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Spatially Localized Rhomboid Is Required for Establishment of the Dorsal-Ventral Axis in Drosophila Oogenesis

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Summary

The establishment of dorsal-ventral asymmetry of the Drosophila embryo requires a group of genes that act maternally. None of the previously identified dorsal-ventral axis genes are known to produce asymmetrically localized gene products during oogenesis. We show that *rhomboid* (*rho*), a novel member of this group, encodes a protein that is localized on the apical surface of the dorsal-anterior follicle cells surrounding the oocyte. Loss of *rho* function causes ventralization of the eggshell and the embryo, whereas ectopic expression leads to dorsalization of both structures. Thus, spatially restricted rho is necessary and sufficient for dorsal-ventral axis formation. We propose, based on these observations and double mutant experiments, that the spatially restricted rho protein leads to selective activation of the epidermal growth factor receptor in the dorsal follicle cells and subsequently the specification of the dorsal follicle cells.

Introduction

The Drosophila embryo acquires polarity along its anterior–posterior and dorsal–ventral axes through the action of a large number of maternal genes (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991; Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988; Steward, 1987, 1989; Rushlow et al., 1989; Roth et al., 1989; for review see St Johnston and Nüsslein-Volhard, 1992; Anderson, 1987; Govind and Steward, 1991). The establishment of the dorsal–ventral axis can be divided roughly into three stages. First, communication between the oocyte and the surrounding somatic follicle cell layer leads to the asymmetric differentiation of the follicle cells along the dorsal–ventral axis (Schüpbach et al., 1991). Second, the follicle cells contribute to the appearance of a ventrally localized signal in the perivitelline space between the follicle cells and the oocyte (Anderson, 1987; Stein et al., 1991). Third, the signal triggers the establishment of the dorsal–ventral pattern of the embryo (Govind and Steward, 1991). The ventrally localized signal, which is thought to be generated from a uniformly distributed precursor by restricted proteolytic processing in the ventral perivitelline space (Stein et al., 1991; Stein and Nüsslein-Volhard, 1992; DeLotto and Spierer, 1986; Chasan and Anderson, 1989; Chasan et al., 1992; D. Schneider and K. Anderson, personal communication), is transmitted to the ventral side of the embryo through the uniformly distributed transmembrane protein Toll (Hashimoto et al., 1988, 1991). This leads to the selective nuclear import of the morphogen dorsal in the ventral region of the embryo (Rushlow et al., 1989; Roth et al., 1989; Steward, 1989).

The first stage of dorsal–ventral axis formation takes place during oogenesis and involves genes required either in somatic cells (Drosophila epidermal growth factor receptor *Egfr*/*DER* alleles include *torpedo* (*top*), *Ellipse*, and *faint little ball*; Schüpbach, 1987; Schejter and Shilo, 1989; Price et al., 1989; Baker and Rubin, 1989) or in the germline (*cappucino*, *spire*, *fs(1)K10*, *cornichon*, *gurken* [*grk*]; Manseau and Schüpbach, 1989; Wieschaus et al., 1978; Schüpbach and Wieschaus, 1986; Schüpbach et al., 1991). Although *fs(1)K10* is required in the germline, a loss-of-function mutation causes dorsalization of both the embryo and the follicle cell layer (Wieschaus et al., 1978; Wieschaus, 1979). In contrast, *Egfr* is required only in the soma, but loss-of-function mutations ventralize both the follicle cell layer and the embryo (Schüpbach, 1987). These and other results have led to a model in which *cappucino*, *spire*, *fs(1)K10*, *cornichon*, and *grk* act in the germline to produce a signal that is received by Egfr in the follicle cells and is required for the determination of the dorsal follicular epithelium as well as the formation of the dorsal–ventral axis of the embryo (Schüpbach et al., 1991).

None of the gene products required for dorsal–ventral axis formation are known to be themselves asymmetrically localized during oogenesis. Thus, where in the pathway the polarity originates and how it is maintained are not well understood.

Besides specification of the dorsal–ventral axis during oogenesis, *Egfr* acts at multiple stages during embryogenesis, often in apparent concert with three other genes: *rhomboid* (*rho*), *spitz*, and *Star* (the *spitz* group; Raz and Shilo, 1992; Rutledge et al., 1992). Mutation in any of these genes leads to a ventral midline defect, including loss of ventral cuticular structures and a failure to separate the axon tracts of two lateral longitudinal connectives in the central nervous system (Mayer and Nüsslein-Volhard, 1988; Klämbt et al., 1991; Raz and Shilo, 1992). These mutations also result in the selective loss of the two lateral chordotonal organs and possibly the precursor of these two sensory organs (Bier et al., 1990; Rutledge et al., 1992). Whereas the *spitz* product shows homology to epidermal growth factor (EGF) and transforming growth factor α and therefore is a potential ligand for *Egfr* (Rutledge et al., 1992), the *rho* product is most likely a transmembrane protein (Bier et al., 1990). Among this group of genes, only *rho* is known to be expressed exclusively in the cells that are affected by the mutations (Bier et al., 1990; Zak et al., 1990; Rutledge et al., 1992). Thus, the rho protein may

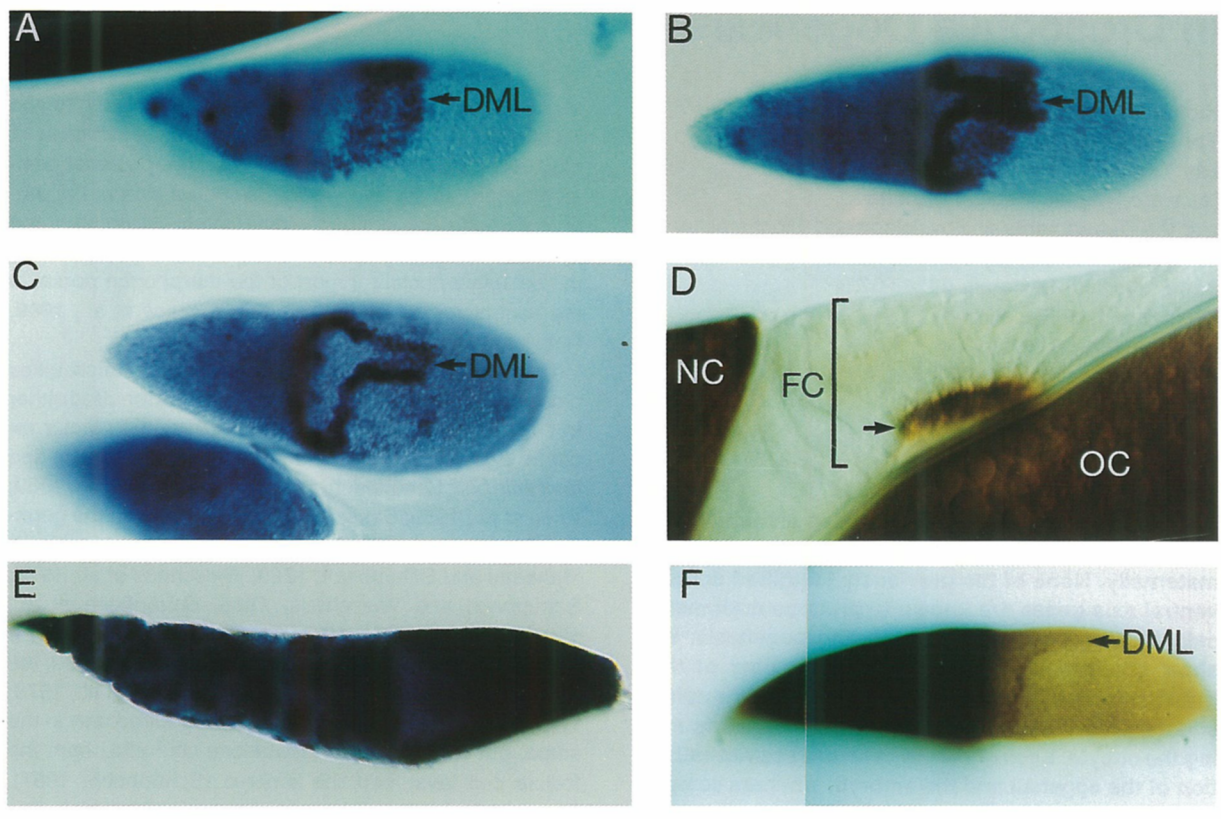


Figure 1. Rho Is Localized on the Apical Surface of Dorsal–Anterior Follicle Cells during Oogenesis

The distribution of *rho* mRNA in wild-type stage 9–10 egg chambers is restricted (A–C) in contrast with that of the HS(*rho*) transformant line (E). Overall expression of rho protein (F) is similar to the *rho* mRNA pattern. The subcellular distribution of rho protein in the dorsal–anterior follicle cells is further restricted to the apical surface (D). Anterior is to the left.

