Development 125, 589-597 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5151

Hyperactivation of the *folded gastrulation* pathway induces specific cell shape changes

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Accepted 17 November 1997: published on WWW 22 January 1998

SUMMARY

During Drosophila gastrulation, mesodermal precursors are brought into the interior of the embryo by formation of the ventral furrow. The first steps of ventral furrow formation involve a flattening of the apical surface of the presumptive mesodermal cells and a constriction of their apical diameters. In embryos mutant for *folded gastrulation* (*fog*), these cell shape changes occur but the timing and synchrony of the constrictions are abnormal. A similar phenotype is seen in a maternal effect mutant, *concertina* (*cta*). *fog* encodes a putative secreted protein whereas *cta* encodes an α-subunit of a heterotrimeric G protein. We have proposed that localized expression of the *fog* signaling protein induces apical constriction by interacting with a receptor whose downstream cellular effects are mediated by the *cta* Gα protein.

In order to test this model, we have ectopically expressed *fog* at the blastoderm stage using an inducible promoter. In addition, we have examined the constitutive activation of *cta* protein by blocking GTP hydrolysis using both in vitro synthesized mutant alleles and cholera toxin treatment. Activation of the *fog/cta* pathway by any of these procedures results in ectopic cell shape changes in the gastrula. Uniform *fog* expression rescues the gastrulation defects of *fog* null embryos but not *cta* mutant embryos, arguing that *cta* functions downstream of *fog* expression. The normal location of the ventral furrow in embryos with uniformly expressed *fog* suggests the existence of a *fog*-independent pathway determining mesoderm-specific cell behaviors and invagination. Epistasis experiments indicate that this pathway requires *snail* but not *twist* expression.

Key words: Morphogenesis, Drosophila, Gastrulation, Invagination, *concertina*, *folded gastrulation*

INTRODUCTION

During gastrulation, the embryo acquires a three-layered structure when mesoderm and endoderm precursors invaginate. In the Drosophila embryo, these primordia form two largely distinct invaginations (Campos-Ortega and Hartenstein, 1985; Costa et al., 1993). The posterior endoderm forms the posterior midgut invagination (PMG). The mesoderm and possibly anterior endoderm form the ventral furrow (VF). These invaginations occur without any mitosis (Foe, 1989) and rely entirely on changes in cell shape (Turner and Mahowald, 1977; Leptin and Grunewald, 1990; Sweeton et al., 1991). Morphological and genetic studies suggest that very similar processes trigger and control the invagination of both the VF and the PMG (Costa et al., 1993). Formation of the VF provides a particularly suitable system to study the process of invagination for several reasons: the relatively simple shape of the invagination, the absence of cell rearrangement during this process, and the basic understanding of the molecular mechanisms of mesoderm specification (Leptin, 1991; González-Crespo et al., 1993).

Initial formation of the VF consists of two distinct phases that are separated by an abrupt transition (Leptin and Grunewald, 1990; Sweeton et al., 1991). Cell shape changes begin only after the completion of cellularization. During the first phase, the cells inside the invagination primordium flatten their apices and some cells begin to reduce their apical diameter. In a given cell, constriction begins only after flattening. Some cells, however, may constrict before all the cells in the ventral domain have completed flattening. The initial apical constrictions occur in a relatively random manner suggestive of cell autonomous regulation. When approximately 40% of the cells in the invagination primordium have both flattened and constricted their apices, a sudden transition occurs. All the remaining cells in the primordium simultaneously begin apical constriction, resulting in a coherent shallow furrow (Leptin and Grunewald, 1990; Sweeton et al., 1991). As apical constriction continues, the nuclei of these cells move basally and the cells increase in length along their apical/basal axis (Kam et al., 1991). When the cells have almost doubled in length, they enter the third phase, during which they start to shorten and widen basally. This change accentuates the wedge-like shape of each cell due to the increased size of the basal surface relative to the

constricted apex. As the cells shorten further, the furrow is pushed inside the embryo and eventually closes. Our long-term goal is to understand how these cell shape changes are triggered and coordinated in order to make an invagination.

Cells in the ventral region of the blastoderm are specified to mesodermal fates by the expression of the two transcription factors, TWIST (Thisse et al., 1987, 1988) and SNAIL (Simpson 1983; Boulay et al., 1987). Although both genes are essential for the formation of mesoderm, they have different effects on VF formation (Leptin and Grunewald, 1990; Costa et al., 1993). In *sna* mutant embryos, none of the ventral cells flatten or constrict their apices, suggesting that wild-type *sna* product is essential for all changes in apical morphology that normally occur in mesodermal cells. In *twi* mutant embryos, apical flattening is observed in a narrower stripe of cells along the ventral midline and some cells even form transient apical constrictions. The domain in which these cell shape changes occur corresponds to the narrowed domain of *sna* expression in *twi* embryos, consistent with a primary role for *sna* in eliciting apical constriction. A more substantial invagination is observed in *twi* embryos in which *sna* expression is artificially restored to its normal width (Ip et al., 1994). The resultant invagination, however, is still extremely irregular in morphology. These defects are thought to reflect a more direct role of TWI in coordinating and speeding up the cell shape changes.

Two other genes affect cell shape changes in the VF. In embryos lacking *folded gastrulation* (*fog*) or *concertina* (*cta*) activity, the non-synchronous flattening and constriction occurring during the first phase are normal but the phase of synchronous apical constrictions never occurs (Parks and Wieschaus, 1991; Costa et al., 1994). As a consequence, the cells continue to initiate apical constriction at the slow rate characteristic of the first phase and the invagination is irregular. The morphology of the furrow formed is similar to the furrows of *twi* embryos in which SNA activity is maintained at its normal width (Ip et al., 1994). This similarity suggests that one role of wild-type TWI activity may be to activate the *fog /cta* pathway.

Although the *fog* and *cta* null mutant phenotypes are similar, *fog* is required zygotically whereas *cta* is a maternal effect gene. *fog* is highly expressed in regions that undergo invagination (Costa et al., 1994), while *cta* mRNA is ubiquitously present in the precellularized embryo (Parks and Wieschaus, 1991). Molecular analysis of these genes has shown that *fog* encodes a putative secreted molecule, whereas *cta* encodes the α subunit of a heterotrimeric G protein (Parks and Wieschaus, 1991; Costa et al., 1994). A model has been proposed in which FOG could bind to an as yet unidentified receptor and thereby trigger CTA activation (Costa et al., 1994). The activation of CTA would induce cell shape changes by a yet unknown mechanism. The secreted nature of the *fog* product in this model would coordinate the formation of the furrow.

