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Sequential activation of the EGF receptor pathway during *Drosophila* oogenesis establishes the dorsoventral axis

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SUMMARY

Previous work has demonstrated a role for the *Drosophila* EGF receptor (Torpedo/DER) and its ligand, Gurken, in the determination of anterioposterior and dorsoventral axes of the follicle cells and oocyte. The roles of DER in establishing the polarity of the follicle cells were examined further, by following the expression of DER-target genes. One class of genes (e.g. *kekon*) is induced by the DER pathway at all stages. Broad expression of *kekon* at the stage in which the follicle cells migrate posteriorly over the oocyte, demonstrates the capacity of the pathway to pattern all follicle cells except the ventral-most rows. This may provide the spatial coordinates for the ventral-most follicle cell fates. A second group of target genes (e.g. *rhomboid* (*rho*)) is induced only at later stages of oogenesis, and may require additional inputs by signals emanating from the anterior, stretch follicle cells. The function of Rho was analyzed by ectopic expression in the stretch follicle cells, and shown to induce a non-autonomous dorsalizing activity that is independent of Gurken. Rho thus appears to be involved in processing a DER ligand in the follicle cells, to pattern the egg chamber and allow persistent activation of the DER pathway during formation of the dorsal appendages.

Key words: EGF receptor, Oogenesis, Rhomboid, Gurken, *Drosophila*

INTRODUCTION

Patterning the follicle cells that cover the developing oocyte determines the polarity of the *Drosophila* embryo. This is achieved by an elaborate communication between the oocyte and the surrounding follicle cells. Eventually, signals from the follicle cells determine the polarity of the future embryonic axes. The specification of at least three subsets of follicle cells, posterior, dorsal and terminal, is responsible for determining the anterioposterior and dorsoventral axes of the embryo, and the terminal structures, respectively (reviewed by Ray and Schüpbach, 1996). Surprisingly, the pathway triggered by the *Drosophila* EGF receptor (DER/Torpedo/EGFR) is responsible for the determination of both posterior and dorsal follicle cell fates, at two discrete stages of oogenesis (González-Reyes et al., 1995; Roth et al., 1995; Price et al., 1989).

The anterioposterior axis is the first to be specified in the developing oocyte. From the initial stages of oogenesis, the population of follicle cells covering the oocyte is not uniform. Situated at both termini is a small group of polar follicle cells that has been determined at an earlier stage (Ruohola et al., 1991; Margolis and Spradling, 1995). There appears to be no distinction at this phase however, between the anterior and posterior follicle cells. Prior to stage 7, *gurken* transcripts, encoding a TGFα homologue that is likely to be a ligand for DER, are localized at the space between the oocyte nucleus and the posterior part of the oocyte (Neuman-Silberberg and Schüpbach, 1993). DER, in contrast, is expressed in all follicle cells (described below). Activation of DER by Gurken at the posterior follicle cells induces posterior follicle cell fates, which are distinct from the default fate that is maintained in the anterior follicle cells. The posterior cells then signal back to the oocyte, through an unknown mechanism. Consequently, reorganization of the microtubule and microfilament system of the oocyte ensues, and the localization of mRNAs (e.g. *bicoid* and *oskar*) along the anterioposterior axis takes place. This system is also responsible for directing migration of the oocyte nucleus to an anterior peripheral position in the oocyte, which will subsequently become the dorsal region.

Migration of the oocyte nucleus is completed by stage 8-9. In parallel to the continuous growth of the oocyte, posterior migration of follicle cells over the oocyte is observed. By stage 10 the nurse cells, which occupy half of the egg chamber, are covered by only approx. 50 thin follicle cells (the stretch cells), while the oocyte itself is surrounded by the remaining approx. 1,000 follicle cells, which are columnar in shape (reviewed by Spradling, 1993).

gurken transcripts maintain their close association with the migrating oocyte nucleus (Neuman-Silberberg and Schüpbach, 1993). After completion of nuclear migration, a second phase of DER activation takes place, this time leading to the induction of dorsal follicle cell fates. The dorsal-anterior cells

express *rhomboid* (*rho*) (Ruohola-Baker et al., 1993), which was shown to be an integral member of the DER signaling cassette in other tissues (Sturtevant et al., 1993; Noll et al., 1994; Schweitzer et al., 1995; Golembo et al., 1996; Gabay et al., 1997). The presence of Rho in these cells appears essential for normal patterning of the follicle cells and embryo (Ruohola-Baker et al., 1993). Ventral follicle cells which do not receive DER-induced signaling will assume the default, ventral cell fate. The differentiated ventral follicle cells generate a ventralizing signal that is mediated by the products of the *pipe*, *nudel* and *wbl* genes. These genes are required at early stages of embryogenesis, for restricting the domain in which Späztle will be proteolytically processed, to produce an active ligand of Toll (reviewed in Chasan and Anderson, 1993; Ray and Schüpbach, 1996).

Several issues regarding patterning of the dorsoventral axis in the oocyte remain open. The mechanism by which activation of the DER pathway in the dorsal follicle cells leads to the correct patterning of the ventral follicle cells and subsequently of the embryo, is not clear. In particular, it is difficult to understand how a region of high DER activity in the dorsal-anterior patch of follicle cells is capable of inducing a putative stripe of ventral follicle cells running along the entire length of the egg chamber.

In addition, the mechanistic basis for the function of Rho in the ovary is not clear. *rho* encodes a protein with multiple putative transmembrane domains (Bier et al., 1990). Several lines of evidence suggest that in the embryo Rho may participate in the processing of the transmembrane form of Spitz to generate a secreted, active ligand. Rho was shown to function non-autonomously, e.g. expression of Rho only in the midline of *rho* mutant embryos, was sufficient to pattern the adjacent ventral ectodermal cells (Golembo et al., 1996). Furthermore, secreted Spitz is epistatic to the *rho* mutant phenotype (Schweitzer et al., 1995). Taken together, these results imply that Rho should be expressed in the same cells as the ligand precursor. The vesicular localization of the Rho protein (Sturtevant et al., 1996) may be consistent with a role in ligand processing. However, the only known ligand for DER in the ovary, Gurken, is expressed in the oocyte, while Rho is expressed in the follicle cells. Thus, the role of Rho in oogenesis is not known. Finally, it is not clear why *rho*, being a DER-target gene, is induced only at the late phases of DER activation in the ovary.