(A) In stage 9 egg chambers, *rho* is expressed in dorsal–anterior follicle cells at both sides of the dorsal midline (early expression pattern).

(B) The early expression pattern of *rho* undergoes dynamic changes. *rho* expression diminishes in the dorsal-most region, resulting in two stripes located on either side of the dorsal midline.

(C) By the end of stage 10 in oogenesis, *rho* is expressed only in two symmetric stripes of dorsal follicle cells and in a thin layer of follicle cells between the nurse cells and oocyte (late expression pattern).

(D) Rho protein is located on the apical surface of the epithelial follicle cells.

(E) Uniform expression of *rho* is detected in the follicle cells and germline in the HS(*rho*) line 30A after the induction of the HS promoter.

(F) Rho protein is expressed in dorsal–anterior follicle cells at both sides of the dorsal midline.

Abbreviations: DML, dorsal midline; NC, nurse cells; FC, follicle cells; OC, oocyte.

serve as a spatial cue for the site of action of these four genes.

Like *Egfr*, *spitz* and *Star* also play a role in oogenesis. While *Egfr* is required in the somatic follicle cells (Schüpbach, 1987), *spitz* and *Star* are required in the germline (Mayer and Nüsslein-Volhard, 1988). Thus, several of the *spitz* group genes appear to function in both embryonic patterning and oogenesis. There are precedents for a group of genes to function as a cassette in different developmental processes (Cagan and Ready, 1989; Hartenstein and Posakony, 1990; Ruohola et al., 1991; Corbin et al., 1991). Since *rho* is likely to provide the spatial cue for the *spitz* group in embryogenesis, we were prompted to examine the possibility that *rho* plays a similar role in oogenesis. Here we show that *rho* expression is both necessary and sufficient for the specification of dorsal follicle cells. We also report studies that suggest that *rho* may function as a positional cue for localized activation of an Egfr-dependent signaling pathway between the oocyte and follicle cells.

Results

Rho Is Expressed on the Apical Surface of Dorsal–Anterior Follicle Cells

In situ hybridization revealed that *rho* was expressed in both the somatic follicle cells and germline during oogenesis (Figures 1A–1C). Previous studies indicate that *rho* is not required in the germline for proper oogenesis because mosaic females with a germline homozygous for the rho^{7M43} mutation had no apparent defects in oogenesis (Mayer and Nüsslein-Volhard, 1988). Since we cannot rule out the possiblity that rho^{7M43} is not a complete loss-of-function mutation, we reinvestigated the possible function of *rho* in germline by generating germline clones with a null mutation $rho^{p\Delta 5}$ (Freeman et al., 1992). Mosaic females

Table 1. *rho* Function in the Germline Is Not Required for Oogenesis

rho^{pd5} GLC	N	NUF	NUH	N − NUF − NUH/N − NUF = Hatching Rate
A. x *yw/Y*				
Clone 1	50	10	3	37/40 = 93%
Clone 2	11	1	0	10/10 = 100%
Clone 3	71	2	2	67/69 = 97%
Clone 4	31	10	1	20/21 = 95%
Clone 5	8	2	0	6/6 = 100%
Clone 6	8	2	1	5/6 = 83%
Total	179	27	7	145/152 = 95%
B. x rho^{pd5}/*TM6B*				
Clone 1	124	11	61	52/113 = 46%
Clone 2	22	5	11	6/17 = 35%
Clone 3	6	2	2	2/4 = 50%
Clone 4	36	1	18	17/35 = 49%
Total	188	19	92	77/169 = 46%

Females carrying rho^{pd5} germline clones (see Experimental Procedures) were crossed either to rho^{+} (*yw;+/+*) (A) or to heterozygous *rho* null (rho^{pd5}/*TM6B*) (B) males. (A) Of the embryos that were derived from six females with a null *rho* mutation in their germline, 95% developed normally (column 5). Thus, *rho* is dispensable in the germline for proper oogenesis. (B) As a control, females having a *rho* null germline were crossed to males heterozygous for the *rho* null mutation. Approximately half of the resulting embryos failed to hatch. Importantly, the nonhatching embryos developed cuticle phenotypes indistinguishable from those of the zygotic *rho* mutant embryos (Mayer and Nüsslein-Volhard, 1988; Freeman et al., 1992). Thus, the germline clones are *rho* null, and the maternal function of *rho* does not appear to have a significant role in specifying the polarity. All eggs showed normal eggshell morphology. N is the total number of embryos produced in 3–6 days (mosaic females that lay fewer than five eggs in about 5–6 days are not counted in this table), NUF is the number of unfertilized eggs, NUH is the number of unhatched embryos (after 24 hr incubation at 25°C), and GLC is the germline clone.

with a germline homozygous for the *rho* null mutation had no defect in oogenesis (Table 1). Thus, *rho* in the germline does not play an important role in oogenesis. The following analysis will therefore focus on the function of *rho* in the follicle cells.

Expression of *rho* messenger RNA (mRNA) in the follicle cells was spatially and temporally restricted. In stage 9 egg chambers (staging is according to King, 1970, and Ashburner, 1989), *rho* was expressed in a broad group of follicle cells on the dorsal–anterior end of the egg chamber (Figure 1A). By stage 10, expression was restricted to two dorsal–anterior stripes corresponding to the position of future dorsal respiratory appendages (Figures 1B and 1C).

The rho protein was also detected in both the germline and the follicle cells. The overall distribution of rho protein in the egg chamber paralleled that of the mRNA (Figure 1F). The subcellular localization of rho protein in the follicle cells is shown in Figure 1D. Rho was localized predominantly on the apical (oocyte facing) surface of the dorsal–anterior cells (Figure 1D, arrow).

Antisense Expression of *rho* during Oogenesis Ventralizes the Eggshell and the Embryo

The early expression of *rho* in dorsal follicle cells suggests that it may play a role in the formation of the dorsal–ventral axis. Since null alleles of *rho* are embryonic lethal (Mayer and Nüsslein-Volhard, 1988; Bier et al., 1990; Freeman et al., 1992), the function of *rho* in the follicle cells was examined by expressing either antisense or sense constructs of *rho* under the control of the heat shock–inducible (*hsp70*) promoter.

To study the function of *rho* in oogenesis, we examined stable transformants containing an antisense *rho* construct under the control of the *hsp70* promoter, HS(anti-*rho*). Shifting adult animals to 39°C for 15 min led to a uniform expression of antisense *rho* RNA in the follicle cells (data not shown). The dorsal–ventral polarity of the follicle cells was determined by analyzing the outer layer of the eggshell, the chorion, which is secreted by the follicle cells and therefore serves as a footprint for these cells. The respiratory appendages, which are located in the dorsal–anterior end of the eggshell, are part of the chorion structure and serve as a marker for the fate of dorsal–anterior follicle cells (Figure 2C, arrows). The eggs laid 5–20 hr after the heat shock showed a ventralized eggshell phenotype (fused appendages in 50%–78% of the eggs; Figures 2A and 2E). These eggs were at stage 8–10 in oogenesis during the antisense RNA induction. This correlates with the onset of *rho* expression in wild-type flies (see Figure 1).