In order to test this model, we have examined the effects on cell shape caused by ubiquitous expression of *fog* under the control of the *hsp70* promoter. We also constructed alleles of *cta* that would be predicted to confer constitutive activity based on homologies to known activated forms of Gα proteins in other organisms. Expression of these constructs during gastrulation has allowed us to demonstrate effects of the *fog/cta* pathway on cell shape that are independent of a cell's programming to the mesodermal or endodermal fates.

MATERIALS AND METHODS

Genotypes and stocks used

The following mutations were used in all experiments where alleles are not stated in the text: fog^{4a6}, svb^{YD390}, cta^{RC10}, dl^{1}, sna^{IIG05}, twi^{ID96}. Each appears to be a null allele. The three in vitro synthesized alleles of *cta* used in this study are designated cta^{Q303R} (a Gln to Arg change at position 303), cta^{R277H} (an Arg to His change at position 277, the cholera toxin-sensitive site) and cta^{R277C} (an Arg to Cys change also at position 277). No phenotypic differences were observed between the three alleles; they are used interchangeably in our experiments and are referred to in the text as cta^{GOF}. Oregon-R, *FM7* males and w^{1118} were used as wild type.

Whole mount *in situ* hybridization and antibody labeling

Double labeling of embryos for RNA using *in situ* hybridization and for protein using immunolabeling with rabbit anti-β-galactosidase antibodies (Vector Laboratories) was performed according to Manoukian and Krause (1992) with the following modifications: after devitellinizing, embryos were rinsed twice for 20 minutes each in PBS, 0.01% Tween-20 and 1.5 mM DTT, then once for 20 minutes in PBS, 0.01% Tween-20 (all solutions were filter-sterilized). Primary antibody was diluted 1:2000 in PBS, 0.01% Tween for incubation. Hybridization to a Digoxigenin-labelled antisense RNA probe (made using the RNA Genius Kit from Boehringer-Mannheim) was performed at 55°C. The following secondary antibodies were used: biotinylated goat anti-rabbit (Vector Laboratories; diluted 1:2000) and alkaline phosphotase-conjugated Fab fragments from sheep anti-Digoxigenin (Boehringer-Mannheim, diluted 1:4000). All antibodies were preabsorbed by overnight incubation with fixed embryos. Labeled embryos were dehydrated using an ethanol series, rinsed in acetone and in acetone:Durcopan™ ACM overnight, then mounted in Durcopan™ ACM (Fluka Chemie AG). Embryos were examined on a Zeiss Axioplan microscope with Nomarski optics. Images were captured using a Sony 3CCCD videocamera and Adobe Photoshop™ software on a PowerMacintosh computer. These images were printed on a dye-sublimation printer (Codonics).

Transgenic flies

All experiments involving conventional uses and manipulations of nucleic acids were performed according to standard protocols (Sambrook et al., 1989). We cloned the *fog* cDNA (Costa et al., 1994) into a pCaSpeR vector that contains a heat-shock inducible promoter and generated transgenic flies carrying this construct. The *cta* cDNA (Parks et al., 1991) was mutagenized by site-directed mutagenesis using the Kunkel method as described in Sambrook et al. (1989). DNA was prepared from plaques and sequenced to see if the proper change was incorporated. Fragments carrying the change were re-ligated in frame to the original vector and subcloned into a Germ8/80 vector (Serano et al., 1994). We tested these constructions by first purifying and capping the in vitro transcription products, then injecting each mRNA into wild type and cta^{RC10} embryos. The same constructs were then injected into w^{1118} embryos to generate transgenic lines.

Heat shock procedure

For SEM analysis, embryos were collected at 30 minute intervals, aged at room temperature for 2 hours then placed at 36°C in halocarbon oil for 30 minutes. They were allowed to recover for 30 minutes before fixation and preparation for morphological analysis. In the rescue experiments, embryos were collected for 1-2 hours and staged in halocarbon oil. Embryos of the appropriate stage (Wieschaus and Nüsslein-Volhard, 1986) were transferred to 35°C for 10 minutes then allowed to develop at room temperature for 2 days. Dead embryos were mounted to examine their cuticles (Wieschaus and Nüsslein-Volhard, 1986). Larvae were collected and fixed in 4% formaldehyde in PBS, 0.01% Tween-20 for 30 minutes, rinsed and mounted in the same manner as the embryos.

Cholera toxin injections

0.25 mg/ml cholera toxin A-subunit (Sigma) was activated with 10 mM DTT and was injected at the syncytial blastoderm stage into embryos, which were then allowed to develop until early gastrulation. At that stage embryos were fixed, hand-peeled and prepared for SEM analysis. In some experiments, injected embryos homozygous for *fog4a6* (a protein null allele) were identified using antibody against the Fog protein (A. E. Christiansen et al., unpublished).

Scanning election microscopy

Scanning electron microscopy was performed according to Sweeton et al. (1991) with several modifications. Embryos were fixed for 20 minutes and devitellinized using either methanol-popping for whole-mount embryos or hand-peeling in PBS for embryos to be viewed by cross section. Embryos for cross-section viewing were cut in half after peeling using a bent tungsten needle. After post-fixing and dehydrating, embryos were dried either using 50% PELDRI II (Ted Pella Inc.) in ethanol for 30 minutes, and in 100% PELDRI II for 1 hour, or using TetraMethylSilane (TMS from Ted Pella Inc.) after a treatment in acetone for 5 minutes. Embryos were then coated with gold palladium in a Denton Desk II sputter coater and photographed in a JEOL 840 SEM. For high magnification analysis, embryos were coated a second time.

Sectioned material

Embryos were stained with rabbit anti-Twi (gift from S. Roth; Roth et al., 1989) and 3 μm-thick sections were generated according to Wieschaus and Sweeton (1988). Sections were photographed on a Zeiss Axioplan microscope with Nomarski optics.

