises anterior to the Broad cells. (B) The *rho-lacZ* cells express FAS3 asymmetrically and reorganize to form the ventral floor of the single dorsal appendage tube. (C,D) Both the Broad and *rho-lacZ* cell populations expand ventrally in eggs from *fs(1)K10* females. Despite the increase in dorsal appendage-forming cells, the *rho-lacZ* cells reorganize to form the ventral floor of the abnormal appendages. (C) Shows merged confocal stacks of external Broad cells and (C',C'') show single slices through the middle of the egg in (C). Arrow shows ectopic dorsal appendage cells and chorion. (D) On the ventral side of *K10* mutants we occasionally observe a row of unpaired *rho-lacZ* cells underneath the Broad cells (arrowhead). A←P shows the direction of tube elongation from the posterior to the anterior.

these two markers continue to define two distinct cell types with stereotypic behaviors.

In *K10; rho-lacZ.8.3/+* egg chambers, the *rho-lacZ* cells flank the anterior and dorsal margins of each large domain of Broad cells. These two cell types do not intermingle and both exhibit wild-type characteristics. The *rho-lacZ* cells express higher levels of FAS3 (data not shown) and usually associate in a pairwise fashion to form the ventral floor of each dorsal appendage (Fig. 4C,D). Occasionally, however, the ventral *rho-lacZ* cells elongate under the Broad cells, yet do not associate in a pairwise manner to form a tube. Rather, they remain in a single elongated row under the Broad cells and form a 'wedge' like lumen (Fig. 4D and data not shown).

Although eggs from *K10* females display a variety of dorsalized phenotypes, the ventral limits of both the *rho-lacZ* and Broad cells precisely mirror one another (data not shown). As noted above for the *Ras1* mutant egg chambers, the spatial arrangement between the *rho-lacZ* cells is occasionally aberrant in this mutant as well (Fig. 4D). Thus, despite the large increase in the number of cells contributing to the dorsal appendage primordia in this mutant, both dorsal appendage cell types are specified and generally rearrange appropriately to form tubes.

2.4.3. UAS-decapentaplegic

decapentaplegic (dpp) is required for patterning anterior eggshell structures (Twombly et al., 1996; Deng and Bownes, 1997; Peri and Roth, 2000). When *dpp* activity is reduced, the two Broad-expressing dorsal appendage primordia expand anteriorly and the dorsal appendages are

shifted correspondingly. Conversely, when *dpp* is expressed ectopically in most follicle cells, the Broad cells and dorsal appendages are positioned more posteriorly (Deng and Bownes, 1997). *dpp* activity also defines the size of the operculum (the larval exit hatch) (Dobens et al., 2000). The size of the operculum expands or contracts, respectively, with the gain or loss of *dpp* activity. *dpp* is expressed in the stretch cells and the first row of centripetally migrating cells from stage 8 onward, indicating that *dpp* acts non-autonomously to regulate the position of the dorsal appendages (Twombly et al., 1996).

Ectopic *dpp* expands the operculum and shifts the dorsal appendages to the posterior; presumably, this gain in anterior fate shifts *rho-lacZ* expression to the posterior as well. Since ectopic *dpp* results in a dramatic increase in the number of operculum cells, it is possible that the single row of *rho-lacZ* cells may expand to several rows as a result of ectopic *dpp* activity. To examine the consequences of altering the *dpp* gradient, we used a stretch-cell GAL4 driver to express higher levels of *dpp* specifically in the cells that normally express *dpp*. *l(1)3At*^{PG150} (PG150, Bourbon et al., 2002) expresses GAL4 exclusively in the stretch cells from stage 8 onwards (EJW and CAB, data not shown). Ectopic expression of *dpp* driven by PG150 reproducibly produces eggs with an expanded operculum and a more posterior placement of the dorsal appendage bases (Fig. 5).

To facilitate a portion of our analysis of the *rho-lacZ.8.3* cells in UAS-*dpp* egg chambers, we used another marker, JUN, which is expressed at a high level in the *rho-lacZ* cells (Fig. 5A). *Jun-related antigen (Jra)* encodes the *Drosophila* homologue of the AP-1 transcription factor JUN (Perkins et al., 1988). *Drosophila* JUN is expressed in the dorsal anterior follicle cells and is required for proper development of the dorsal appendages (Dobens et al., 2001; Suzanne et al., 2001). Interestingly, a subset of the cells in each group expresses higher levels of JUN; double labeling reveals that these cells correspond to the *rho-lacZ* cells (Fig. 5A). Conversely, cells expressing high levels of Broad express low levels of JUN (data not shown).