The heat shock treatment at stage 8–10 during oogenesis also resulted in embryonic lethality (Figure 2B). The polarity of the embryo is easily discerned in the larval cuticle, which is secreted by the ectoderm. The wild-type ventral cuticle contains eight abdominal denticle bands, while the dorsal cuticle contains fine hairs (Figure 2D). Most (85%–89%) of the HS(anti-*rho*) embryos collected 5–20 hr after the heat shock were either unfertilized or arrested very early in embryogenesis. However, a small group of the embryos (11%–15%) developed a cuticle. These cuticles were ventralized with an excess of ventral denticle band material at the expense of dorsal structures (Figure 2F). These phenotypes are reminiscent of those of two other maternal ventralizing mutations, *grk* and *top* (Schüpbach, 1987; see Figure 6), and suggest that *rho* is required for the formation of the dorsal–ventral axis of both the follicle cell layer and the oocyte.

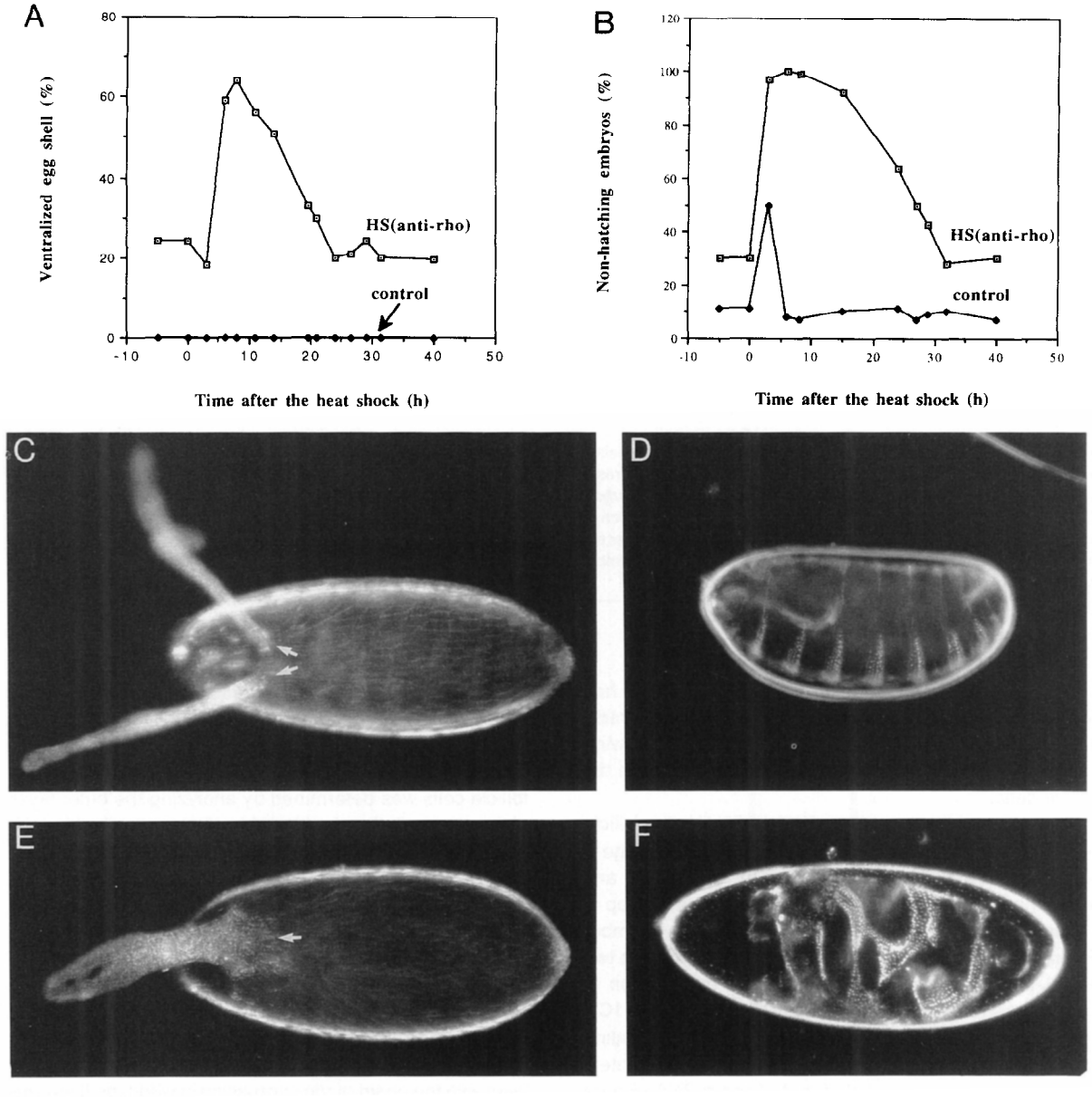


Figure 2. Antisense Expression of *rho* RNA Ventralizes the Eggshell and Embryo

(A and B) Time zero refers to the time when heat shock was performed. (A) The percentage of eggshells displaying the ventralization phenotype at different times after 15 min heat shock induction of HS(anti-*rho*) females (18-1/18-1;18-6/18-6). In the absence of heat shock, the ventralized eggshell phenotype was detected in 20% of the eggs, probably owing to a small amount of constitutive expression of antisense RNA in this line. No ventralized eggshells were detected in eggs laid by control animals (wild type) with or without the heat shock. (B) The percentage of eggs arrested in embryonic development at different times after heat shock of HS(anti-*rho*) (18-1/18-1;18-6/18-6) or control (wild-type) lines. In the first 20 hr after heat shock, only ~20% of the embryos develop to form a cuticle. A substantial fraction of the cuticles that did form 6–15 hr after heat shock showed a ventralized phenotype (at 6 hr, 67%; at 8 hr, 73%; at 15 hr, 89%).

(C–F) Dark-field micrographs of chorion (C and E) and embryonic cuticles inside vitelline membrane (D and F). (C) Characteristic features of the dorsal–anterior portion of the wild-type eggshell are the two respiratory appendages (arrows) flanking the dorsal midline. (E) Fusion of the two appendages, indicative of the loss of dorsal midline follicle cells, was detected in the eggs laid 5–20 hr after the heat shock. Of the eggs laid by 18-1/18-1;18-6/18-6 females 5–20 hr after the heat shock induction, 11%–15% were ventralized to varying degrees. An extreme case is shown in (F); an excess of ventral denticle band material was detected at the expense of dorsal structures (see wild-type embryo in [D]). The complete absence of dorsal structures differentiates the HS(anti-*rho*) phenotype from the ventralization phenotype observed in *Egfr* and *grk* mutants (Schüpbach, 1987). Anterior is to the left. (C) and (E) show the dorsal view and (D) and (F) the lateral view. Dorsal is up.

Ectopic Expression of *rho* during Oogenesis Dorsalizes the Eggshell and the Embryo

To date, the *rho* gene product is the only known component that is localized during oogenesis and required for dorsal–ventral axis formation (Figures 1 and 2). To determine whether this localization per se is important for dorsal–ventral asymmetry, we altered the expression pattern of *rho*. A *rho* cDNA encoding the full-length protein under