RESULTS

Ectopically expressed *fog* can induce apical flattening

To determine the consequences of ubiquitous *fog* expression outside the mesodermal and endodermal primordia, we used transgenic flies carrying the *fog* cDNA under the control of the *hsp70* promoter (*hsfog*). When embryos carrying this transgene are heat shocked during late cellularization, all cells express *fog* mRNA (data not shown). The most immediate response of this expression is an apical flattening of all cells in the early gastrula, such that these cells appear tightly cohesive and lack their typical dome-shaped apical surfaces. (Fig. 1B,D) This effect on surface morphology is not observed in heat-shocked embryos that do not carry the *hsfog* transgene (Fig. 1A,C). Flattening occurs very soon after heat shocked embryos have cellularized: it is always observed by the time heat-shocked embryos have begun gastrulation, but never in embryos fixed during the cellularization process itself. Simultaneous with apical flattening, ectopic expression of *fog* disrupts the ordered hexagonal arrangement of cells on the surface on the embryo. The cells appear to be stretched or pulled in an isotropic fashion (Fig. 1C,D). Their overall appearance suggests that the forces that cause apical flattening also pull on adjacent cells. These forces appear to be uniform over the surface, such that no cells at this time are able to fully constrict.

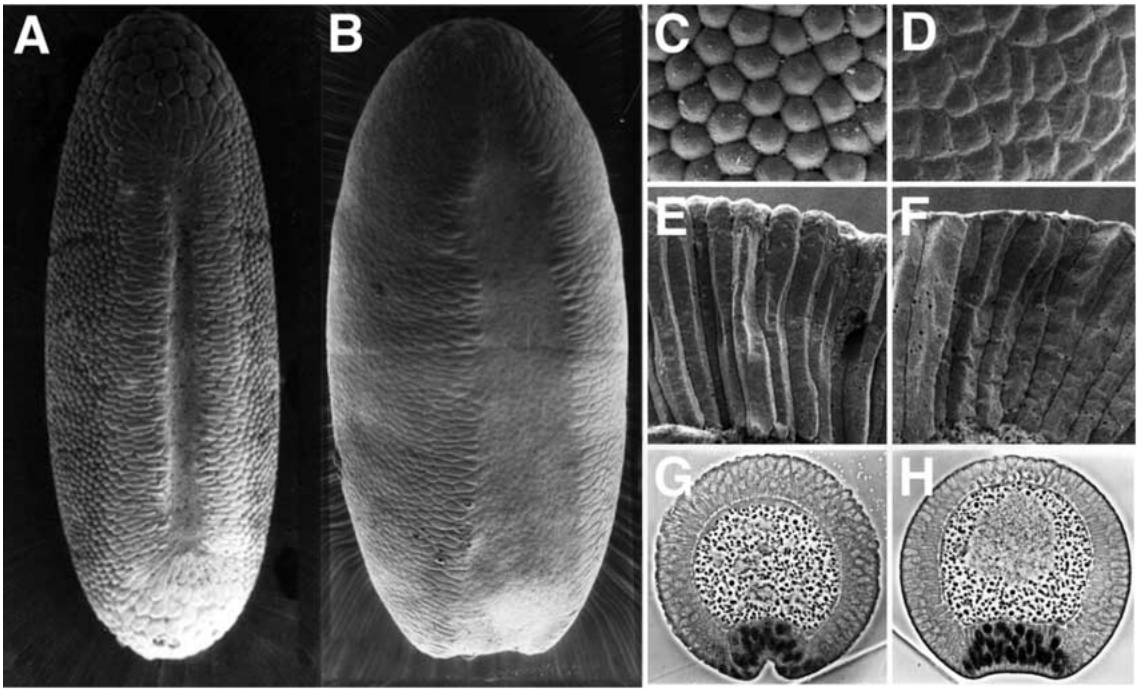

Fig. 1. Heat-shock induced *fog* expression induces apical flattening. (A) Ventral view of heat-shocked Ore-R gastrula. Only the cells on the ventral side of the embryo have lost their cobblestoned appearance; the most ventral of these have formed a shallow groove or furrow. (B) Ventral view of *hsfog* gastrula. The lateral cells are flattened and the ventral furrow is wider than normal. (C) High magnification surface view of heat-shocked Ore-R gastrula showing the dome-shaped apex of each cell. (D) High magnification surface view of heat-shocked *hsfog* gastrula. Cell apices are flattened and the cells have lost the regular hexagonal outlines seen in wild type. (E) Lateral view of cells in broken heat-shocked Ore-R gastrula. Cell apices are rounded. (F) Lateral view of cells in broken heat-shocked Ore-R gastrula. Cell apices are flattened and appear stretched. (G) Sectioned heat-shocked Ore-R gastrula stained with anti-TWI antibody. About three TWI-expressing cells in the right and left regions of the mesodermal primordium do not undergo apical constriction. (H) Sectioned *hsfog* gastrula stained with anti-TWIST antibody. All TWIST-expressing cells have undergone apical constriction.