In wild-type egg chambers the anterior most 2–3 rows of columnar follicle cells and the dorsal midline cells migrate centripetally between nurse cells and oocyte and contribute to the future operculum. These cells express high levels of FAS3 (Fig. 3). Under our ectopic *dpp* conditions, the domain of operculum-producing cells expands to ~5–6 rows (Fig. 5B). A single curved row of cells expressing higher levels of FAS3 surrounds a cluster of cells expressing lower levels of FAS3 (presumably the Broad cells). Double labeling shows that the FAS3 cells in the curved row also express higher levels of JUN, indicating that these cells correspond to the *rho-lacZ* cells. Importantly, although the operculum expands dramatically, only one row of *rho-lacZ*

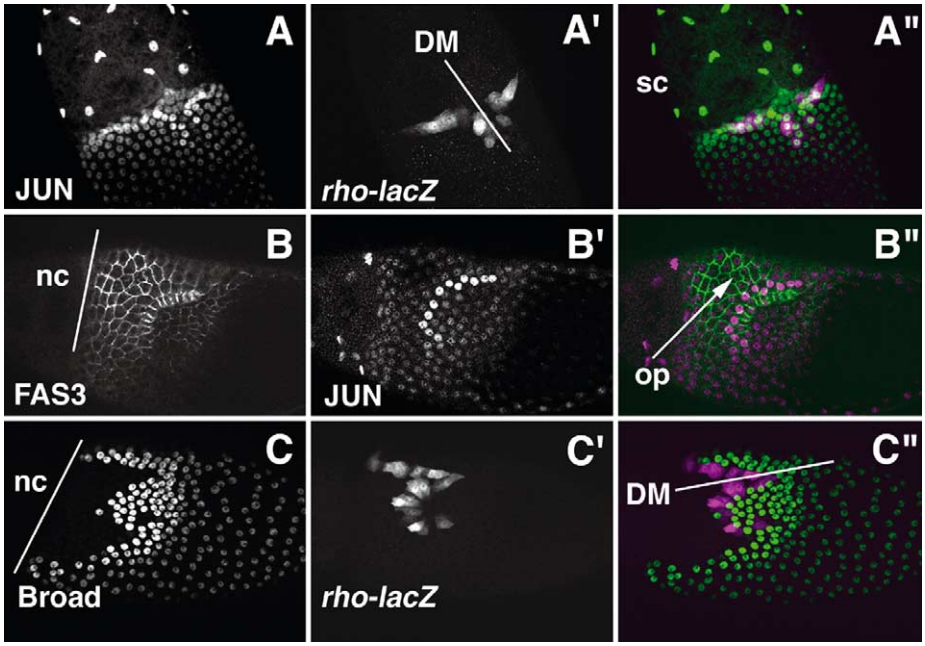

Fig. 5. Ectopic *dpp* produces a single row of *rho-lacZ* cells. (A) Shows dorsal views of a wild-type egg chamber; (B,C) show lateral views of egg chambers in which stretch-cell GAL4 is driving *UAS-dpp*. (A,B) Stained for JUN, (B) for FAS3, (A,C) for anti-β-galacactosidase (*rho-lacZ*) and (C) for Broad expression. (A) Stage 10B and (B,C) stage 11 egg chambers. (A) At stage 10B the stretch cells (sc) and *rho-lacZ* cells express high levels of JUN. *Note*: At this stage β-galactosidase is just beginning to accumulate in the cytoplasm of the *rho-lacZ* cells. (B) In this stage 11 egg chamber, ectopic *dpp* has expanded the operculum from a normal width of 2–3 cells (see Fig. 3A) to 5–6 cells. Operculum cells extend from the nurse-cell boundary (white line) to just anterior of the hinge of floor cells. (B′) A single row of JUN-expressing (*rho-lacZ*) cells arises adjacent to the operculum cells. (B,B″) These cells express FAS3 asymmetrically. The dramatic elongation of the *rho-lacZ* cells shifts the position of the floor-cell nuclei, marked by high JUN, to the basal end of the cell relative to FAS3 in the apical end of the cell. (C) The *rho-lacZ* and Broad cells rearrange to form a tube positioned more posterior in the egg chamber. Abbreviations: DM, dorsal midline; nc, nurse cells; op, operculum; sc, stretch cells.

cells is adjacent to each cluster of Broad cells. As in all of the mutants described thus far, the *rho-lacZ* and Broad cells do not intermingle and go on to rearrange and move appropriately to produce the ventral floor and roof, respectively, of the dorsal appendage tube (Fig. 5C).