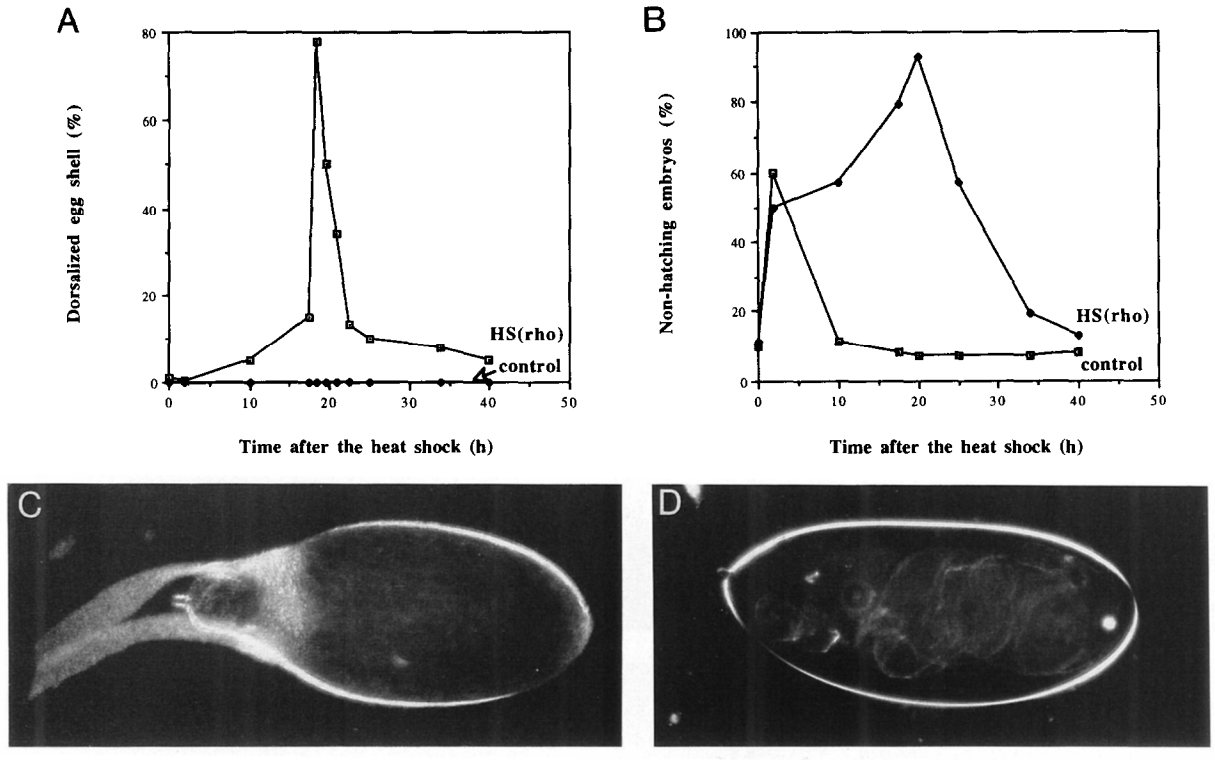


Figure 3. Ectopic Expression of *rho* during Oogenesis Dorsalizes the Eggshell and the Embryo

The eggshell (C) and embryonic (D) phenotypes are detected in the eggs laid by HS(*rho*) line 30A/30A about 20 hr after the 15 min heat shock. Of the eggs laid 18–20 hr after the heat shock, 50%–80% displayed dorsalized chorion (A), and either the dorsal respiratory appendages were further apart or the appendage material was expanded around the whole anterior part of the egg (C). Most of the embryos derived from these eggs did not hatch (B), and only dorsal cuticle was secreted in these embryos (D). Two groups were found among the nonhatching embryos (between 18 and 25 hr; shown in [B]). The first group (I) exhibited extreme dorsalized chorion and unfertilized embryonic structures (probably owing to the defective micropyle in the chorion), and the second group (II) had mildly dorsalized or wild-type chorion structures and dorsalized cuticle. The distribution between these two groups is as follows; 18 hr, 77% (I) and 23% (II); 20.5 hr, 62% (I) and 38% (II); 25 hr, 8% (I) and 70% (II). At the 25 hr time point, 22% of the nonhatching embryos had no apparent cuticle or eggshell defects. Anterior is to the left.

the control of the *hsp70* promoter HS(*rho*) was introduced into flies. Without heat shock, a normal pattern of *rho* expression was detected during oogenesis. After a 15 min heat shock to activate the *hsp70* promoter, strong uniform expression of *rho* was detected (see Figure 1E). Eggs laid 18–20 hr after the heat shock had dorsalized eggshells (Figures 3A and 3C) and gave rise to dorsalized embryos (Figures 3B and 3D). Of these eggs, 50%–80% exhibited expansion of the dorsal eggshell material at the expense of the ventral region (Figure 3C). Most of the eggs with a strong dorsalized eggshell phenotype were not fertilized, probably owing to defects in the micropyle, an anterior eggshell structure required for fertilization (a phenotype comparable to that of *fs(1)K10*). Where fertilization was successful, the embryos were dorsalized (Figure 3D). In some cases, stronger dorsalization was detected in the anterior region of the embryo than in the posterior (this may reflect the dorsal–anterior location of the oocyte nucleus; see Discussion). A similar anterior–posterior gradient in the strength of dorsalization has been observed in *fs(1)K10* mutant embryos (Wieschaus, 1979). The large percentage of nonhatching embryos (Figure 3B) reflects both the failure of fertilization and the dorsalization of the embryo.

Expression of antisense *rho* had a more prolonged effect on dorsal–ventral axis formation than ectopic *rho* expression did (compare Figures 2 and 3). It may be necessary to have ectopic *rho* expression early (say, during the triggering of an autofeedback loop; see Discussion) to alter dorsal–ventral axis formation, while rho protein function may be required for a much longer period so that a mutant phenotype would result from antisense expression throughout that period.

The eggshell and cuticle patterns represent terminally differentiated phenotypes due to defects in the follicle cell layer and the embryo, respectively. The earliest stage at which ectopic *rho* expression caused a detectable phenotype was revealed by examining the β-galactosidase expression patterns in the *l(3)6D1* enhancer trap line. *l(3)6D1* is a P-lacW-induced lethal line (Bier et al., 1989). Heterozygous females of this line show β-galactosidase staining in the dorsal follicle cell population. Compared with the normal pattern of a group of dorsal–anterior follicle cells expressing β-galactosidase at stage 10 in oogenesis (Figure 4A), heat shock–induced expression of *rho* led to an expansion of these β-galactosidase-expressing follicle cells at stage 10 (Figure 4C). Since molecular markers for the ventral follicle cells do not exist at present, the fate of