In this work we dissect the roles of the DER signaling pathway at large, and Rho in particular, during oogenesis. We show that immediately after the movement of the oocyte nucleus to the future dorsal pole, during the posterior migration of the follicle cells, a broad activation of the DER pathway takes place. As a result, all follicle cells, except the ventral-most rows, express DER-target genes. This stage may be responsible for the establishment of follicle cell fate, as the default state. After completion of cell migration, transcription of *rho* in the dorsal-anterior follicle cells is achieved by activation of the DER pathway, in conjunction with signals that may emanate from the anterior, stretch follicle cells. Like its embryonic functions, the role of Rho in the ovary is non-autonomous. Ectopic expression of Rho in the stretch follicle cells, positioned around the nurse cells, can lead to activation of the DER pathway in the follicle cells covering the oocyte. These results suggest that Rho is responsible for triggering the production or processing of a DER ligand that is expressed in the follicle cells. Mutant follicle cell clones for *rho*, *Star* and *spitz*, as well as genetic interactions, confirm the requirement for these genes in the follicle cells, to form and pattern the egg chamber.

MATERIALS AND METHODS

Fly lines

The following lines were used: *55B-Gal4* (obtained from A. Brand), *AN296* (obtained from T. Schüpbach), *AA69* (obtained from S. Crews), 2.2*rho* (obtained from M. Levine), *HS-ras** (obtained from N. Perrimon), *UAS-rho 24-2* (on the 2nd chromosome) and *UAS-sSpitz 4a*. grk^{HK36}, grk^{WG41}, top^{1}, top^{CJ} and *fs(1)K10* were obtained from T. Schüpbach. For a homozygous *grk* or *top* background, females carrying two different alleles were generated. The following mutations in the *spitz* group genes were used: spi^{OE92} (obtained from N. Perrimon), spi^{IIT25}, spi^{IIA14}, S^{IIN23} (obtained from C. Nüsslein-Volhard), *Df(2L)S-3* (removing the *S* locus). We also used $argos^{l\Delta7}$ (obtained from M. Freeman) and Sos^{e4G} (obtained from M. Simon). For follicle cell clones *fs(2)Ugra* and *fs(3)Apc* were used (obtained from J. Szabad).

Ovary staining and antibodies

X-Gal staining was according to Margolis and Spradling (1995). Fixation of ovaries was in 0.5% glutaraldehyde. Antibody staining was according to Hsu et al. (1996), and fixation of ovaries was in 4% paraformaldehyde. The following antibodies were used: mouse polyclonal anti-DER was generated against the C-terminal *Eco*RI fragment inserted into pRSET, and rabbit anti-β-Gal (Cappel). Specificity of anti-DER antibodies was verified by the expected embryonic expression pattern, and by absence of staining in embryos homozygous for a deficiency uncovering the *DER* locus. To visualize nuclei, egg chambers were treated with RNaseH (400 μg/ml) for 15 minutes after staining, washed and incubated with Oli green (Molecular Probes $^{1}/_{5,000}$). Secondary antibodies were purchased from Jackson laboratories. Chorion morphology was monitored by mounting in Hoyers, and embryonic cuticles were visualized following dechorionation and devitellinization, after mounting in Hoyers/lactic acid 1:1.

Induction of constructs and mosaic clones

For all Gal4/UAS inductions, flies were grown at 18°C until hatching, and transferred to 25°C. Heat-shock based constructs were induced twice at 37°C for 20-30 minutes in a water bath, with a 2-hour interval. Four hours after the second heat shock, ovaries were dissected for staining, and 15-30 hours after heat shock eggs were collected.

For generation of mosaic follicle cell clones, males carrying *fs(2)Ugra* or *fs(3)Apc* were crossed to females carrying the mutation of interest. Second to third instar progeny were X-irradiated with 1,500 Rad. Hatched females (at least 15) carrying the dominant female sterile allele over the mutation of interest were collected and their capacity to lay eggs was analyzed.

RESULTS

DER expression in the ovary

Pole cell transplantation experiments have demonstrated that DER/Torpedo is required in the follicle cells but not in the germ line, in contrast to Gurken which is required only in the germ line (Schüpbach, 1987). In view of the diverse roles of DER in oogenesis, it was important to determine its expression pattern.

DER antibodies were used to stain ovaries. Membranal staining, that is restricted to the follicle cells was detected. It

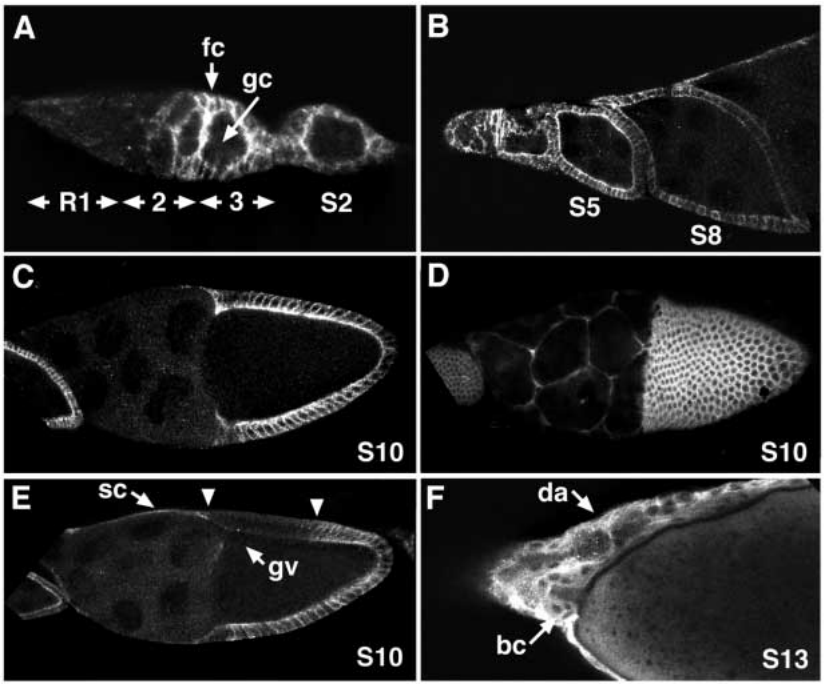

Fig. 1. Expression of DER during oogenesis. (A) In the germarium, membranal expression of DER first appears in region 2 of the germarium, when the follicle cells (fc) are surrounding the germ line cyst (gc). (B-D) Expression of DER in all follicle cells continues until stage 10. (E) In some stage 10 egg chambers, reduced amounts of DER are detected in the dorsal-anterior follicle cells (arrowheads), defined by the position of the germinal vesicle (gv). Note the expression of DER also in the stretch cells (sc). (F) During the final stages of oogenesis, DER is expressed in the dorsal appendage cells (da), as they migrate anteriorly over the border cells (bc).

initiates in the germarium in the middle of region 2, where inwardly migrating follicle cells cover the 16-cell germ line cyst (Fig. 1A). DER continues to be expressed uniformly in the follicle cells when individual egg chambers are formed at stage 2 of oogenesis (Fig. 1A), until stage 10 (Fig. 1B-E). Most egg chambers displayed uniform staining in all follicle cells at stage 10 (Fig. 1C,D). In some egg chambers however, a clear reduction in the level of DER was detected, specifically in the dorsal follicle cells above the oocyte nucleus (Fig. 1E). This reduction appears to result from decreased transcription of *DER*, as a similar pattern is seen by *DER* RNA in situ hybridization (not shown). Reduction in *DER* transcription that is induced by DER activation was reported for several tissues (Sturtevant et al., 1994), and may also take place in the ovary. DER expression is detected at stages 11-14 in the follicle cells that form the dorsal appendages, as they migrate anteriorly away from the egg (Fig. 1F).