2.5. *Ectopic* rhomboid *induces* rho-lacZ *and Broad cells in the anterior of the egg chamber*

In wild type and mutants, we always find a single row of *rho-lacZ* cells adjacent to a cluster of Broad cells, suggesting that the juxtaposition of these two cell types is tightly regulated during development and is required for tube formation. To test this hypothesis, we generated ectopic dorsal appendages, reasoning that such tubes would form only when both cell types were present. We took advantage of previous studies that showed that ectopic *rhomboid* expression produces ectopic dorsal appendages in the anterior of the egg chamber (Ruohola-Baker et al., 1993; Sapir et al., 1998) but *rhomboid* alone is insufficient to produce appendages in the posterior of the egg chamber (Peri and Roth, 2000). Interestingly, ectopic expression of Broad does not produce ectopic dorsal appendages (Tzolovsky et al., 1999), presumably because Broad is just one of many transcription factors whose activity is regulated in specifying roof-cell fate. These results suggest that in the anterior of the egg chamber, ectopic *rhomboid* expression induces both the *rho-lacZ* and Broad cell types. We therefore used the flip-out technique (Pignoni and Zipursky, 1997) to ectopically express *rhomboid* in random cells throughout the follicular epithelium. We assayed ectopic *rho-lacZ* and Broad expression and compared the nature, size and location of these patterns with the ability to produce chorionic structures. We predicted that tube formation would require the juxtaposition of *rho-lacZ* and high-Broad expressing cells.

Clones of cells ectopically expressing *rhomboid* produced two different types of eggshell defects: (1) ectopic dorsal appendages and (2) ectopic chorionic material resembling warts. The warts were restricted to the ventral/lateral region in the middle of the egg chamber (Fig. 6A). In this area ectopic *rhomboid* induced high levels of Broad; such clones, however, never expressed *rho-lacZ* ($n=50$, Fig. 6C). Furthermore, cells adjacent to the clones also expressed high levels of Broad, indicating that *rhomboid* activates Broad non-autonomously. *rhomboid* encodes a serine protease that cleaves the EGFR ligand Spitz (Urban et al., 2001; Lee et al., 2001); thus, non-autonomous Broad expression presumably resulted from this feature of the pathway. Ectopic *rhomboid* represses *pipe-lacZ* expression non-autonomously in this region as well (Peri et al., 2002), indicating that other downstream events in patterning these cells do occur. Although the high-Broad cells secreted additional chorion to produce a wart, these cells failed to constrict apically or exhibit other morphological behaviors of roof cells (data not shown).

In contrast, the ectopic dorsal appendages were restricted to the ventral/lateral region around the anterior circumference of the egg chamber, that is, arising from the collar (Fig. 6B). Unlike the wart-producing clones, each ectopic dorsal appendage tube always contained both roof and floor cells ($n=35$). Within each clone, the GFP-marked cells expressed either *rho-lacZ* or Broad, never both. The *rho-lacZ* cells elongated and moved under the Broad cells normally to produce an ectopic tube (Fig. 6D,E). Amazingly, *rhomboid*-expressing clones of only two cells could organize neighboring cells to produce a tube (Fig. 6E). Thus, in the anterior of the egg chamber, ectopic *rhomboid* can induce production of roof and floor cells and the two cell types change shape and reorganize appropriately to form ectopic dorsal appendage tubes. When ectopic expression adjoined the wild-type primordium, the excess *rho-lacZ* and Broad cells simply enlarged the lateral domains of the floor and roof cells (data not shown).