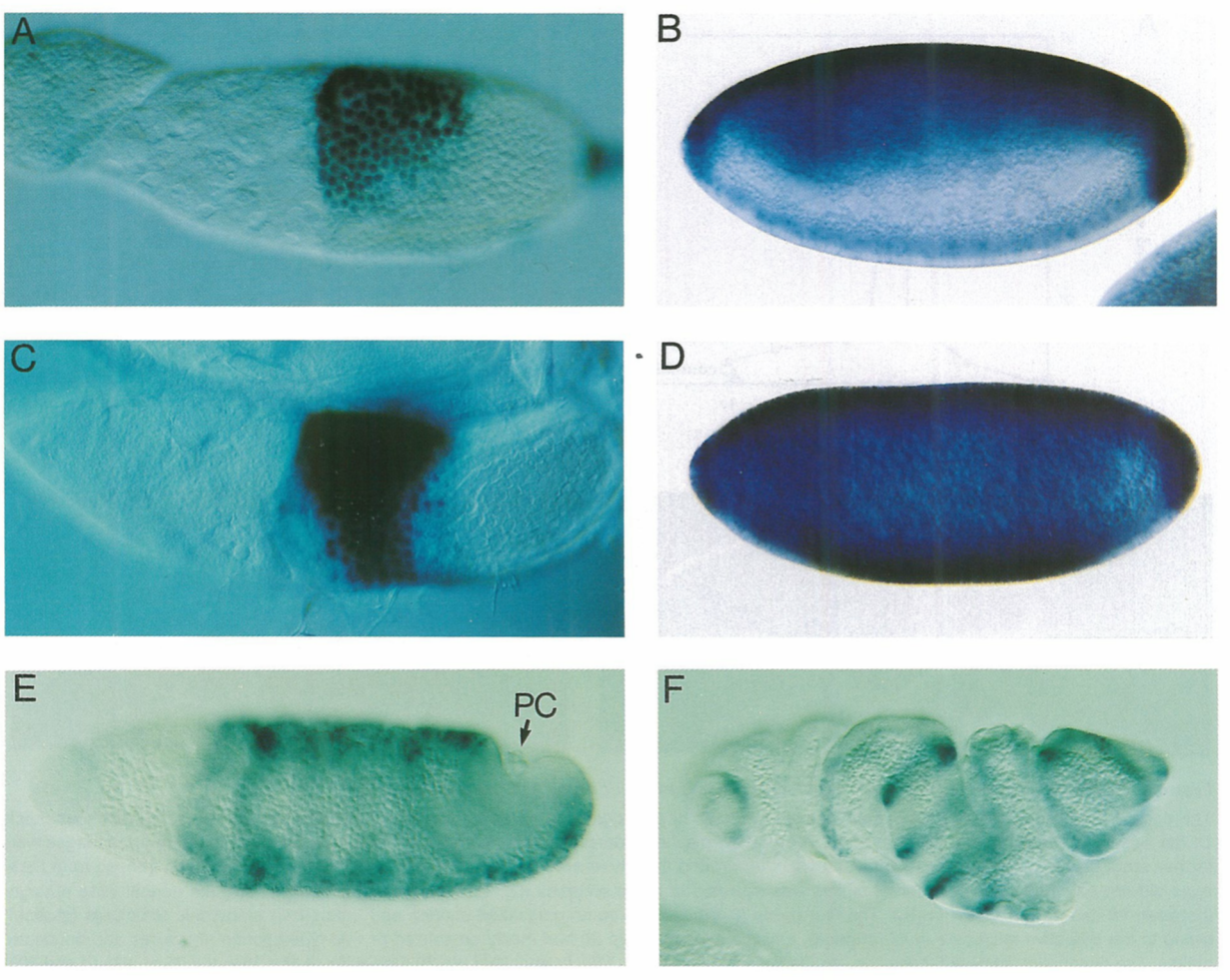


Figure 4. Ectopic Expression of *rho* Dorsalizes the Follicle Cells and the Early Embryo

Dorsal–anterior follicle cells in stage 10 egg chambers are marked by X-Gal staining in the enhancer trap line *l(3)6D1* (A). In an egg chamber of the HS(*rho*)*30A*/*l(3)6D1* line, an expansion of the X-Gal staining cells was detected 10 hr after the heat shock (C). At this stage, the X-Gal staining dorsal follicle cells surrounded the whole egg chamber. (B and D–F) The expression pattern of *dpp* was revealed by whole-mount in situ hybridization in embryos laid by wild-type (B) or HS(*rho*)*30A* females (D–F) after the heat shock induction (18–20 hr). The typical dorsal expression of *dpp* was detected in the wild-type embryos (B). However, the region of *dpp*-expressing cells was already expanded in the blastoderm stage in HS(*rho*) embryos (D). During early gastrulation and pole cell (PC) migration (stage 6), the anterior and posterior transverse furrows were expanded to the ventral side in HS(*rho*) embryos (E), resulting in a totally dorsalized embryo (F). Anterior is to the left and dorsal to the top of the page.

the ventral domain cannot be addressed independently. In any case, ectopic *rho* must act at or before stage 10 in oogenesis.

Early markers for the dorsal–ventral axis of the embryo include *tolloid* (*tld*), *decapentaplegic* (*dpp*), and *twist* (*twi*) (Shimell et al., 1991; St Johnston and Gelbart, 1987; Ray et al., 1991; Thisse et al., 1987). These zygotic patterning genes are initially expressed either dorsally (*tld* and *dpp*) or ventrally (*twi*) and are downstream of *dorsal*. In HS(*rho*) embryos, the normally dorsal expression of *dpp* (Figure 4B) was expanded into the ventral region of early blastoderm and later embryos (Figures 4D–4F). Similar results were obtained when *tld* was used as the marker for the dorsal cells (data not shown). The expansion of the dorsal tissue was attained at the expense of the ventral region of the embryo as revealed by the loss of *twi* staining (data not shown). Thus, ectopic *rho* expression during oogenesis alters the dorsal–ventral axis in the early embryo.

rho Acts Upstream of *Toll*

To determine whether *rho* acts in the known pathway for dorsal–ventral axis formation (Anderson, 1987; Govind and Steward, 1991), we constructed a double mutant of HS(*rho*) and a dominant ventralizing allele of *Toll*, *Toll*^{9Q} (Anderson et al., 1985; Schneider et al., 1991). Although some of the eggs laid by the double mutant 18–20 hr after the heat shock treatment had dorsalized eggshells (the HS(*rho*) phenotype; Figure 5C) that were clearly different from those derived from *Toll*^{9Q} mothers (Figure 5A), the resulting embryos were either ventralized (the *Toll*^{9Q} phenotype; Figure 5B) or lateralized (Figure 5D). Lateralized embryos are also obtained in double mutants of *Toll*^{9Q} and

the upstream gene *nudel*, *pipe*, or *snake* (Anderson et al., 1985). The gain-of-function mutation in *Toll* thus renders the embryo largely insensitive to the changes in the follicle cell fates induced by ectopic *rho* expression. These results indicate that *rho* functions upstream of *Toll*.

rho Action Requires Functional *grk* and *Egfr*

Three genes are known to act upstream of *Toll* and exert early maternal effects on the dorsal–ventral axis of both the eggshell and the embryo, namely *fs(1)K10*, *grk*, and *Egfr* (*top* is an allele of *Egfr* that specifically affects oogenesis). *top* and mutations in *grk* cause a ventralization of the follicle cell epithelium. These mutations block the dorsalization normally observed in *fs(1)K10* mutants, suggesting that they act downstream of *fs(1)K10*. Whereas *fs(1)K10* and *grk* act in the oocyte, *Egfr* acts in the follicle cells. Thus, the dorsal–ventral pattern appears to depend on the transfer of spatial information from the germline to the somatic follicle cells (Wieschaus et al., 1978; Schüpbach, 1987; Cheung et al., 1992; Prost et al., 1988).

To determine whether ectopically expressed *rho* requires the function of *grk*, *Egfr*, or both to dorsalize follicle cells, we investigated the HS(*rho*) phenotype in *grk* or *top* mutant backgrounds. The eggs laid by *grk;*HS(*rho*) and *top;*HS(*rho*) females showed ventralization of both eggshell (Figures 5G and 5K) and embryo (Figures 5H and 5L). These phenotypes are similar to those observed in *grk* (Figures 5E and 5F) and *top* (Figures 5I and 5J) single mutants and very different from the HS(*rho*) mutant phenotype (see Figures 3C and 3D). This epistatic relationship indicates that either *rho* is upstream of *grk* and *Egfr* or that *rho* acts together with *grk* and *Egfr* in receiving the dorsal–ventral positional information provided from the oocyte to the follicle cells. In any event, *rho* action clearly requires the *grk–top* signaling pathway from the oocyte to the follicle cells.

Effect of *fs(1)K10*, *grk*, and *Egfr* on the *rho* Expression Pattern

Genes that act upstream of *rho* might alter the expression pattern of *rho* RNA. To look for such effects, we examined *rho* expression in *fs(1)K10*, *grk*, and *Egfr* mutants. Only 1 of the 3 mutants, *fs(1)K10*, affected the early *rho* expression pattern. The *fs(1)K10* mutation is known to cause an expansion of follicle cells that assume the dorsal fate, as indicated by the chorion phenotype (Wieschaus et al., 1978; Figure 6C). A similar expansion of the early *rho* expression pattern (83% of the early egg chambers; Figure 6A) as well as an expansion of the two stripes of *rho* expression later during oogenesis was observed (Figure 6B). This result suggests that *fs(1)K10* acts upstream of *rho*.

In contrast with *fs(1)K10*, the *grk* and *Egfr* mutations did not affect the early dorsal expression of *rho*, but caused considerable restriction of the late expression pattern. Instead of the two stripes (see Figure 1C), only one or no dorsal stripes were observed (Figures 6D and 6F). A similar restriction of the dorsal region of the eggshell was also detected (Figures 6E and 6G). These results suggest that the early and late *rho* expression patterns are differentially regulated. The later, more restricted expression pattern may play a more specialized role in the formation of the dorsal appendages. Since the *grk* and *Egfr* alleles used in this study are not null alleles, however, it is unclear whether the early *rho* expression pattern is completely independent of these genes. (The effects of stronger *grk* and *Egfr* alleles on *rho* expression could not be tested because these alleles affect oogenesis at much earlier stages.) Importantly, the changes in the *rho* expression pattern mimic the changes in the anterior eggshell phenotype in *fs(1)K10*, *top*, and *grk* mutants, extending the correlation between *rho* expression and the dorsal fate.