AN296 (kekon) transcription – a sensitive marker for DER activity in follicle cells

Since the DER pathway plays a dynamic role in patterning the follicle cells, it is important to identify markers that will accurately reflect the temporal and spatial pattern of DER activation. The *AN296* enhancer trap, inserted next to the *kekon* gene, was previously reported to represent a target for DER activation in the dorsal-anterior follicle cells (Roth and Schüpbach, 1994a; Brand and Perrimon, 1994; Musacchio and Perrimon, 1996). Closer examination of the expression pattern of this line shows that it may also provide a marker for other phases in which DER is activated.

AN296 expression is first detected at stage 6 in the posterior follicle cells. By stage 8-9, as the follicle cells begin to migrate over the oocyte nucleus, the expression pattern of *AN296* becomes asymmetric with respect to the dorsoventral axis (Fig. 2A,B). Broad expression is detected over the entire dorsal and lateral groups of follicle cells, while expression of the marker is excluded only from a narrow stretch of ventral follicle cells. While this phase of DER activation was not described previously, it is well correlated with the dynamics of *gurken* expression. The *gurken* transcripts are already localized at the dorsal anterior edge of the ooctye in this stage. A dorsoventral gradient of the Gurken protein traversed by the migrating follicle cells could, therefore, account for the transient activation of DER in this stage. The demarcation of the ventral cells as AN296 non-expressors, is the earliest manifestation of a difference between the dorsal and ventral follicle cells. It is therefore likely that this signaling event establishes the dorsoventral coordinates which will direct the cells to the different fates when they are induced to differentiate.

At stage 10, after completion of cell migration, *AN296* is expressed in a triangle of dorsal-anterior follicle cells, lying immediately above the oocyte nucleus (Fig. 2C). *AN296* continues to be expressed in the dorsal appendage cells throughout their migration away from the egg, until the completion of stage 14 (Fig. 2D).

To verify that *AN296* is induced by the DER pathway, we checked its expression under conditions that modify DER activation. Expression of *AN296* has been shown to expand upon ubiquitous expression of activated Raf, suggesting that it is indeed a downstream target of the DER pathway (Brand and Perrimon, 1994). A similar uniform expansion can be observed upon ectopic expression of activated Ras (Fig. 2E). These results demonstrate that activation of the DER pathway is sufficient to induce *AN296* expression. This could be demonstrated directly in egg chambers derived from *fs(K10)* females, where the *grk* transcripts are distributed as a ring around the circumference of the egg (Neuman-Silberberg and Schüpbach, 1993), and a parallel expansion of *AN296* expression is observed (Fig. 2F). Conversely, a significant reduction in the level of *AN296* expression is observed in egg chambers derived from females homozygous for *grk* or *top* mutations. Expression in the posterior follicle cells is not

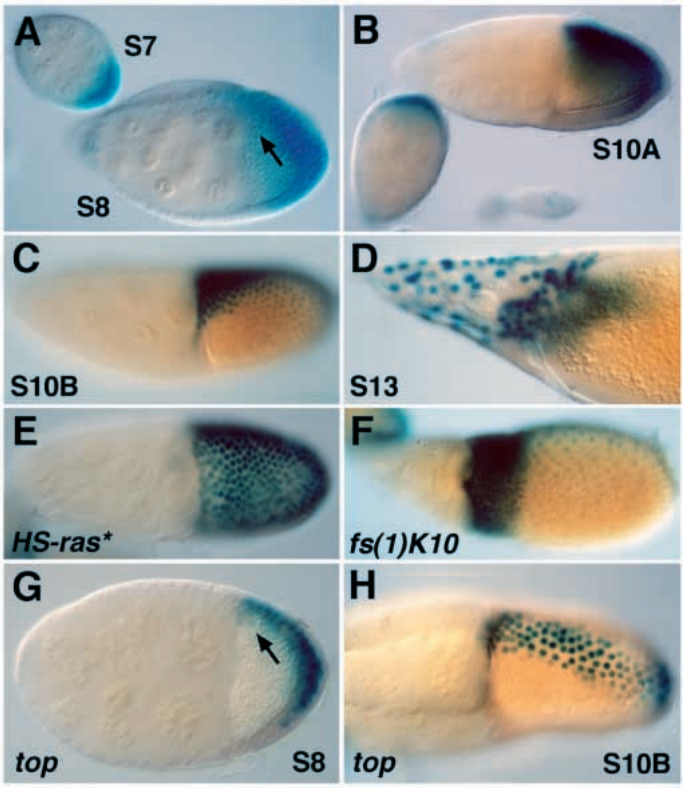

Fig. 2. Expression of *AN296* (*kekon*) in oogenesis. (A) Expression of *AN296* begins at stage 6-7, in the posterior follicle cells. At stage 8-9, after completion of the dorsal anterior migration of the oocyte nucleus (arrow), expression follows the position of the nucleus. Only the ventral-anterior follicle cells do not express the marker. (B) At stages 9-10A, as the follicle cells migrate posteriorly over the oocyte, expression is induced in a broad pattern covering the dorsal and dorsolateral cells. Again, only the ventral-most cells do not express *AN296*. (C) At stage 10B the expression pattern is refined to a triangle of dorsal-anterior follicle cells, above the oocyte nucleus. (D) Expression continues in the dorsal appendage precursor cells, as they migrate anteriorly away from the egg. (E) Following ubiquitous expression of activated Ras, *AN296* is expressed in all follicle cells. (F) In *fs(1)K10* egg chambers, expression of the marker follows the distribution of the *gurken* transcript, as an anterior ring. (G,H) In *top*^{1} mutant egg chambers, posterior expression of *AN296* is not affected, but the dorsal-anterior expression is significantly reduced. By stage 10B the marker is seen only as a stripe of five cell rows, representing the cells that were directly above the oocyte nucleus during their posterior migration.