Our data reveal three key points about the patterning and morphogenesis of the dorsal-appendage-forming cells. First, ectopic *rhomboid*, which activates EGFR signaling (Sapir et al., 1998; Wasserman and Freeman, 1998), induces both dorsal-appendage cell types. Ectopic *rhomboid*, however, cannot turn on *rho-lacZ* expression in more posterior follicle cells (Fig. 6C), suggesting that *rho-lacZ* expression is determined by multiple factors. This restriction of ectopic *rho-lacZ* expression to the anterior of the egg chamber presumably reflects a requirement for sufficient levels of *dpp* (Peri and Roth, 2000). These results are consistent with previous studies demonstrating that combinatorial signaling specifies dorsal appendage fates (Queenan et al., 1997; Deng and Bownes, 1997; Peri and Roth, 2000; Dequier et al., 2001). Second, along the dorsal/ventral axis, *rho-lacZ* can only be activated in the ventral/lateral cells (Fig. 6D,E), suggesting that some mechanism in the dorsal appendage primordium prevents aberrant activation of *rho-lacZ*. Presumably, *argos* activity inhibits *rho-lacZ* expression along the dorsal midline (Wasserman and Freeman, 1998). Argos or some other downstream process may restrict *rho-lacZ* expression within the primordium itself. Finally, even in the ventral/lateral anterior, *rho-lacZ* is NOT expressed in all cells of the flip-out clone (Fig. 6D,E). That is, GAL4 induces expression of *UAS-rhomboid* in all clone cells but the *rho-lacZ* marker is expressed only in floor cells, and these floor cells ALWAYS reside anterior to the Broad cells. Recall that the *rho-lacZ* promoter fusion does not contain all elements necessary for *rhomboid* expression; it does not replicate the stage 10A saddle pattern in which *rhomboid* and Broad overlap but portrays only the later hinge pattern. These data suggest that additional patterning events must occur to resolve the initially common region of EGFR activation into two domains of non-overlapping *rho-lacZ* and Broad cells.

In summary, both the *rho-lacZ* and high-Broad cells are required for dorsal appendage formation. All wild-type, mutant and ectopic dorsal appendage tubes are composed of