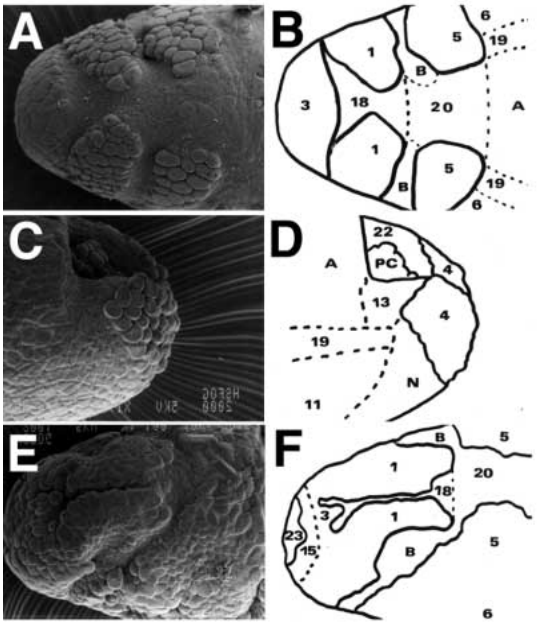

Fig. 2. Mitosis blocks the apical flattening produced by ectopic expression of *fog* during early gastrulation. (A) Dorso-anterior view of *hsfog* gastrula. The cells in mitotic domains MD 1, MD 3 and MD 5 (see B) have a rounded shape. The cells comprising other mitotic domains (MD A, B, 6, 18, 20, 19) show constricted apices and have lost their outlines. When the size of the constricted area is sufficiently small and it is surrounded by nonconstricting cells, it forms a shallow groove, e.g. MD B and 18. (B) Outlines and number designations of mitotic domains in A. (C) Posterior view of the same embryo in A. At this stage only MD4 is dividing, all other cells are quiescent and show a flattened aspect. Note the rounded appearance of cells in mitotic domain 4. (D) Outlines of the mitotic domains in C. PC, pole cells. (E) An older *hsfog* gastrula. Mitotic domains 18 and 20, 23 and B are surrounded by unconstricted cells and form furrows. MDA only comprises quiescent cells that have a flattened aspect; it does not form a furrow, possibly because of its larger size. The lateral ectopic furrows mark the boundaries between different clusters of MD11. The MD 1, 3, 4, 5, 6 and 15 comprise active mitotic cells. (F) Outlines of the mitotic domains in E.

Ectopically expressed *fog* can induce apical constriction

Although expression was induced at the blastoderm stage, the surface of *hsfog* embryos remains flattened until midgastrulation when groups of cells on the dorso-anterior side of the embryo acquire a round shape. (Fig. 2A). These groups of cells appear in the same sequence and spatial pattern as the mitotic domains described by Foe (1989) (Fig. 2B,D,F). In all the regions where two domains of dividing cells are separate but close to each other, the intervening cells constrict their apices and form shallow grooves with a morphology reminiscent of the VF. We propose that the increased apical surface area of the dividing cells as they round up releases the tension in the surface of the embryo, such that the cells located next to these mitotically active domains are able to fully constrict their apices. The furrows that are formed by these constrictions are transient and persist only until the cells forming them begin to divide.

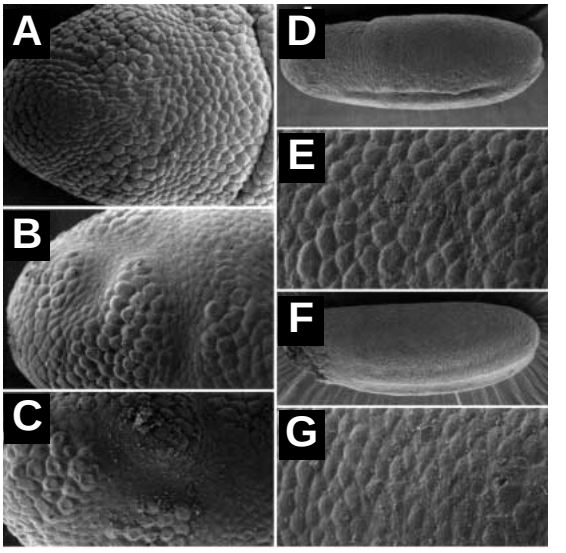

Fig. 3. Hyperactivation of *cta* mimics the *hsfog* phenotype. Embryos from wild-type mothers (A), mothers homozygous for a gain of function allele of *cta* (B, D, E) and wild-type embryos injected with cholera toxin (C, F, G). B and C are dorso-anterior views; D and F are lateral views; E and G show higher magnifications of the surface.

The *fog*-induced cell shape changes disrupt the morphogenetic movements that rely on cell shape changes

Although all cells in a heat-shocked embryo flatten in response to *fog* expression, other aspects of their behavior are not uniform. Mesodermal cells on the ventral side of the embryo still form a VF and the posteriormost cells still form a PMG. The morphology of these invaginations are subtly different from wild type, presumably reflecting the early, more uniform distribution of *fog* transcripts in each primordium after heat shock. Apical constrictions appear in the PMG primordium almost immediately after the completion of cellularization and, in contrast to wild-type embryos, invagination of the PMG is well advanced at a stage when the VF is still open. The VF is wider than in normal embryos and encompasses the entire width of the *twi*- or *sna*-expressing domain, rather than just the most medial 12 cells that normally express *fog* at high levels (Fig. 1G,H).

During early stages of gastrulation, wild-type embryos form additional folds including the cephalic furrow and the posterior and the anterior transverse folds. These are not classical invaginations because the cells that form these furrows stay at the surface of the embryo. However, like the VF these morphogenetic movements are triggered by cell shape changes (Turner and Mahowald, 1977; Costa et al., 1993). In embryos where *fog* is ubiquitously expressed, a shallow groove forms in the region of the cephalic furrow, but it does not form a proper furrow (compare Fig. 1A and B). Such embryos also fail to form anterior and posterior transverse folds on their dorsal sides. We suspect that the surface forces generated by *hsfog* induced apical flattening and constriction prevent the closure of these furrows. On the other hand, ubiquitous expression of *fog* does not appear to affect germ band extension, a process which involves cell rearrangement rather