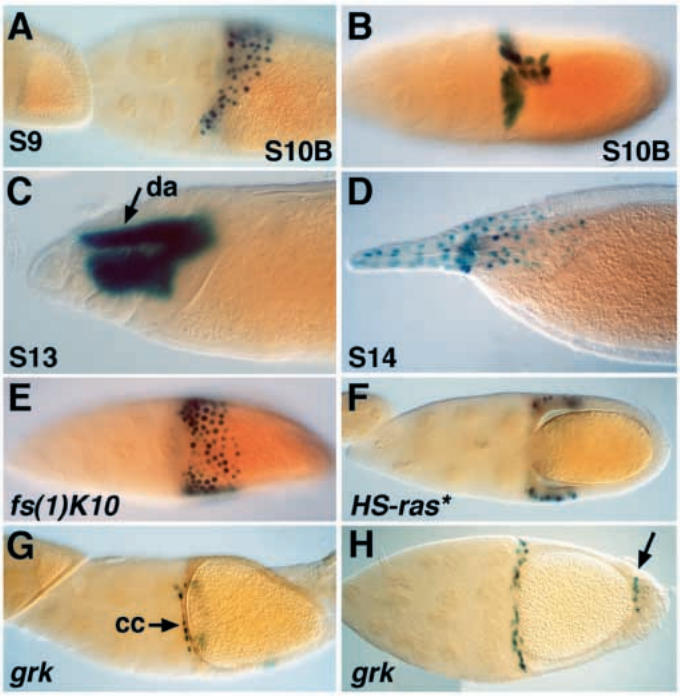

Fig. 3. Expression of *rhomboid* in oogenesis. *rho* expression was followed by an enhancer trap marker (*AA69*; A,D-H) and a promoter reporter (2.2*rho*; B,C). (A) Expression of *AA69* begins only at stage 10B, and includes the triangle of dorsal-anterior follicle cells, and the centripetal cells. (B-D) Expression is refined to the sub-population of follicle cells forming the dorsal appendages. B and C depict the 2.2*rho* enhancer trap line. This line is not expressed at stage 10A, therefore expression in the forming dorsal appendages represents de novo induction of *rho*, rather than the stability of β-Gal which was expressed at stage 10. (E) In *fs(1)K10* mutant egg chambers, the expression of *rho* follows the distribution of the *gurken* transcript, as an anterior ring. (F) Following ubiquitous expression of activated Ras, expression of *AA69* is detected only as an anterior ring, similar to E. (G) In *gurken* mutant egg chambers, only the expression in the centripetal cells (cc) is retained. (H) In a small fraction of *grk* egg chambers, ectopic expression of *AA69* in the posterior follicle cells (arrow) is observed.

significantly reduced, but at stage 9/10, only a dorsal stripe of expression is observed, in the follicle cells that were immediately over the oocyte nucleus during the posterior migration of these cells (Fig. 2G,H). Since *grk* and *top* may not represent null alleles, the remaining expression of *AN296* correlates with the site of maximal *gurken* concentration, and therefore with the highest residual DER activation. Taken together, these observations suggest that activation of the DER signaling pathway is necessary and sufficient to induce *AN296* transcription.

Expression of Rhomboid in the ovary

Rho expression in the ovary begins only at stage 10, as a triangle of follicle cells positioned directly above the oocyte nucleus (Ruohola-Baker et al., 1993), and is also observed in the centripetal follicle cells positioned between the oocyte and nurse cells. Subsequently, *rho* expression in the dorsal-most cells becomes confined to two smaller triangles at stage 10-11 (Neuman-Silberberg and Schüpbach, 1994). This group of cells will give rise to the two dorsal appendages. Anterior migration of these cells over the growing end, and subsequent secretion of chorion gives rise to the typical structure of the dorsal appendages. An enhancer trap in the *rho* gene (termed *AA69*) faithfully represents the reported pattern of the *rho* transcripts. It is first expressed in the triangle over the oocyte nucleus, and subsequently in the migrating dorsal appendage cells (Fig. 3A,D). A *rho* promoter reporter (2.2*rho*) is initially expressed at stage 10B in a subset of follicle cells that will give rise to the dorsal appendages, and continues to be expressed in these cells at subsequent stages (Fig. 3B,C).

The DER pathway participates in the initial induction of *rho*. In egg chambers derived from females containing multiple

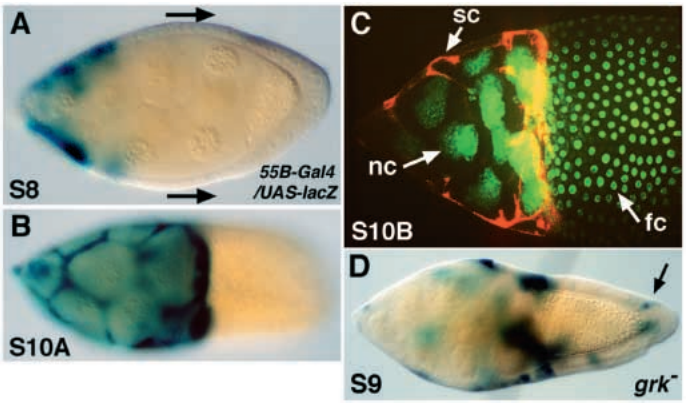

Fig. 4. Expression pattern of the *55B-Gal4* line. Expression of the *55B-Gal4* line was followed by crossing to a line carrying the *UAS-lacZ* chromosome, and staining with X-Gal or anti-β-Gal. (A) At stage 8, Gal4 is induced in the group of approx. 50 stretch follicle cells, which represent the anterior-most cells. Arrows show direction of the posterior migration of the follicle cells. (B) Upon completion of migration at stage 10, the stretch cells, which become thin and extended, cover the nurse cells. (C) Confocal image showing β-Gal, in red, only in the stretch cells (sc), and a nuclear Oli green stain (green). nc, nurse cells; fc, follicle cells. (D) In a *grk* mutant background, expression of *55B* is also observed in clusters of posterior follicle cells (arrow), probably due to the failure of these cells to assume the posterior cell fate.