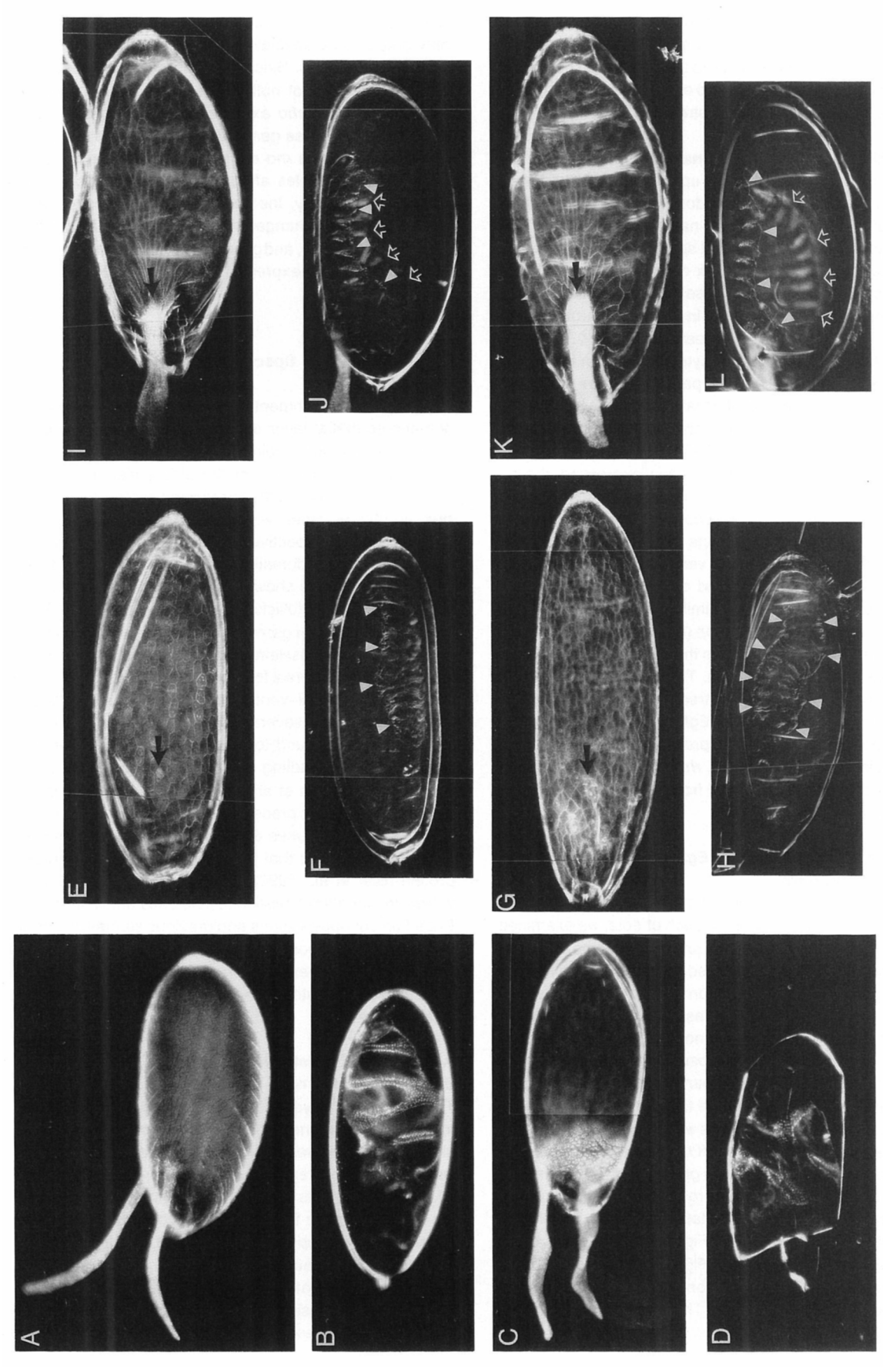
Discussion

rho Is Required to Specify the Dorsal Follicle Cell Fate

The proper establishment of dorsal–ventral polarity in the somatic epithelial layer of follicle cells is a prerequisite for proper embryonic polarity. We have shown that *rho* is necessary and sufficient for the differentiation of dorsal follicle cells. Antisense *rho* expression ventralizes the embryo and the eggshell (which derive from the oocyte and the follicle cells, respectively). Conversely, ectopic expression of *rho* causes dorsalization of both embryo and eggshell. We have also shown that *rho* is expressed both in the dorsal–anterior follicle cells and the germ cells. Since the function of *rho* in germ cells is not required for oogenesis (Mayer and Nüsslein-Volhard, 1988; this study), the *rho* expression in dorsal follicle cells is likely to be required to specify the dorsal–ventral axis of both follicle cells and the embryo. While several genes required in the follicle cells have been found to affect eggshell formation (the chorion genes; Spradling, 1992) or embryonic polarity (Anderson, 1987; Stein et al., 1991), only *Egfr* is previously known to affect both processes (Schüpbach, 1987). Given that *rho* action requires *Egfr* in the follicle cells and *grk* in the germline and that *rho* encodes a transmembrane protein (Bier et al., 1990), it seems likely that rho is involved in signaling between follicle cells and the oocyte. Based on our observations and previous studies, we present the following models for the establishment of the dorsally restricted *rho* expression pattern (Figure 7A) and for the action of rho protein in the specification of dorsal follicle cells (Figure 7B).

A Model for the Establishment of the *rho* Expression Pattern

The first sign of asymmetry along the oocyte dorsal–ventral axis is the asymmetric positioning of the oocyte nucleus near what eventually becomes the dorsal–anterior surface of the oocyte (King, 1970; Ashburner, 1989; Figure 7A, diagram 1). This initial asymmetry may originate simply from stochastic fluctuation and could be maintained by microtubule trapping (Gutzeit, 1986). The fs(1)K10 protein in the oocyte nucleus (Prost et al., 1988; Montell et al., 1991) may negatively regulate a diffusible factor that induces *rho* expression in the follicle cells (Figure 7A, diagram 2). In the presence of wild-type fs(1)K10, only the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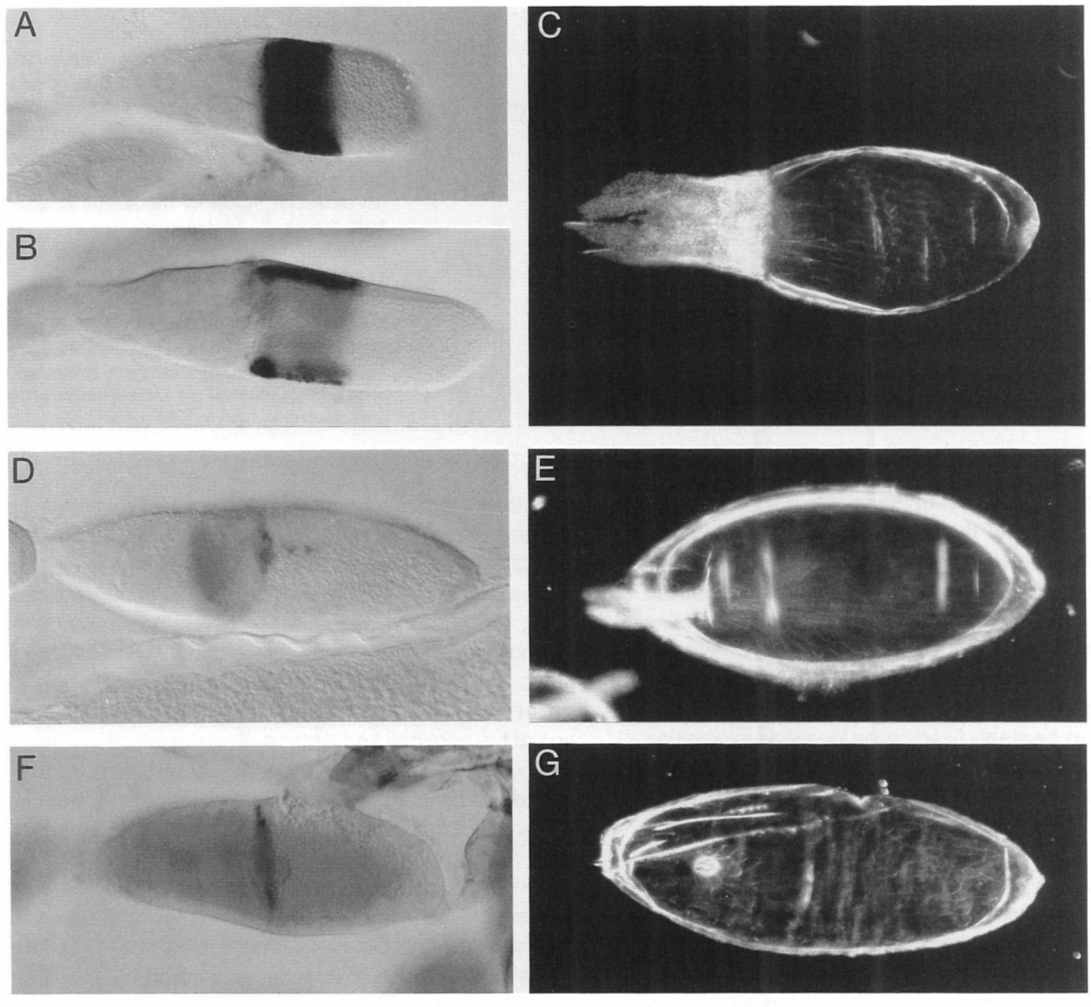