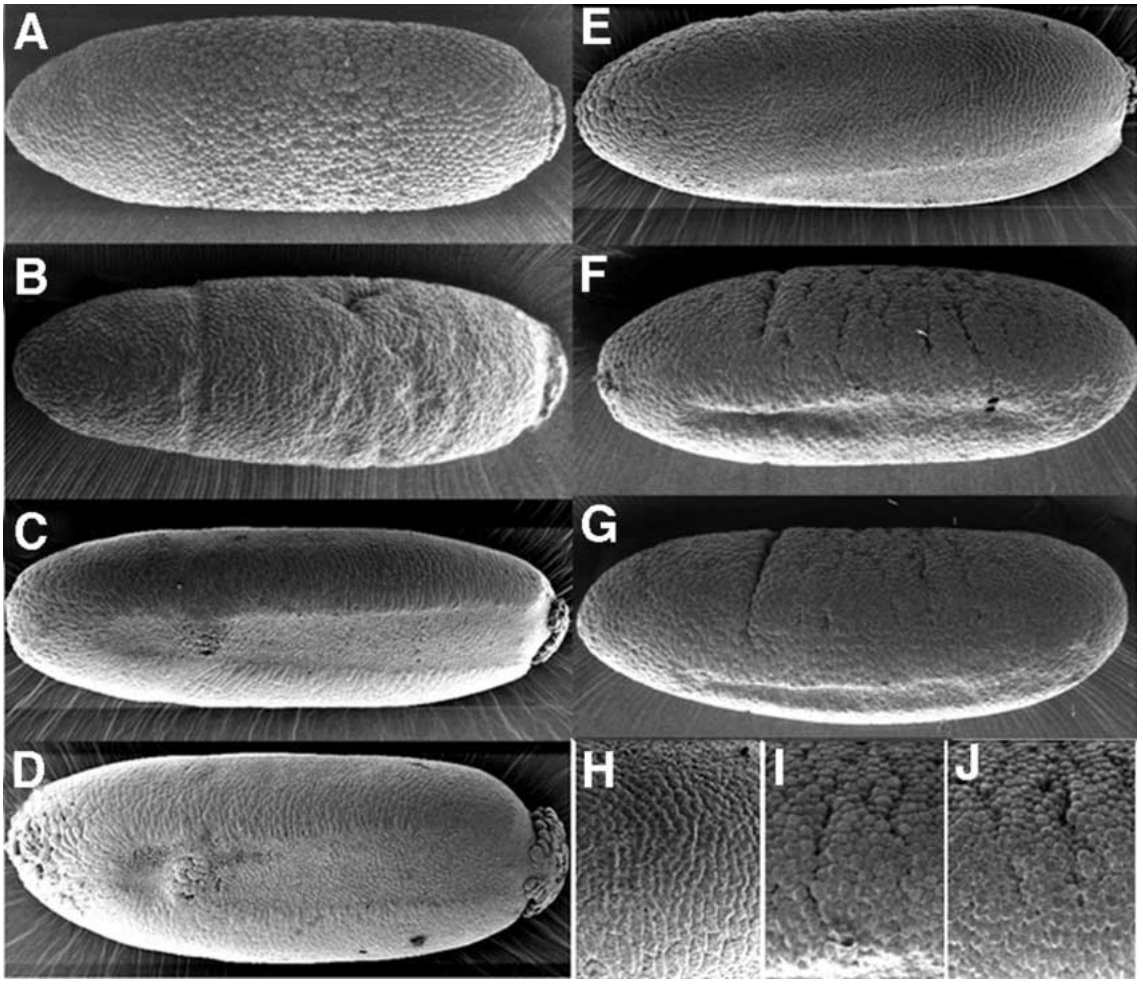

Fig. 4. *Hsfog* phenotype requires wild-type *cta* activity but does not require the presence of cells programmed to mesoderm fates. (A) Embryos laid by homozygous *dorsal* mothers lack mesoderm and all cells show a rounded appearance even upon heat shock. (B) Heat-shocked embryos carrying the *hsfog* transgene from *dorsal* mothers show a flattened stretched appearance around their entire circumference. (C) Wild-type embryos injected with cholera toxin show the features of *hsfog* phenotype. (D) *fog*– embryos injected with cholera toxin also show the hsfog. The efficiency of the cholera toxin injection in these experiments was 80%; the mutant *fog* embryos from the cross were identified by antibody staining. (E) *hsfog* embryos obtained by crossing fog^{4a6}/FM7 females with *hsfog* homozygous males. Upon heat shock, all the progeny from this cross show the characteristic *hsfog* phenotype, indicating that endogenous, zygotic *fog* activity is not required for the *hsfog* phenotype. (F) Heat-shocked embryos from homozygous *cta* mothers showing the *cta* phenotype. (G) Heat-shocked *hsfog*/+ embryos from homozygous *cta* mothers. Ectopic expression of *fog* has no morphological consequences in *cta* embryos. (H,I and J) Magnified views of lateral cells from embryos shown in E, F and G, respectively.

than changes in cell shape (Hartenstein and Campos-Ortega, 1985; Irvine and Wieschaus, 1994).

The constitutive activation of *cta* phenocopies the ectopic expression of *fog*

cta encodes an α-subunit of a heterotrimeric G protein of the G_α class 12 and 13 (Strathman et al., 1989; Strathman and Simon, 1990; Parks and Wieschaus, 1991; Wilkie and Yokoyama 1994). Little is known about this class of Gα proteins, but several studies have shown that they can be converted into a constitutively active form by single amino-acid changes that block the endogenous GTPase activity of the protein (Xu et al., 1993, 1994; Voyno-Yasenetskaya et al., 1994). We used in vitro mutagenesis to produce homologous changes into the *cta* cDNA (cta^{GOF}), which were then introduced into the fly as transgenes under the control of a female germ line specific promoter (Serano et al., 1994).

We examined these transgenes for effects on embryos during gastrulation. These alleles of *cta* induce all the features seen with ubiquitous expression of *fog* (overall flattening of the surface of the embryo, disruption of the regular array of cells, interference with the cephalic furrow formation and non responsiveness of mitotic cells), although the frequencies are low and somewhat variable (Fig. 3B,D,E). These phenotypes are observed in progeny of homozygous cta^{GOF} females at frequencies of about 10%, but were increased to 20% in embryos from females carrying four transgenes. Phenotypic frequencies were independent of the genotype of the father. Since these females also carry two wild-type copies of the