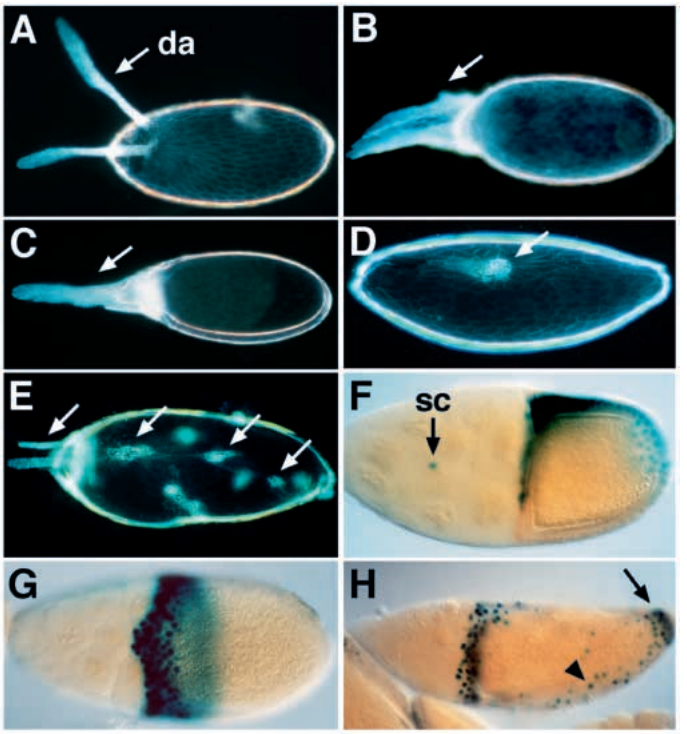

Fig. 5. Non-autonomous effects of Rho in the ovary. (A) Wild-type egg. Note the dorsal position of the two dorsal appendages (da). (B) When secreted Spitz is expressed in the stretch cells in *55B/UAS-sSpi* females, dorsal appendages are produced around the anterior circumference of the egg (arrow). (C) Similar results are obtained when Rho is expressed in the stretch cells in *55B/UAS-rho* females. (D) Egg chamber of homozygous *gurken* female. (E) When Rho is similarly induced in homozygous *gurken* females, dorsal appendage material is produced not only in the anterior part of the egg, but also in the posterior and central parts (arrows). (F) Expression of Rho in the stretch cells in *55B/UAS-rho* females leads to ectopic induction of *AN296* (*kekon*) only in scattered stretch cells (sc). (G) In contrast, the *AA69* (*rho*) marker is induced in an anterior ring of follicle cells positioned above the oocyte. (H) When Rho is similarly induced in homozygous *gurken* females, *AA69* is induced not only in an anterior ring, but also in the posterior follicle cells (arrow) and in scattered follicle cells covering the central part of the egg (arrowhead).

copies of the normal *grk* gene, an expanded expression of *rho* was monitored (Neuman-Silberberg and Schüpbach, 1994). In *fs(1)K10* mutant egg chambers, or following ectopic expression of activated Ras in the follicle cells, an expansion of *rho* expression forming an anterior ring is observed (Fig. 3E,F). Finally, in *grk* or *top* mutant egg chambers, *rho* is not expressed in the dorsal follicle cells. Normal expression is retained, however, in the centripetal follicle cells, positioned between the oocyte and nurse cells (Ruohola-Baker et al., 1993; Fig. 3G,H).

Non-autonomous activity of Rho in the ovary

Expression of *rho* in the dorsal-anterior follicle cells is essential for dorsoventral patterning by DER (Ruohola-Baker et al., 1993). Since DER and Rho are expressed in the follicle cells and the ligand Gurken in the oocyte, it was inferred that the activity of Rho is required for cells expressing the receptor to receive the signal. However, we have recently proposed that Rho is involved in posttranslational processing of the DER ligand Spitz (Schweitzer et al., 1995; Golembo et al., 1996). To check if this is also true in the egg chamber, it was important to see if in this system Rho can also exert a non-autonomous effect. To address this issue, we wanted to express Rho in cells that do not participate in dorsoventral patterning, such that resulting dorsoventral defects will clearly be attributed to a non-autonomous activity.

The group of ~50 stretch follicle cells do not normally experience the activation of DER, since they are never in direct contact with the oocyte, which is the source for Gurken. To induce Rho expression in the stretch cells, the *55B* Gal4 inducer line expressing Gal4 exclusively in these cells was used. Gal4 is first expressed at stage 7 in the anterior follicle cells. Expression continues during stages 9-10, as these cells extend over the nurse cells. By stage 10, Gal4 is expressed in the follicle cells covering all nurse cells and in the centripetal cells, but is not expressed in the follicle cells positioned above the oocyte, or in the nurse cells (Fig. 4).

Expression of secreted Spitz in the stretch follicle cells induced by the *55B* line, gave rise to non-autonomous effects, as monitored by the appearance of dorsal appendages throughout the anterior circumference of the egg (Fig. 5B). A small fraction of eggs was fertilized, and developed into dorsalized embryos (not shown). Thus, a secreted DER ligand produced only in the stretch follicle cells, has the capacity to diffuse and pattern the follicle cells covering the oocyte. The system was then used to express *rho* ectopically in the stretch follicle cells, and the results were very similar to the ones monitored following expression of secreted Spitz (Fig. 5C).

The observed effects following ectopic expression of Rho in the stretch cells, which are not in contact with the oocyte,

suggested that Grk is not involved in the process. To address this issue directly, the same induction of *rho* was monitored in *grk* mutant egg chambers. In a *grk* mutant background, expression of the *55B*-Gal4 line which is typically restricted to the anterior follicle cells, is also extended to patches of posterior follicle cells (Fig. 4D). This may be explained by the failure to induce posterior follicle cell fates and the retention of a default anterior fate in a *grk* mutant background (González-Reyes et al., 1995; Roth et al., 1995; Twombly et al., 1996). Induction of Rho by *55B*-Gal4 in *grk* females resulted in deposition of dorsal appendage material around the anterior circumference of the egg chamber (Fig. 5E). In addition, dorsal appendage material was also deposited at the posterior end of the egg chamber, according to the expression pattern of the *55B* inducer line. This indicates that the effects of ectopic Rho are not mediated by Grk.

The consequences of ectopic *rho* expression in the stretch cells were further monitored with molecular markers. The effect on the expression of *AN296* was subtle, as no pronounced expansion of expression in the follicle cells over the oocyte was observed. However, induction of the marker could be detected in several of the stretch cells (Fig. 5F). This is consistent with the expression of DER in these cells (Fig. 1D). Expression of the *rho AA69* marker provided a more compelling indication for the non-autonomous effects of Rho expression in the stretch cells. Expansion of the marker in a ring-like structure surrounding the anterior part of the egg chamber was detected (Fig. 5G). In *grk* mutant egg chambers, a similar anterior ring of *AA69* expression was induced by ectopic Rho (Fig. 5H). We found again that under these conditions, additional follicle cells also express the marker (see Discussion).