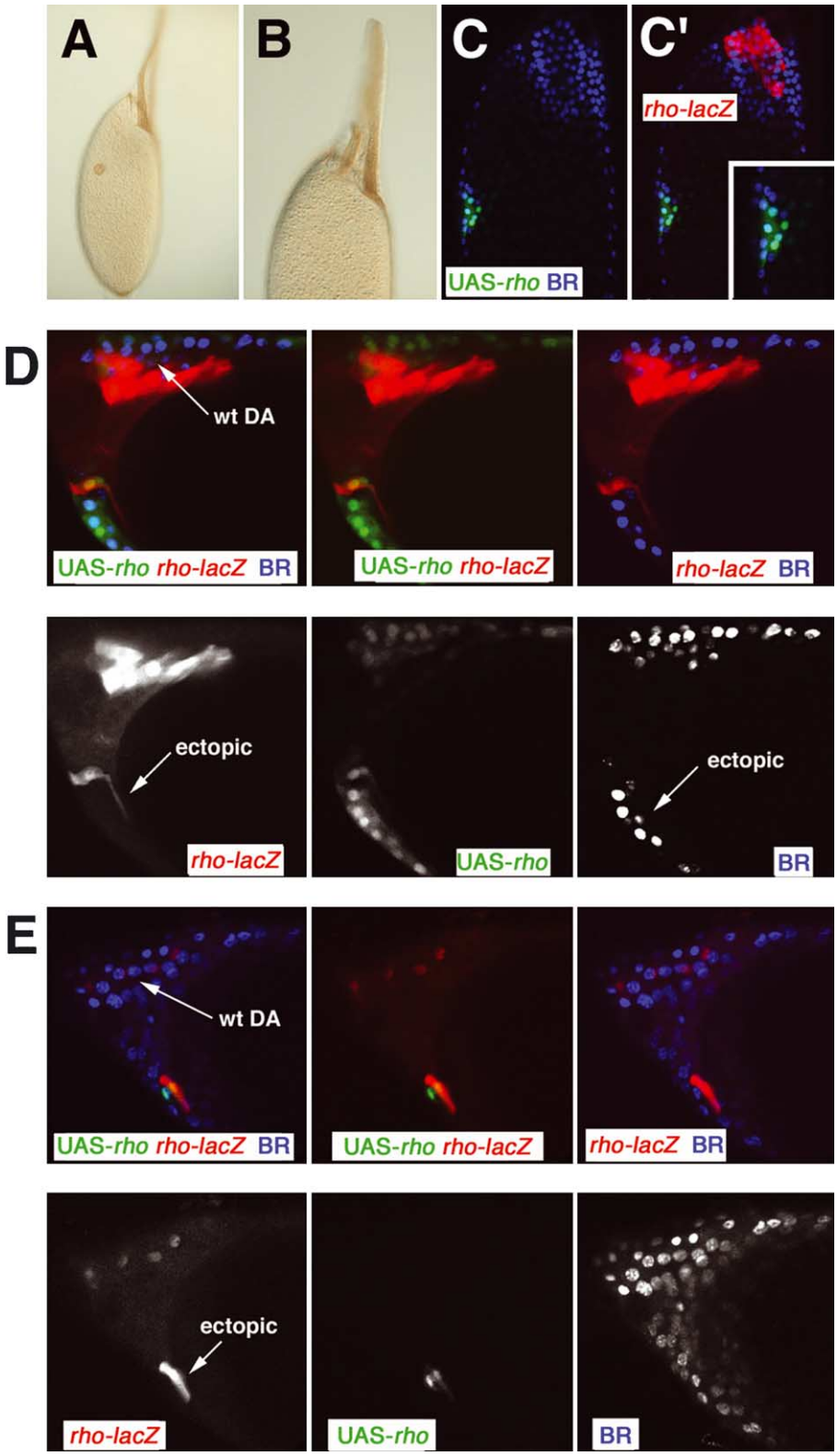

Fig. 6. Ectopic *rhomboid* induces *rho-lacZ* and Broad cells in the anterior follicle cells. (A–E) Egg chambers with clones expressing ectopic *rhomboid*. (A–E) Lateral views of (A,B) stage 14, (C,D) stage 12 and (E) stage 13 egg chambers. (A,B) Bright-field images of eggshells and (C–E) single confocal slices of triply labeled egg chambers. (A) Example of ectopic dorsal appendage material resembling a 'wart'. (C) Warts are found in the ventral/lateral half of the egg and are formed by cells expressing high levels of Broad. Inset shows higher magnification view of wart-producing clone. (B) Example of an ectopic dorsal appendage. (D,E) Ectopic dorsal appendages are found along the ventral/lateral portion at the anterior of the egg and are formed by ectopic *rho-lacZ* and Broad cells. (D) A large clone (20 GFP-positive cells) in the ventral anterior produces an ectopic *rho-lacZ* cell under a cluster of high-Broad cells. (E) A two-cell clone in the ventral anterior produces an ectopic *rho-lacZ* cell and high Broad cells (out of the plane of focus).

both cell types. In the anterior, ectopic *rhomboid* is sufficient to induce *rho-lacZ* and high-Broad cells whereas ectopic Broad is insufficient (Tzolovsky et al., 1999). Normally, a combination of EGFR and DPP signaling produces the two dorsal appendage primordia (Peri and Roth, 2000). Since *rhomboid* is a component of the EGFR pathway, cells expressing ectopic *rhomboid* presumably have elevated EGFR signaling. Thus, ectopic *rhomboid*, coupled with normal DPP levels in the anterior, induces ectopic *rho-lacZ* and high Broad cells. In ventral follicle cells outside the collar, however, elevated EGFR signaling only induces the high-Broad cells. These cells alone cannot form tubes. These results suggest that both cell types are required to form dorsal appendage tubes. The *rho-lacZ* cells may provide a signal to the high-Broad cells to constrict and reorganize during tube formation. In addition, or alternatively, an anterior signal such as DPP may instruct the Broad cells during tube formation. In the latter case the *rho-lacZ* cells may play a structural, rather than a functional, role during tube formation.

3. Discussion

3.1. Mechanistic features of dorsal appendage formation

Each dorsal appendage is made from a population of cells that reorganizes from an initially flat epithelium into a tube. This process most closely resembles wrapping, one of a variety of mechanisms that produce epithelial tubes (Lubarsky and Krasnow, 2003). Dorsal appendage tube formation exhibits all three characteristics of the wrapping mechanism. First, the dorsal appendage cells maintain epithelial contacts during tube formation (this work; Dorman et al., 2004; James and Berg, 2003). Second, the Broad cells constrict apically, causing the flat epithelium to curve (Dorman et al., 2004). Finally, the dorsal appendage tubes are parallel to the follicular epithelium, rather than perpendicular as observed in budding tubes (Lubarsky and Krasnow, 2003). These features also characterize vertebrate neural tube and *Drosophila* ventral furrow formation (Costa et al., 1993; Wallingford and Harland, 2002).