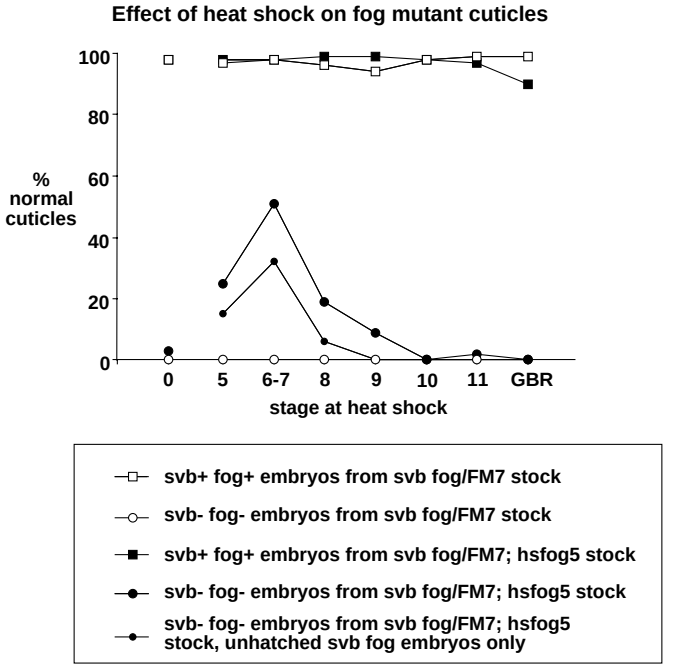

Fig. 5. Ubiquitous *fog* expression during late blastoderm and early gastrula stages rescues the final cuticle phenotype of *fog* embryos. Embryos were collected from *svb fog/FM7* and *svb fog/FM7; hsfog5* stocks and the experimental series subjected to a 10-minute heat shock. Cuticle phenotypes were scored in *svb* and *svb*$^{+}$ embryos and hatched larvae. Heat shocking during stages 6 or 7 provided the greatest degree of rescue. Rescued larval appeared normal. *svb fog* embryos without the *hsfog* transgene never showed any rescue.

endogenous gene, the transgenic alleles appear to be dominant, consistent with their proposed constitutive activity.

Some Gα proteins can be constitutively activated by cholera toxin. This toxin irreversibly modifies the protein by ADP-ribosylating a specific arginine residue required for its GTPase activity (Moss and Vaughan, 1977). A homologous arginine residue exists in the *cta* sequence, suggesting that *cta* is cholera toxin-sensitive. We have found that Drosophila embryos injected with cholera toxin at the syncytial blastoderm stage show a flattened cell surface and other morphological changes during gastrulation similar to those observed previously following heat shock induced expression of *fog* (Fig. 3C,F,G). The fraction of embryos showing apically flattened morphology (between 80% and 100%) is higher than that obtained with the in vitro mutagenized *cta* alleles but is qualitatively similar. Injection of cholera toxin does not induce apical flattening in *cta* null mutant embryos, suggesting that *cta* may be the only target of the toxin at this stage with respect to this phenotype.

Ubiquitous activation of the *fog/cta* pathway rescues mutant embryos

In the experiments reported so far, the effects of ectopic *fog* and constitutively active *cta* were assayed in embryos that carried wild-type alleles of each gene. Such embryos continue to express the endogenous *fog* gene in the ventral furrow and thus activation of pathway may not be uniform around the circumference. To exclude the possibility that the stretched appearance of cells in more lateral regions is a secondary consequence of the behavior of the ventral furrow cells, we examined *hsfog* embryos from mothers homozygous for *dorsal*. Although such embryos lack mesoderm and form no

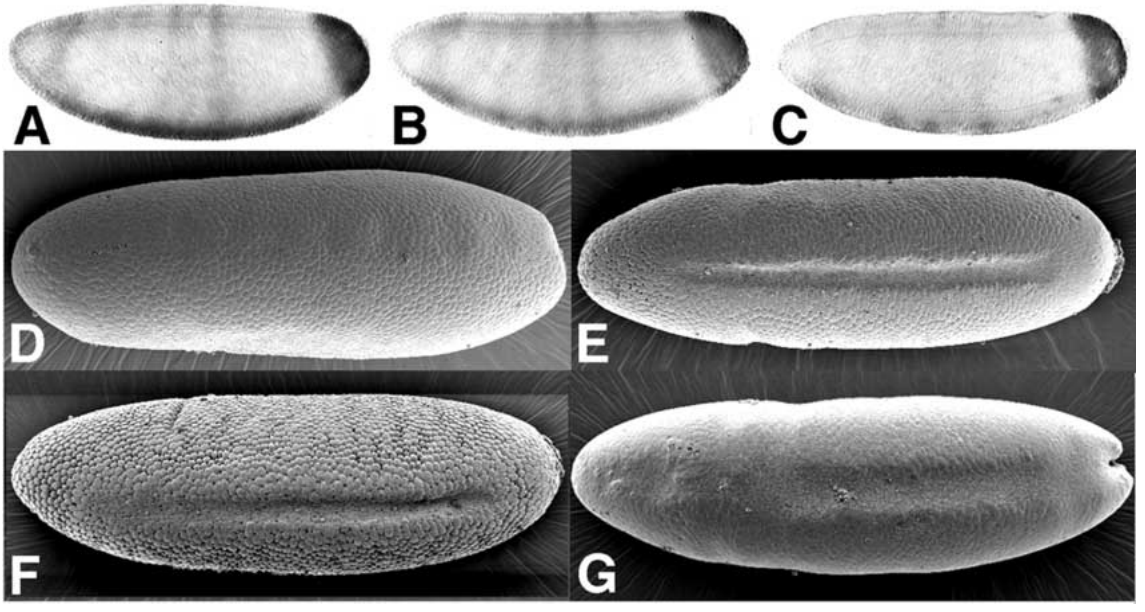

Fig. 6. Expression of *fog* and the consequences of ubiquitous *fog* expression in *twi* and *sna* embryos. *fog* RNA accumulation in wild-type (A), *sna* (B) and *twi* (C) embryos. In *sna* embryos, the level of *fog* RNA is similar to wild type, while the expression is reduced in *twi* embryos. (D) *hsfog* embryos mutant for *sna*. (E) *hsfog* embryos mutant for *twi*. Following heat shock, one quarter of the embryos from the *sna hsfog/Cyo* or *hsfog twi/Cyo* stocks show no phenotype and were assumed to be of the genotype *Cyo/Cyo* (F); one half of the embryos show the characteristic hsfog phenotype (genotype *sna*^{IIG05}, *hsfog/Cyo* or *twi*^{ID96} *hsfog/Cyo* (G); the remaining one quarter were assumed to be homozygous for sna or twi (see Results). *hsfog* embryos mutant for *twi* form a narrow ventral furrow (compare E with G) while *hsfog* embryos mutant for *sna* form no ventral furrow at all.

VF at all, uniform apical fattening and disorganization of the ordered arrangement of cells are still observed following heat-shock induced *fog* expression (Fig. 4A,B).