Requirement of *spitz* group genes for Rhomboid effects

While many of the egg chambers formed following ectopic Rho expression could not be fertilized due to excess dorsal appendage material around the micropile, some fertilized eggs were obtained. Cuticle preparation of these embryos revealed that they were dorsalized, as can be inferred from the absence or almost complete elimination of denticle bands, and the expansion of dorsal hairs (Fig. 6A). This result indicates that ectopic expression of Rho in the stretch cells has the capacity not only to alter the pattern of marker gene expression in the follicle cells, but also to interfere with dorsoventral patterning of these cells and of the embryo.

Apart from *rho*, the sites of expression in oogenesis and possible roles for other genes in the DER signaling cassette are not known. The effects of ectopic Rho expression were used to provide a sensitized genetic background, to determine the involvement of other members of the DER signaling pathway. Heterozygosity for the *Star* gene was previously reported to suppress the phenotypic effects of ectopic *rho* in many tissues, including the egg chamber (Noll et al., 1994). However, these experiments did not indicate whether Star is required during the activation of DER by Gurken, or at subsequent stages. In females heterozygous for a *Star* mutation, dramatic suppression of the ectopic rho phenotype is observed. The dorsal appendages appeared more normal, and the embryo displayed reduced but distinct ventral denticle bands (Fig. 6B). To test if Spitz may participate in the Rho-induced effects, the phenotype was monitored in embryos laid by females heterozygous for a *spitz* mutation. However, no significant effects on the Rho-induced phenotype were observed in these embryos. These results do not prove that *spitz* is not involved in the process, since the levels of the Spitz precursor in heterozygous females may be high enough to promote the full biological response.

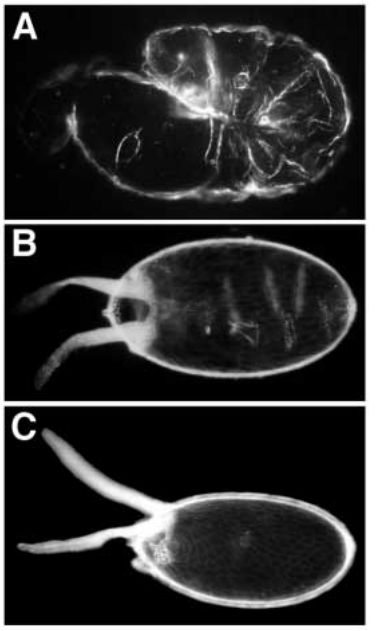

Fig. 6. Genetic interactions between ectopic Rho and the *Star* and *grk* genes. (A) Eggs generated by *55B/UAS-rho* females that are fertilized, give rise to dorsalized embryos. (B) Halving the gene dosage of *Star* in *55B/UAS-rho* females partially suppressed the phenotype. (C) Halving the gene dosage of *gurken* partially suppressed the phenotype.

Genetic interactions were also tested in females heterozygous for *gurken*. Under these conditions, the level of Grk is halved but not eliminated, and expression of the *55B* inducer line remains restricted to the stretch cells. The severity of the phenotype induced by ectopic Rho is reduced. Although some ectopic dorsal appendage material is still produced, the two dorsal appendages are prominent, and the embryos form denticle bands (Fig. 6C). Thus, a lower level of activation by Grk in the heterozygous females, reduced the capacity to form ectopic dorsal appendages. This indicates that the normal activation of DER by Gurken during stages 9 through 10B conditions the follicle cells, and collaborates with ectopic activation by Rho in the formation of dorsal appendages. In the homozygous *grk* females, the expanded expression of the *55B* inducer may compensate for the absence of Grk activity, and allow the extended appearance of dorsal appendages.

Follicle cell clones of *spitz* group mutants

Genetic interactions described above suggested that the *Star* gene participates in DER signaling in the ovary. The involvement of the different *spitz* group genes was analyzed by generating follicle cell clones. The method is based on dominant female-sterile mutations termed *Ugra* and *Apc*, which map to the left arm of the second and third chromosomes, respectively (Szabad et al., 1989; Szabad and

Hoffmann, 1989; Szabad et al., 1991). Pole cell transplantations have demonstrated that the *Ugra* and *Apc* gene products are required in the follicle cells and not in the germ line. Females heterozygous for the *Ugra* mutation arrest the development of the oocyte at stage 10 and do not lay eggs, while females heterozygous for the *Apc* mutation lay eggs which are abnormal in structure and lack dorsal appendages. Generation of wild-type clones of follicle cells by X irradiation of heterozygous females at the third instar larval phase, rescues the mutant phenotype efficiently: up to 80% of the females lay normal eggs, which undergo proper embryonic development.

Mutations of interest on the left arms of the second and third chromosomes were tested. On 2L, clones for *spitz*, *Star* and *Sos* were generated. No eggs were laid following generation of homozygous mutant clones for these genes. The failure to obtain eggs is not an indirect consequence of additional recessive female-sterile mutations on the chromosome, since different alleles for each locus gave identical results. The initial requirement for *spitz*, *Star* and *Sos* may take place at the early stages of oogenesis, as no eggs (rather than eggs with abnormal polarity) were laid. Examination of ovaries of these females did not reveal any egg chambers that were arrested at a particular stage. We cannot conclude whether these genes are also required subsequently, at the phase in which dorsoventral polarity is established. Mutations on the left arm of the third chromosome were also examined. Again, no rescue was observed by follicle cell clones homozygous for *rho* and *argos* mutations, no ventralized or dorsalized eggs were laid, and no defective egg chambers were detected in the ovaries. Thus, Rho and Argos may also participate in an early phase of DER signaling.

The inability to rescue the female sterile mutations *Ugra* and *Apc* by follicle cell clones homozygous for *spitz*, *Star*, *Sos* and *rho* mutations demonstrated that these DER pathway genes are required in the follicle cells at the early stages of oogenesis. An early DER function for the initial recruitment and spreading of the follicle cells around the 16-cell germ line cyst was indeed previously described (Goode et al., 1996). Interestingly, a requirement for a second DER ligand in this process had also been suggested, based on the observation that double mutants of *brainiac* with *grk* give a phenotype which is less severe than *brainiac/torpedo* double mutants (Goode et al., 1996). This early requirement for the DER pathway may involve the initial spreading of the follicle cells around the 16-cell germ line cyst. Interestingly, mutations in *argos* also failed to produce eggs, suggesting that Argos may also be playing a role early in oogenesis as an inhibitory ligand of DER.

DISCUSSION

Sequential activation of the DER pathway in oogenesis

The EGF receptor is highly pleiotropic, regulating a large number of processes during all stages of *Drosophila* development (Schweitzer and Shilo, 1997). Oogenesis represents an extreme case, where combined data from previous reports and this work, point to five independent stages in which the DER pathway is activated, all within the monolayer of the follicular epithelium.