Dorsal appendage tube formation differs from neural tube/ventral furrow formation in one key respect. The neural tubes and ventral furrow are each made by a symmetric fold in the epithelium. In contrast, asymmetric shape-changes and movements produce each dorsal appendage tube. Prior to tube formation, the *rho-lacZ* rows are perpendicular to one another in a pattern resembling an open hinge. Then, during tube formation the anterior row of *rho-lacZ* cells moves posterior, thereby closing off the ventral midline of the dorsal appendage tube. Cells in the medial (dorsal) row elongate concomitant with the apical constriction of the roof cells, but these floor cells do not swing anteriorly. Thus, during dorsal appendage formation the perpendicular rows of *rho-lacZ* cells do not move equivalent distances to seal the tube. Evidently, this process is both robust and malleable, as normal tubes can still form in patterning mutants with altered primordia.

What mechanism ensures proper tube closure? Recall, during tube formation the Broad pattern simultaneously shortens and lengthens along two perpendicular axes via likely convergent-extension rearrangements. Since the *rho-lacZ* and Broad cells maintain epithelial contacts with one another during tube formation, we propose that the rearrangements among the Broad cells contribute to a reorganization of the adjacent, underlying *rho-lacZ* cells. Thus, the anterior-medial movement of the Broad cells simultaneously lengthens the tube and draws the anterior row of *rho-lacZ* cells posteriorly. This process allows the *rho-lacZ* cells on either side of the hinge to associate with one another in a pair-wise fashion to close off the ventral midline of the tube. Similarly, convergent extension during neural tube development narrows the distance between the neural folds, allowing them to meet and fuse (Wallingford and Harland, 2002).

3.2. Both dorsal appendage cell types are specified in patterning mutants

Additional insight into patterning and morphogenesis is provided by our analysis of loss of function (*Ras1* and *K10*) and gain of function (UAS-*dpp* and UAS-*rho*) mutants (Figs. 4–6). In each mutant, the *rho-lacZ* and Broad cell types are specified and occupy their stereotypical locations within the otherwise defective tubes. Four features of these aberrant tubes are noteworthy. First, the number of cells contributing to each primordium can vary widely, from a few cells in the *UAS-rho* clones to as many as hundreds in *K10* egg chambers. Nevertheless, the *rho-lacZ* and Broad cells coordinate their movements to form a tube. Second, the position of the primordium within the egg chamber is not restricted to the dorsal anterior. Dorsal appendages shift posteriorly when *dpp* expression is greatly expanded, and ventral/lateral tubes may form when *UAS-rho* is expressed in the collar. Apparently, as long as both cell types form, other factors are not limiting in tube formation. Third, the posterior and ventral limits of both *rho-lacZ* and Broad expression precisely mirror one another. Even when ectopic *rhomboid* enlarges the normal domain of the dorsal appendage primordium, Broad and *rho-lacZ* expression expand coordinately. These results suggest that the patterning of these two cell types is a linked process. Finally, in some *K10* egg chambers and in the *Ras1* hypomorphs and UAS-*rho* mutants, the *rho-lacZ* cells flank only the anterior margin of each Broad domain. Although the *rho-lacZ* cells are not arranged in a hinge pattern, the dorsal appendage cells reorganize appropriately to form a tube. Thus, the hinge pattern of *rho-lacZ* cells is not essential for tube formation.

These results indicate that the juxtaposition of *rho-lacZ* and Broad cells is necessary for tube formation and suggests

that communication between the two cell types promotes the cell shape changes and rearrangements necessary to make a tube from an initially flat epithelium.

3.3. Boundary between rho-lacZ and Broad cells coordinates tube formation

How do the dorsal appendage cells reorganize in a coordinated manner to form a tube? We propose that the *rho-lacZ* and Broad cells are separated by a 'boundary' and that signaling across this boundary choreographs the cell shape-changes and rearrangements necessary to make a tube from an initially flat epithelium. Boundaries between two different cell types occur frequently in developing tissues (reviewed by Irvine and Rauskolb, 2001). Importantly, cells on one side of a boundary are free to mix with one another, but do not mix with cells on the other side of the boundary. This fence-like property of boundaries may be maintained by differences in cell adhesion. Although this hypothesis provides a satisfying explanation for cell behaviors, the adhesive mechanisms that prevent intermingling between different cell types are not understood (reviewed by Wolpert, 2003). Finally, a boundary can function as an 'organizer' to instruct cells about their position and fate within a developing tissue.