To investigate whether the localized endogenous activation of the *fog/cta* pathway contributes to the formation of the *hsfog* ventral furrow, we induced *hsfog* expression in *fog* mutant embryos. Such embryos show the same widened ventral furrow seen in wild-type embryos subjected to *hsfog* expression (Fig. 4E), indicating that endogenous *fog* expression does not contribute to the localized cell shape changes that occur following uniform heat shock induction (see below). We also scored embryos at the end of embryonic development using the marker *shavenbaby* to unambiguously distinguish genetically *fog* embryos from their heterozygous siblings. All three separately isolated *hsfog* transgenes partially suppress *fog* cuticle defects even without heat shock, suggesting that the lines have some low level expression at all temperatures. When expression is induced by heat shock during late cellularization or early gastrulation (stages 6 and 7), 30% of the *fog* mutants survived to hatching larvae and an additional 20% were of near normal morphology (Fig. 5). Despite the low efficiency of the transgene, the gain-of-function *cta* alleles also appear to rescue the sterility of *cta* homozygous females. A few of the embryos laid by these females even survive to adulthood (data not shown). Taken together, these results indicate that uniform activation of the *fog/cta* pathway in the absence of endogenous activity is not lethal to the embryo. In fact, uniform activation appears to compensate for activities missing in the mutant.

To position *fog* and *cta* relative to each other, we analyzed the effect of ectopic expression of *fog* on embryos mutant for *cta*. In such mutants, all aspects of the phenotype induced by ectopic expression are absent (Fig. 4F-J). These results indicate that CTA protein is required for cell shape changes induced by *hsfog* and thus may operate downstream of *fog* signaling in wild-type embryos. Although this model predicts that cta^{GOF}-induced cell shape changes should be FOG independent, the low efficiency of our cta^{GOF} alleles prevented our analyzing apical flattening in the fraction of embryos from the appropriate crosses that would be *fog*. We have however examined the effect of removing FOG on the artificial activation of CTA by cholera toxin injection. Cholera toxin induces apical flattening and all other aspects of *hsfog* phenotype with equal efficiently in wild type and *fog* embryos (Fig. 4C,D), indicating that the cell shape changes induced by cholera toxin do not require the presence of Fog protein and thus may function downstream of the FOG signal.

The VF observed in *hsfog* embryos is more sensitive to loss of *snail* than to loss of *twist*

Mesodermal cell fate in a wild-type gastrula depends on activity of two transcription factors, TWI and SNA. Although mutations in either gene affect *fog* transcription, they do so in different ways. *fog* transcripts normally accumulate at highest levels along the ventral midline and grade out towards the edge of the primordium (Costa et al., 1994). A similar graded distribution in the mesoderm is observed for *twi* (Alberga et al., 1991; Kosman et al., 1991; Leptin, 1991), and in *twi* mutant embryos, the mesoderm-specific expression of *fog* is abolished. The only remaining *fog* expression on the ventral side is continuous with the low level striped expression observed in lateral ectoderm (A. E. Christiansen, unpublished), as though the ventral cells have been shifted to a more lateral pathway. In *sna* mutant embryos, the pattern of *fog* expression seems unchanged, although the levels are clearly reduced (Fig. 6A-C). The stronger effect of *twi* on *fog* expression is consistent with the view that TWI functions as a positive regulator of transcription required for mesoderm specific gene expression, whereas SNA functions primarily to prevent expression of non-mesodermal gene in the primordium (Leptin, 1991; Ip et al., 1992; Gray et al., 1994).

In embryos with ectopic, heat-shock induced *fog* expression, a widened furrow forms on the ventral side of the embryo and mesodermal cells ultimately invaginate into the interior. The same phenotype is seen in *fog* mutant embryos subjected to ectopic *fog* expression, arguing that an additional component dictates the location and subsequent cell shape changes involved in mesoderm invagination. This additional component requires wild-type SNA activity, since no furrow forms in *sna* embryos with ectopic *fog* expression (Fig. 6C). Around the circumference of the embryo, cells show the same uniform apical flattening induced by *hsfog* in the lateral regions of wild-type embryos; no differences are observed between ventral and more laterally situated cells. The results obtained in *sna* embryos contrast with ability of uniformly expressed *hsfog* to rescue VF formation in *twi* embryos (Fig. 6E). The VF formed is narrower than that formed in wild-type embryos subjected to ectopic *fog* expression (Fig. 6G) and may correspond to the narrower domain of *sna* expression observed in *twi* mutants (Leptin, 1991). Thus it is possible that the *fog*-independent component important for VF formation is affected by *twi* mutants only to the extent that *twi* activity is required to maintain full *sna* expression.

DISCUSSION

The ability of Fog misexpression to produce apical flattening was somewhat surprising, given that cells of the ventral furrow flattened normally in *fog* mutants (Sweeton et al., 1991; Costa et al. 1994). The most obvious phenotype reported previously for *fog* was its effects on the subsequent apical constriction of the flattened cells. That *fog* affects both apical flattening and constriction suggests that the two processes may both involve the same fundamental mechanism, namely a contraction of the cortical actin-myosin network. In our favored model, the relevant actin cytoskeletal components for both flattening and constriction are initially organized under the dome-shaped apical surface of the cell at the completion of cellularization. As this dome is induced to constrict by the *fog* signaling pathway, the first morphological consequence is apical flattening, and the dome-like network resolves into a more disc-like shape. Further movement of myosin molecules leads to a progressive reduction of the diameter of the network resulting in apical constriction. Although both flattening and constriction would thus involve contraction of the same filamentous network, apical constriction is slower because it involves a greater displacement of the underlying cytoplasm, and because the forces exerted by neighboring cells on the individual cell make it harder to achieve than apical flattening.

This model is consistent with the results obtained with the ubiquitous activation of *fog/cta* pathway. In order for a cell in an epithelial sheet to constrict its apex, it must exert force on

its neighbors. If all cells in the sheet exert equal forces, a stable equilibrium will be established that should prevent any further changes in shape. Consistent with this view, ubiquitous *fog* expression initially produces no reduction in apical diameter. The irregular cellular outlines in such embryos, and the distortion of the cephalic furrow and dorsal transverse folds, suggest that their surfaces are under tension. In such embryos, every single cell is able to flatten its dome-shaped cortex, but none is able to reduce its apical diameter. Later, some cells are able to fully constrict their apices because they are located near a dividing mitotic domain and thus under less resistance from their neighbors.