Figure 6. *fs(1)K10* Is Required for the *rho* Early Expression Pattern

(A, B, D, and F) The distribution of *rho* RNA as revealed by the whole-mount in situ hybridization of stage 9–10 *fs(1)K10* (A and B), *top* (D), and *grk* (F) mutant egg chambers. The chorion structures of the *fs(1)K10* (C), *top* (E), and *grk* (G) embryos are shown in dark-field micrographs. Early (A) and late (B) expression patterns of *rho* are expanded in *fs(1)K10* mutant egg chambers (compare with Figures 1A and 1C, respectively) in a manner similar to the expansion of the dorsal appendage region in the dorsalized *fs(1)K10* mutant chorion ([C]; Wieschaus et al., 1978). The late expression pattern of *rho* in *top* mutant egg chambers is restricted to a discontinuous stripe of the dorsal follicle cells that is one cell layer wide (D). The ventralization of the follicle cell layer is detected as fused appendages in the chorion structure ([E]; Schüpbach, 1987). The *rho* stripes are missing from the dorsal follicle cells in *grk* mutant egg chambers (F), indicating the loss of the dorsal-most follicle cells. This loss is also detected as missing respiratory appendages in the chorion preparation ([G]; Schüpbach, 1987). (A–G) shows a dorsal view, with anterior to the left.

Figure 5. *Toll*, *grk*, and *Egfr* Are Required for the Action of Ectopic *rho*

Chorion and cuticle preparations of the eggs laid 18–25 hr after the heat shock induction by Tl^{pQ}/TM3 (A and B), Tl^{pQ}/HS(*rho*)*30A* (C and D), grk^{HG21}/grk^{HK36} (E and F), grk^{HG21}/grk^{HK36};HS(*rho*)*30A*/+ (G and H), top^{1}/top^{1} (I and J), and top^{1}/top^{1};HS(*rho*)*27A*/+ (K and L) females. Anterior is to the left. Some of the eggshells are dorsalized in (C) but not in (A), indicating the dorsalizing effect of ectopic *rho* on follicle cells. Of the embryos laid by Tl^{pQ}/HS(*rho*)*30A* females, 59% are somewhat lateralized (L1; Anderson et al., 1985), exhibiting stronger lateralization in the anterior than in the posterior end (D). The remainder of the embryos were ventralized, similar to the Tl^{pQ} phenotype (D). The eggs laid by *grk;*HS(*rho*) and *top;*HS(*rho*) females showed ventralization of both eggshell (G and K) and embryo (H and L). These phenotypes are similar to those observed in *grk* (E and F) and *top* (I and J) single mutants and very different from the HS(*rho*) single mutant (Figures 3C and 3D) phenotype. No apparent dorsalization was detected in the eggs laid by *grk;*HS(*rho*) and *top;*HS(*rho*) females at any time point between 0–48 hr after the heat shock. Thus, the dorsalizing effect of ectopic *rho* on the oocyte requires the function of *Toll*, *grk*, and *Egfr* (*top* is an allele of *Egfr* that specifically affects oogenesis). In the case of *top* and *top;*HS(*rho*) mutants, similar variability of the embryonic phenotypes was detected: ~60% of the embryos showed extreme ventralization (L and J), and ~40% of the embryos showed milder ventralization (smaller or no holes on the ventral side). The arrows indicate a fused appendage (I and K) or largely reduced appendage material (E and G). The cuticles in (F), (H), (J), and (L) are not wide enough to surround the entire embryonic circumference, presumably owing to the expansion of mesoderm (Schüpbach, 1987). A stripe of dorsal cuticle is flanked on either side by bands of ventral setae. The closed arrowheads indicate focused cuticle edges (F, J, H, and L); the open arrowheads show edges on deeper focal planes (J and L).

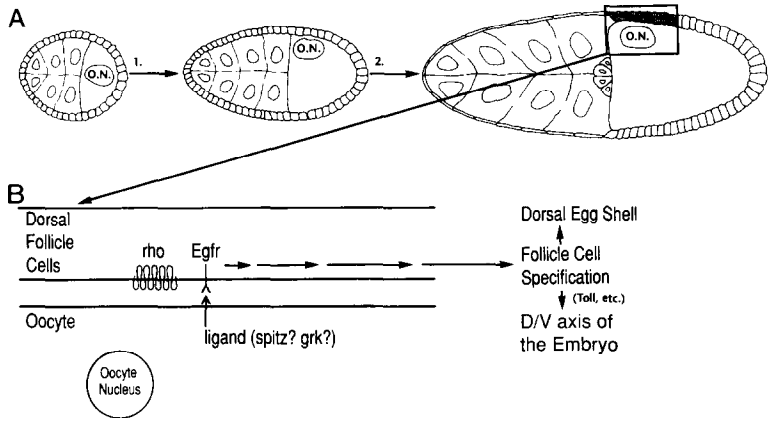

Figure 7. Model for the Role of Rho in Dorsal–Ventral Axis Formation

(A) The establishment of the *rho* pattern. Stochastic fluctuation brings the oocyte nucleus close to the follicle cell layer. A signal regulated by *fs(1)K10* then induces *rho* expression in the nearest follicle cells. These follicle cells subsequently become committed to the dorsal fate.

(B) Molecular model for rho action. A ligand from the oocyte (possibly grk [Manseau and Schüpbach, 1989] or the EGF homolog spitz) interacts with Egfr on the follicle cells. Rho on the surface of the dorsal follicle cells increases the strength of this interaction. High levels of signal cause the follicle cells to adopt the dorsal fate and subsequently to secrete dorsal eggshell and to influence the establishment of the dorsal–ventral (D/V) axis of the embryo.

follicle cells that are closest to the oocyte nucleus receive sufficient quantities of this *rho*-inducing factor, but in a *fs(1)K10* mutant much larger amounts of the factor are made so that most or all anterior follicle cells are induced to express *rho*. The expression of *rho* then serves to define the dorsal follicle cells and, as a consequence, the dorsal region of the oocyte. The weak dorsal–ventral asymmetry in the oocyte, which may originate in stochastic fluctuation, would thus be stably fixed by the localized expression of *rho* in the dorsal follicle cells.

The dorsal–ventral asymmetry in *rho* expression in the follicle cell layer is more extreme than might be expected from the relatively small offset in the position of the oocyte nucleus. Rho may function as part of a positive feedback loop that serves to accentuate the initial asymmetry of the oocyte. We hypothesize that rho, once transported to the follicle cell plasma membrane opposing the oocyte, could increase the strength of signaling generated by the *rho*-inducing ligand emanating from the oocyte. Such a positive feedback mechanism could convert a weak gradient in the strength of the signal from the oocyte into a much steeper gradient in the follicle cells (Schüpbach et al., 1991).