By generating follicle cell clones, we have demonstrated that different elements in the DER signaling pathway are essential for the early stages of oogenesis. In the absence of *spitz*, *rho* and *Star*, egg chambers fail to develop. We assume that absence of the DER pathway leads to loss of the integrity of the follicle cell layer covering the germ line cells. The second phase of DER activation takes place prior to stage 7, and is responsible for induction of posterior follicle cell fates triggered by Gurken (González-Reyes et al., 1995; Roth et al., 1995). In the third cycle of DER activation, determination of the dorsoventral axis takes place, after migration of the oocyte nucleus to the future dorsal-anterior corner of the oocyte. While the essential role of the DER pathway in the induction of dorsal cell fates has been widely recognized (Schüpbach, 1987; Price et al., 1989; Brand and Perrimon, 1994; Roth and Schüpbach, 1994b), the precise phase in which this process takes place was not defined.

The expression of *AN296* described above, provides a sensitive reporter for DER activation, and clarifies the spatial and temporal aspects of dorsoventral patterning by the pathway. At stages 8-9, when the follicle cells migrate posteriorly over the oocyte, expression of *AN296* is very broad. This pattern is induced along the two axes. First, posterior migration of the follicle cells over the source of high Grk induces activation of the DER pathway in all follicle cells passing over the oocyte nucleus. Second, lateral diffusion of the Grk signal leads to a symmetrical lateral activation which decreases towards the ventral follicle cells. Consequently, expression of the marker is induced in all follicle cells, except in the ventral-most rows (Fig. 2A,B). This pattern has the capacity to define, by default, the fate of the ventral-most rows of cells.

The next cycle of DER activation takes place at stage 10, when the follicle cells have completed their posterior migration. A critical event is the induction of *rho* expression, triggered by Gurken-mediated DER activation. *Rho* expression is essential for dorsoventral patterning, since expression of antisense *rho* in all follicle cells can lead to the generation of ventralized egg chambers and embryos (Ruohola-Baker et al., 1993). However, as previously noted, it is implausible that this dorsoanterior expression domain can define the dorsal and ventral regions of the egg chamber. What then is the function of this wave of DER activation with respect to dorsoventral patterning?

One possibility is that this phase has an additive effect to the previous activation of DER, which took place during follicle cell migration. The combined effects of both phases would determine the capacity of the follicle cells to become dorsal. Normally, activation of the DER pathway in the Rho-expressing cells does not seem to extend beyond these cells, as monitored by expression of the DER-target gene *kekon*. It is thus possible that relay mechanisms extend a second, unknown dorsalizing signal from the Rho-expressing cells to the more lateral and posterior follicle cells.

Finally, Rho-dependent signaling appears to be important for patterning the dorsal appendages. This is supported by the persistent expression of *kekon* and *rho* in the precursors of the dorsal appendages until the final stages of oogenesis (Figs 2D, 3D), and by the induction of multiple dorsal appendages following ectopic Rho expression. Patterning of the dorsal appendages may thus represent another distinct DER-dependent process. The stages of DER signaling in the ovary are summarized in Fig. 7.

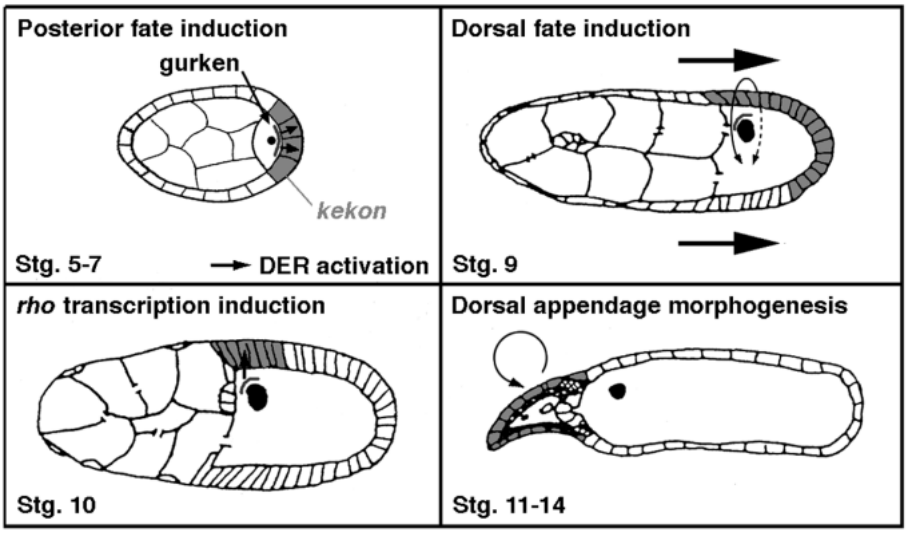

Fig. 7. Sequential roles for DER during oogenesis. The DER pathways is utilized multiple times during oogenesis, to pattern different aspect of the egg chamber. Mosaic analysis of follicle cell clones has demonstrated an early requirement for different components of the pathway. Egg chambers do not develop in the absence of this activity. Subsequently, activation of DER in the posterior follicle cells induces posterior cell fates prior to stage 7. During stage 9, when the follicle cells migrate posteriorly over the oocyte, the cells encounter high levels of Gurken. As a result, all cells except the ventral-most rows, induce the DER pathway. This may allow the ventral rows to retain the ventral (default) cell fate. Determination of distinct ventral follicle cell fates may be essential during the early stages of embryogenesis, for correct dorsoventral patterning of the embryo through the Toll pathway. At stage 10, *rho* expression is induced in the dorsal-anterior follicle cells by the combined activity of the DER pathway and another putative signal emanating from the stretch cells. The activity of Rho may be important for establishment of dorsoventral polarity of the follicle cells through an unknown mechanism. In addition, Rho appears to be crucial for processing a DER ligand which is expressed in the follicle cells. Thus, once *rho* is induced, DER activation no longer requires accessibility to Gurken. The cells expressing Rho are the precursors of the dorsal appendage cells, which continue to activate the DER pathway as they migrate anteriorly to form the dorsal appendages.

Dual input for *rho* expression

Throughout development *rho* expression prefigures and induces DER activation (Gabay et al., 1997). Induction of *rho* expression in the ovary by DER is thus unique and represents a positive feedback loop, where DER activity induces *rho* expression, which in turn will induce DER activation.

While the *kekon* gene is induced at each phase of DER activation, *rho* expression is triggered only from stage 10 onwards. A mechanism must exist to prevent *rho* and possibly other genes (e.g. *bunched*) (Dobens et al., 1997), from being triggered by the same pathway at earlier stages. One option is that induction by the DER pathway is not sufficient to trigger *rho*, and an additional input provided by a different group of cells, is required. The second input may be a signal emanating from the stretch follicle cells. Dpp is expressed in these cells (Twombly et al., 1996) and may provide a likely candidate. Multiple requirements for triggering *rho* expression may thus assure that it will normally be induced in a restricted point in space and time, only when the Gurken-induced signal emanating from the oocyte nucleus can be combined with a signal originating from the stretch follicle cells (summarized in Fig. 8).