In this model, *fog* expression in the VF primordium may contribute to apical flattening of the VF cells. The fact that *fog* activity is not required for this flattening suggests that some other partially redundant pathway also contributes to this cell shape change. Both the *fog* pathway and the alternate pathway may be essential for efficient apical constriction, but either may be sufficient for the initial flattening. Genes involved in the alternate pathway have not yet been identified but we suspect that, like *fog*, at least some may be regulated in ventral cells by mesodermal programming.

Mitosis prevents cell shape changes

The first 13th nuclear cycles of embryogenesis are more or less synchronous. In contrast, the mitoses which occur during gastrulation follow a precisely programmed spatial and temporal pattern (Foe, 1989). These mitotic domains are generally restricted to regions of the embryo not undergoing major morphogenetic movements, possibly allowing specific cell shape changes to occur in the nondividing cells. Support for this view was obtained by Edgar and O'Farrell (1990), who found that induction of mitosis in mesodermal cells by ectopic *string* expression temporally blocks invagination of the VF. Here, we show that cells lose the flattened appearance induced by ectopic *fog* expression when they enter mitosis, suggesting that apical constriction is only possible in nondividing cells. Mitosis may not be the only cellular process that takes precedence over apical constriction, since cellularizing embryos also do not appear to respond to ectopic *fog*. Both mitosis and cellularization involve major localized activities of the actin cytoskeleton. It is possible that *fog/cta*-induced cell shape changes compete for the same cytoskeletal components as cellularization and mitosis and that the latter processes take precedence. In contrast, the *fog*-induced apical flattening interferes with the cell shape changes that normally occur in the cephalic furrow and dorsal transverse folds. These two morphogenetic events involve concerted changes in individual cell shape; they contrast with germ band extension that involves cell rearrangement and is not affected by ectopic *fog* expression.

FOG is upstream of CTA in a pathway that is responsible for specific cell shape changes

During gastrulation in wild-type embryos, *fog* expression is predominantly localized to cells that will undergo cell shape changes and invagination (Costa et al., 1994). Here, we have shown that *fog* expression outside the invagination primordia is sufficient to induce apical flattening and constriction in cells that would not undergo those cell shape changes. For those cells to respond to ectopic *fog*, any downstream components necessary for the constriction must be ubiquitously expressed in the gastrula. This conclusion is consistent with the expression patterns of *fog* and *cta* (Costa et al., 1994; Parks and Wieschaus, 1991) and suggests, for example, that localized transcription of *fog* determines where in the embryo the *cta* Gα protein is activated.

The downstream position of *cta* in this model predicts that artificially activated forms of *cta* should produce cell shape changes independent of *fog*. To test this model we have constructed potential gain-of-function alleles of *cta*. Although these alleles have only a low variable penetrance, their dominant behavior and the fact that they can be phenocopied by injection of cholera toxin is consistent with their being gain-of-function mutations. The cell shape changes produced by these *cta* alleles occur in regions of the embryo where *fog* levels are normally very low or absent, consistent with the possibility that their activity may be downstream and therefore independent of *fog*. Cholera toxin produces a similar phenotype in the early embryo and appears to function by activation of CTA. By injecting the toxin into *fog* mutant embryos, we have found that these cell shape changes do not require Fog protein. These results suggest that activated CTA may also not require zygotic *fog* and thus may function downstream of FOG to control cell shape changes.

fog is sufficient to induce a furrow but cannot make an invagination

Our results suggest that all somatic cells of the early gastrula are able to respond to *fog* and flatten their apices. At the end of cellularization, high *fog* levels are normally restricted to the two regions that will undergo apical constriction and form the VF and PMG. This expression may facilitate local apical constriction and thus contribute to forming a normal invagination. In *fog* mutant embryos that express *fog* ubiquitously from the *hsfog* transgene, a widened furrow still forms on the ventral side of the embryo. Since *fog* expression is uniform in these embryos, the position and size of this furrow presumably reflects the localization of some other component involved in cell shape changes. These other localized components require SNA but not TWI, since ubiquitously expressed *fog* can induce a VF in a *twi* mutant but not in a *sna* mutant. Since SNA transcription factor acts as a repressor (Gray et al., 1994), the alternative pathway may be localized to the VF primordium by the repression of a gene or genes normally expressed in the ectoderm. Loss-of-function mutations in this gene might therefore not affect VF formation, although they might affect ectodermal differentiation.

Fog misexpression can also induce furrows in regions of an embryo where the associated cell shape changes are not normally programmed. Cells between active mitotic domains find themselves in a context similar to that existing in the VF primordium; i.e. a stripe of cells that can respond to *fog* surrounded by cells that do not. These conditions allow the cells to form furrows that can be quite deep (see MD18 in Fig. 2A), but cells in the furrow do not stay inside the embryo. In most cases reversal of the furrows occurs when cells in the furrow enter mitosis (MD18 in Fig. 2A-C). However, even in regions of the embryo where cells do not divide (e.g. MD B), furrows are still transitory and do not result in a permanent invagination. This suggests a second difference between the invagination primordia and other regions on the embryo. In

addition to multiple pathways that drive apical constriction, they possess components that allow those initial constrictions to result in an internalization of the constricted cells.

Uniform activation of *fog/cta* pathway is not lethal and does not alter the patterning of the blastoderm or the ultimate fate of affected cells. Different regions of the blastoderm do respond differently to ectopic *fog* expression, but these differences appear to reflect the morphogenetic events in which they are involved or the state of their cytoskeleton. Our results indicate that the *fog/cta* pathway does not define cell type identity but is rather directly involved in generation of a specific cell shape. We suspect that the other postulated components that account for flattening, apical constriction and permanent invagination in the absence of fog activity may be equally specific for cell shape and behavior rather than cell fate. Identifying these components represents the next major step in understanding how patterning at the blastoderm stage controls the cell behaviors observed at gastrulation.

We gratefully acknowledge the assistance of Joe Goodhouse in the SEM analysis and Gordon Gray in preparing fly food, as well as our colleagues at Princeton and Paris for their valuable comments on the manuscript. This research was supported by NIH grant 5R37HD15587 to E. F. W., as well as support from the Howard Hughes Medical Institute. A. E. C. and M. C. were supported in part by NIH training grant 5T32GM07312.

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