Boundaries are of two types: lineage restricted (compartment) and non-lineage restricted. Since the patterning processes that define the dorsal appendage primorida occur after the cessation of cell division, a boundary between the *rho-lacZ* and Broad cells would be of the non-lineage-restricted type. Previous researchers have proposed that another boundary exists in the dorsal anterior follicle cells. This boundary is established by differential Bunched activity and lies between operculum/non-operculum cells (Dobens et al., 2000). The boundary described in this paper is between the *rho-lacZ* and Broad cells.

What is the evidence that a boundary separates the *rho-lacZ* and Broad cells? First, the roof and floor cells express unique cell-fate markers, display differential levels of cell-adhesion proteins, and exhibit distinct behaviors such as directed elongation and convergence/extension. Clearly they are different cell types. Second, throughout the elaborate cell shape-changes and rearrangements of dorsal-appendage morphogenesis, the *rho-lacZ* and Broad cells coordinate their behaviors and never intermingle, even when patterning goes awry. Finally, the membrane(s) between the *rho-lacZ* and Broad cells accumulates high levels of phosphorylated proteins, consistent with signaling between the two cell types (reviewed by Schlessinger, 2000).

Signaling via an organizer established at the boundary may direct the cell shape-changes and rearrangements necessary to make a tube, perhaps by instructing the *rho-lacZ* cells to elongate and the Broad cells to constrict apically. The boundary could also direct the convergent-extension rearrangements of the Broad cells. Consistent with an organizer acting at the boundary between the *rho-lacZ* and Broad cells, ectopic expression of *rhomboid* in the anterior of the egg chamber produces an ectopic boundary capable of reorganizing the *rho-lacZ* and Broad cells into a tube. Domains of high-Broad-expressing cells merely produced warts, whereas clusters of cells containing both the Broad and *rho-lacZ* cell types reorganized properly and synthesized dorsal appendage tubes.

Altogether, our wild type, mutant, and ectopic *rhomboid* studies indicate that the juxtaposition of *rho-lacZ* and Broad cells is necessary to make a dorsal appendage tube. These two sub-populations of cells express many different cell-fate and adhesion markers, exhibit distinct behaviors, and never intermingle. We hypothesize that a boundary exists between these two cell types and that signaling across the boundary coordinates the cell shape-changes and rearrangements that form the tube. Our studies offer insight into the processes that regulate tubulogenesis, reveal mechanistic links between patterning and morphogenesis, and provide a foundation for inquiry in other systems.

4. Experimental procedures

4.1. Drosophila strains and crosses

We employed the following fly stocks: Canton-S, $Ras85D^{05703}$ (Schnorr and Berg, 1996); $Ras85D^{E62K}$ (Simon et al., 1991); $fs(1)K10^{1}$ (Wieschaus et al., 1978); *P{w+mC=*UAS-*dpp.S}42B.4* (Staehling-Hampton et al., 1994); *CyO*, $Fas3^{A183.1F2}$ (Bellen et al., 1989; Wilson et al., 1989); $Fas3^{E25}$ (null allele, A. Chiba, personal communication) and $P\{GawB\}l(1)3At^{PG150}$ (PG150; Bourbon et al., 2002).

The *rho-lacZ.2.2* and *rho-lacZ.8.3* strains carry promoter fusion constructs encoding β-galactosidase under the control of the *rhomboid* promoter (Ip et al., 1992). Flies homozygous for the *rho-lacZ.2.2* insertion on the third chromosome exhibit a slightly ventralized eggshell phenotype (EJW, data not shown). To avoid this phenotype we documented *rho-lacZ.2.2* expression in wild type and mutant flies carrying only one copy of the *rho-lacZ.2.2* chromosome. For our analysis of *rho-lacZ* expression in *K10*, $Ras85D^{05703}$/$Ras85D^{E62K}$ and UAS-*dpp*/PG150 mutants we used the *rho-lacZ.8.3* insertion on the second chromosome. For the purpose of equivalent comparisons, we also used flies heterozygous for the *rho-lacZ.8.3* chromosome.

4.2. Ectopic expression studies

To over express *dpp* in the stretch cells, we crossed $l(1)3At^{PG150}$ flies (carrying stretch-cell GAL4 driver PG150) to UAS-*dpp* flies at 18 °C. To expand anterior follicular cell fates, we shifted adult females to 25 °C for two days prior to dissection. To express *rhomboid* ectopically in random clones, we crossed *y hsp70-FLP;*

Act>CD2>GAL4, UAS-GFP (Pignoni and Zipursky, 1997) to *P{UAS-ve.(rho)G}11-1* flies (Golembo et al., 1996). We heat shocked the F_1 progeny at 37 °C for 30 min, reared the flies at 25° 2–3 days, then dissected and analyzed ovaries.