The requirement for a second input is supported by the experiment in which ubiquitous expression of activated Ras in the follicle cells leads to the induction of *rho* expression only in an anterior ring of follicle cells, rather than in all follicle

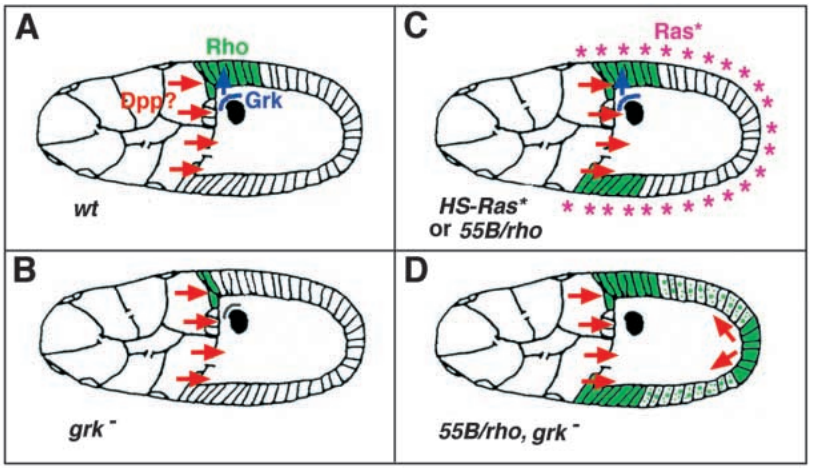

Fig. 8. Dual activation is required to trigger *rhomboid* expression. The *rho* gene is induced by activation of DER, yet it is only expressed at stage 10 and onwards, in the dorsal-anterior follicle cells, and not at earlier stages in which DER is induced. (A) A mechanism that accounts for this observation may be a dual input for *rho* transcription (green), involving not only Gurken-induced (blue) DER activation, but also an additional signal (red arrows) emanating from the stretch cells, e.g. Dpp. The model is supported by the following observations: (B) In *grk* mutant females, *rho* transcription in the dorsal-anterior follicle cells is absent, and is retained only in the centripetal cells. (C) Following ubiquitous activation of the DER pathway (in *HS-Ras** or *55B/UAS-rho* egg chambers), *rho* transcription is ectopically induced in an anterior ring. However, it does not include the entire egg chamber, indicating that DER activation is not sufficient and another signal generated by the stretch cells may be necessary. (D) Following the same ubiquitous activation of DER (*55B/UAS-rho*) in *grk* mutant females, *rho* transcription is induced not only in an anterior ring, but also in a posterior patch and in a scattered fashion in the follicle cells positioned between them. In this background the signals normally emanating from the anterior cells are also produced by the posterior follicle cells, which retain their anterior fate in the *grk* mutant background.

cells (Fig. 3F). This model may also explain why in a *grk* mutant background, we observed a broader response to ectopic activation of the DER pathway than in a wild type background. In *grk* egg chambers, the induction of *rho* expression was also observed in the posterior and central follicle cells, and the widespread formation of ectopic dorsal appendage material was monitored (Fig. 5E,H). In *grk* mutant egg chambers, the posterior follicle cells were shown to develop as anterior cells, and express Dpp, among other anterior markers (Twombly et al., 1996). The defective posterior cells may thus provide a second source for the signals necessary to collaborate with DER, leading to expression of *rho* in all follicle cells. A similar formation of ectopic dorsal appendage material covering all parts of the oocyte was observed when constitutively activated DER was ubiquitously expressed in a *grk* mutant background (Queenan et al., 1997).

In *grk* mutants, a small fraction of egg chambers expressed *rho* in the posterior follicle cells (Fig. 3H). A similar result was obtained for expression of the *bunched* gene (Dobens et al., 1997). Again, ectopic signals in the posterior follicle cells may occasionally collaborate with residual DER activation in the same cells, to induce expression of *rho* or *bunched*.

CF2 is a zinc finger protein shown to function as a repressor of *rho* transcription in the ovary. Expression of an antisense construct of CF2 resulted in ubiquitous expression of *rho* in all follicle cells (Hsu et al., 1996). Thus, CF2 degradation or inactivation may represent a stage which integrates the dual input from DER and the additional pathway.

The dual requirement for a signal emanating from the stretch cells combined with DER activation, ensures that *rho* expression will be induced only once the posterior migration of the follicle cells has been completed. This provides a temporal separation between the two phases of DER activation, namely the early Gurken-mediated activation and the subsequent Rho-mediated activation.

The roles of Rho in oogenesis

Two possible mechanisms for Rho activity in the ovary are ruled out by this work. One option was that Rho functions in a cell-autonomous manner to facilitate activation of DER in the follicle cells. The other scenario was that Rho may support cleavage of Gurken which is located on the oocyte membrane. These notions appears less likely now, since Rho was shown to exert its activity in the stretch cells, which are not in contact with the oocyte. Furthermore, the effect of *55B-Gal4/UAS-rho* is manifested even in a *grk* background.

We remain with a model for the function of Rho which is based on the corollaries to the embryonic roles of the protein. It is possible that the follicle cells express a precursor for another DER ligand (Spitz or an unknown ligand). Normal expression of *rho* in the dorsal-anterior cells or ectopic expression in the stretch cells, may allow processing of this putative ligand, followed by its diffusion.

We suggest that this molecule is a ligand for the DER pathway rather than for another receptor system. First, in all other tissues where Rho is expressed during development, it is intimately linked to the activation of the DER pathway (Gabay et al., 1997). Second, ectopic Rho expression in the egg chamber triggers the expression of downstream markers for DER activation, such as *kekon* and *rho* itself. Induction of Rho expression in the dorsal follicle cells may allow processing of a ligand expressed in these cells. This should generate a loop of DER activation which is independent of Gurken signal emanating from the oocyte nucleus. It is thus possible to maintain DER signaling in the dorsal appendage cells, even after they migrate from their original position and are no longer in contact with the egg that provides Gurken.

In conclusion, DER signaling emerges as a central pathway for morphogenesis of structural components of the egg chamber such as the follicle cell layer and the dorsal appendages. In addition, it is responsible for establishment of polarity in both anterioposterior and dorsoventral axes. To further understand patterning of the follicle cells by the DER pathway, it will be necessary to decipher how successive activation cycles are integrated in the developmental continuum, and coordinated with other signaling pathways.

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