Spatial Regulation of Egfr Activation by Rho?

We propose the following molecular model for the rho-dependent signaling between follicle cells and oocyte (Figure 7B). An EGF homolog is produced by the oocyte and interacts with Egfr in the follicle cells. The presence of rho in the dorsal–anterior follicle cells is required to initiate or potentiate this ligand–receptor interaction. Interestingly, rho protein is detected in vesicular structures (Freeman et al., 1992), reminiscent of the vesicles carrying internalized boss–sevenless ligand–receptor complexes in the R7 precursor cells (Krämer et al., 1991). Other components of this signal transduction pathway include grk and possibly the EGF homolog spitz (Rutledge et al., 1992). Although *spitz* function in the germline is required for oogenesis (Mayer and Nüsslein-Volhard, 1988), it is not yet known whether it is required for dorsal–ventral axis formation.

The wild-type *rho* expression pattern, the phenotypes generated by antisense and ectopic *rho* expression, and the double mutant phenotypes are all consistent with the hypothesis that *rho* functions by spatially regulating a signaling pathway involving *Egfr*. The exclusive expression of *rho* in dorsal follicle cells of wild-type egg chambers allows only those cells to take on the fate of dorsal follicle cells, owing to the ligand–receptor interaction mediated by rho and Egfr. Ectopic expression of *rho* in all follicle cells, on the other hand, causes all follicle cells to take on the dorsal fate and consequently leads to the dorsalization of the eggshell and embryo. This dorsalization phenotype requires the action of *grk* for the production of the ligand and *Egfr* for the function of the receptor. As a consequence, ectopic expression of *rho* in *grk* or *Egfr* mutants does not affect the ventralization phenotype of these mutants. Removal or reduction of *rho* function by antisense expression weakens the ligand–receptor interaction required for the specification of dorsal follicle cells, resulting in the ventralization of eggshell and embryo.

Does *Egfr* also function in the initial signaling pathway from oocyte to follicle cells that leads to the establishment of the *rho* expression pattern? One scenario is that the signal from the oocyte activates Egfr to induce *rho* expression. The induced rho protein in turn increases the strength of signaling through Egfr, resulting in a positive feedback loop. The higher level of *rho*-inducing signal at the dorsal side of the oocyte (due to the dorsal location of the oocyte nucleus) could lead to weak activation of Egfr and subsequently the positive feedback loop in the dorsal but not in the ventral follicle cells. Only the dorsal follicle cells would therefore express high levels of rho protein and receive sufficient signal to take on the fate of dorsal follicle cells. One prediction of this model is that the early *rho* expression depends on *Egfr* function. Although the early *rho* expression was not significantly altered in *top* mutants, it is possible that a null mutation, unlike the hypomorphic *top* allele, affects early *rho* expression. This remains to be tested in mosaic studies. At the moment we cannot distinguish between this model and the possibility that a hitherto unidentified pathway is involved in the establishment of the *rho* expression pattern and that *Egfr* functions only in the subsequent signaling pathway required for follicle cell fate specification.

How might rho spatially regulate the EGF receptor? One possibility is that rho protein increases the adhesion be-

tween oocyte and follicle cells and thus indirectly facilitates signaling through Egfr. A second possibility is that rho is directly involved in EGF receptor–dependent signaling, either by increasing the affinity for ligand or by increasing the kinase or effector binding activity of ligand-bound EGF receptor. It would be interesting to know whether proteins similar to rho serve to regulate signaling through the EGF receptor in other organisms.

Experimental Procedures

Stocks

Drosophila melanogaster stocks were raised on standard cornmeal–yeast–agar medium at 25°C. The following fly strains were used: Oregon R, w^-, *l(3)6D1* (maps into 69D; Bier et al., 1989), $Toll^{09}$ (Anderson et al., 1985), *fs(1)K10* (Wieschaus et al., 1978), $capu^{G7}$, $spir^{PJ56}$ (Manseau and Schüpbach, 1989), $grk^{(HK36,\ HG21,\ WG41)}$, $Egfr^{(1\sim QYI,\ 3C81)}$, and *Df(2R)3F18* (Schüpbach, 1987; T. Schüpbach, personal communication; Clifford and Schüpbach, 1989). Single females of *Egfr* allele used in this study produced eggs and embryos in which phenotypes varied from weak to strong ventralization (Schüpbach, 1987; Schüpbach et al., 1991). Most of the eggs laid by *fs(1)K10*, *grk*, and *top* alleles are not fertilized, probably owing to the defects in the chorion structure.

HS(*rho*) Transformation

The sense version of the *rho* cDNA clone under the control of the heat-inducible promoter in the hs-CaSpeR P element transformation vector HS(*rho*) (M. Sturtevant, M. Roark, and E. Bier, submitted) was injected into w^- host embryos according to the procedure of Rubin and Spradling (1982), with the modification of using a constitutive source of transposase activity as described in Spradling (1986).

Twenty-eight stable HS(*rho*) (2 on the X chromosome, 7 on the second chromosome, and 19 on the third chromosome) P element transformant lines were generated. In addition, two stable HS(anti-*rho*) lines (one on the second and one on the third chromosome) were provided by John Emery and Ethan Bier. The HS(*rho*) lines 1D/CyO, 27A/TM3, 30A/TM3, and 30A/30A were used for the ectopic expression experiments. The HS(anti-*rho*) line 18-1/18-1;18-6/18-6 with four copies of the antisense *rho* cDNA (provided by Ethan Bier) was used for the loss-of-function studies.

Heat Shock Treatment and Egg Collections

To induce the expression of *rho* by *hsp70* promoter, glass vials containing 3- to 5-day-old animals (fed with yeast at room temperature) were incubated in a 39°C (measured with a calibrated thermometer) water bath for 15 min. Thereafter, either the ovaries were dissected immediately for in situ hybridization procedures as described or timed egg collections were taken from these flies at 25°C.

Germline Clones

Germline clones of a *rho* null allele, $rho^{p\Delta 5}$, were generated by X-ray irradiation (Perrimon et al., 1984) using a dominant female sterile mutation, $P[ovo^{D1}]^{2X48}$, introduced via P element transposon insertion onto the left arm of the third chromosome (T.-B. C. et al., unpublished data). Progeny from a cross between $rho^{p\Delta 5}$/TM6B virgin females and $w;P[ovo^{D1}]^{2X48}$/TM3 males were irradiated during the first instar larval stage with 1000 rads (using a Torrex 120 X-ray machine at 100 kV, 5 mA, 3 mm aluminum filter) to induce germline clones. Under these conditions, approximately 1.5% of the resulting $rho^{p\Delta 5}$/P[ovo^{D1}]^{2X48} females carried germline clones. These mosaic females were crossed to either $rho^{p\Delta 5}$/TM6B or *yw;+/+* males and raised at 25°C.

Staining Procedures, In Situ Hybridization, and Other Methods

Immunocytochemistry, in situ hybridization, and X-Gal staining procedures were performed as described earlier (Ruohola et al., 1991) with minor modifications. The antibodies used in this study were anti-rho monoclonal antibody (1:1 dilution; Freeman et al., 1992) and anti-twi antibody (1:500 dilution). Ovaries for the anti-rho immunocytochemistry were fixed for 20 min at room temperature, washed with buffer, and thereafter used for staining as a fresh preparation without dehydration or storage at −20°C. The DNA fragments used for in situ hybridization were *tld* cDNA (Shimell et al., 1991), *dpp* cDNA (St Johnston and Gelbart, 1987), and *rho* cDNA (Bier et al., 1990).

For microscopic examination of chorions and embryonic cuticular structures, eggs and embryos were treated according to Wieschaus and Nüsslein-Volhard (1986). Embryos were mounted in lactic acid: Hoyer's (1:1, after 48 hr incubation at 25°C) and allowed to clear overnight at 65°C. The analyses of the chorions and embryonic cuticular structures were made according to Schüpbach (1987) and Anderson et al. (1985). Lateralization was defined as expansion of the region bearing the lateral-most fine ventral hair and loss of most dorsal and ventral structures.

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