4.3. Antibodies

The primary antibodies were: mouse anti-Broad core (Emery et al., 1994, 1:500), mouse anti-Phosphotyrosine (Zymed, 1:20), rabbit anti-β-galactosidase (Cappel, 1:6000), mouse anti-β-galactosidase (Sigma, 1/3000), rabbit anti-GFP (Molecular Probes, 1/500) and mouse anti-JUN (Hou et al., 1997, 1:100). The mouse monoclonal antibodies anti-FAS3 (C. Goodmann, 1:35) and anti-α-spectrin (D. Branton and R. Dubreuil, 1:20) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Primary antibodies were detected using fluorescently labeled secondary antibodies (Molecular Probes, 1:200) or biotinylated secondary antibodies (Vector Laboratories, 1:300).

4.4. Immunofluoresence and immunohistochemistry

Ovaries from two-day-old females raised in vials on wet yeast were dissected in PBSTwn (1X phosphate buffered saline, 0.1% Tween-20) and fixed in PBSTwn plus 4% EM grade paraformaldehyde (Ted Pella) at room temperature for 20 min. Ovaries were washed with three 10-min washes (referred to as 'washed' from hereon). To permeabilize, the ovaries were incubated with 1% Triton-X100 in PBSTwn for one hour at 25 °C. Following this procedure, the ovaries were dissociated into individual egg chambers by pipetting gently through a P-1000 pipet tip 10 times (Jackson and Berg, 1999).

Then, the tissue was washed, blocked with 5% Normal Goat Serum (Vector) in PBSTwn 30 min at 25 °C followed by incubation with diluted primary antibodies overnight at 4 °C. After washing, the eggs were incubated with diluted secondary antibodies one hour at 25 °C, washed, cleared sequentially with 50% glycerol/1X PBS then 80% glygerol/1X PBS and mounted with a drop of Vectashield (Vector). Images were obtained using a BioRad MRC600 confocal microscope and processed using Adobe Photoshop. Images from triply labeled egg chambers were obtained using a BioRad Radiance 2000 MP.

Immunohistochemically stained egg chambers were treated with the following modifications of the above protocol: after permeabilization the tissue was washed and then treated with 3% hydrogen peroxide in methanol to inactivate the endogenous germline peroxidase activity. The ovaries were washed, blocked and incubated with primary antibodies and washed again as described above. Then, the tissue was incubated with diluted biotinylated secondary antibodies at 25 °C for 1 h, washed, treated with an ABC solution prepared according to the manufacture's instructions (Vector) and washed. Horseradish peroxidase activity was detected using a DAB kit (Vector). Egg chambers were viewed under DIC optics on a Nikon Microphot FXA compound microscope. Images were collected with a Nikon Coolpix camera and processed using Adobe Photoshop.

4.5. Analysis of Broad cells

To determine the number of Broad cells per primordium, on photographic prints, we drew a border around each cluster of cells expressing high levels of Broad and counted the number in each primordium. We counted at least 10 primordia per stage (stages 10B-13, $n=51$) and found an average of 53 ± 4 Broad cells per dorsal appendage. Since the number of Broad cells remains constant during appendage formation, it is likely that the Broad pattern observed at stage 13 represents rearrangements between the cells originally specified at stage 10B. Since we have not labeled individual cells and followed their movement to produce a fate map, however, it remains possible (albeit unlikely) that dorsal appendage cells rapidly turn Broad on and off.

4.6. Analysis of dorsal appendage stalk diameters

To estimate tube lumen size in wild type and $Fas3^{null}$ mutants, we first separately photographed the right and left appendages of laid eggs that had been mounted in Hoyer's medium. We then employed ImageJ (http://rsb.info.nih.gov/ij/) to determine stalk width at five sites along each appendage stalk and used the mean of these measurements as a 'stalk width' for each appendage. We used the sum of such analyses for 10 wild-type egg chambers and 10 $Fas3^{null}$ egg chambers to calculate the mean and standard deviation for each strain (Canton $S=23.5\pm4.16$ μm; *Fas3* $=36.7\pm4.1$ μm). A *t*-test demonstrates that these means differ significantly at $P<0.001$.

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Appendix. Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2004.10.006.

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