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Combined activities of Gurken and Decapentaplegic specify dorsal chorion structures of the *Drosophila* egg

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SUMMARY

During *Drosophila* oogenesis Gurken, associated with the oocyte nucleus, activates the *Drosophila* EGF receptor in the follicular epithelium. Gurken first specifies posterior follicle cells, which in turn signal back to the oocyte to induce the migration of the oocyte nucleus from a posterior to an anterior-dorsal position. Here, Gurken signals again to specify dorsal follicle cells, which give rise to dorsal chorion structures including the dorsal appendages. If Gurken signaling is delayed and starts after stage 6 of oogenesis the nucleus remains at the posterior pole of the oocyte. Eggs develop with a posterior ring of dorsal appendage material that is produced by main-body follicle cells expressing the gene *Broad-Complex*. They encircle terminal follicle cells expressing variable amounts of the TGFβ homologue, *decapentaplegic*. By ectopically expressing *decapentaplegic* and clonal analysis with *Mothers against dpp* we show that Decapentaplegic signaling is required for *Broad-Complex* expression. Thus, the specification and positioning of dorsal appendages along the anterior-posterior axis depends on the intersection of both Gurken and Decapentaplegic signaling. This intersection also induces *rhomboid* expression and thereby initiates the positive feedback loop of EGF receptor activation, which positions the dorsal appendages along the dorsal-ventral egg axis.

Key words: Oogenesis, TGFβ, EGF, Nuclear migration, Follicle cell patterning, *Broad Complex*, *kekkon*, *pipe*, Pathway synergy

INTRODUCTION

The anterior-posterior (AP) and dorsal-ventral axes (DV) of the *Drosophila* egg and embryo are established during oogenesis by two consecutive steps of cell signaling from the germline to the somatic cells (González-Reyes et al., 1995; González-Reyes and St Johnston, 1994; Roth et al., 1995). Both steps are mediated by Gurken (Grk), a TGFα-like molecule present in the oocyte, the *Drosophila* homologue of the EGF receptor (DER) expressed by the follicle cells and Cornichon (Cni), a small hydrophobic protein (Neuman-Silberberg and Schüpbach, 1993; Price et al., 1989; Roth et al., 1995; Schejter and Shilo, 1989). First, DER activation by Grk leads to the acquisition of posterior cell fates by one subgroup of cells within the follicular epithelium, and later the same ligand-receptor interaction specifies dorsal fates in another subgroup of cells of the epithelium (González-Reyes et al., 1995; Roth et al., 1995). This raises the question of how an identical inducing signal can elicit two different cellular responses.

The two induction events are separated by a change in cytoskeletal polarity of the oocyte occurring at stage 8, which leads to the migration of the oocyte nucleus from a posterior central to an anterior asymmetric position (Clark et al., 1994; Lane and Kalderon, 1994; Micklem et al., 1997; Theurkauf et al., 1992). Since *grk* mRNA is associated with the oocyte nucleus, signaling occurs first to the posterior and later to a group of anterior follicle cells (Neuman-Silberberg and Schüpbach, 1996, 1993). The change in oocyte polarity is induced by a signal of unknown nature, which emanates from posterior follicle cells. This event, which we will refer to as 'back signaling', can only occur after posterior follicle cell fates are specified by early Gurken signaling (González-Reyes et al., 1995; Roth et al., 1995); therefore, lack of Gurken signaling leads to a polarity defect in the oocyte and thus prevents the normal migration of the oocyte nucleus.

The fact that early and late Grk signaling are both directed to a distinct region of the follicular epithelium appears to explain the different responses. Mutant phenotypes, overexpression studies and clonal analysis suggest that the follicular epithelium is subdivided into two major cell populations (González-Reyes et al., 1995; González-Reyes and St Johnston, 1998; Keller Larkin et al., 1999; Lee and Montell, 1997; Roth et al., 1995). Both ends of the egg chamber are believed to harbor terminal follicle cells, which display default anterior fates unless early Grk signaling induces them to adopt posterior fates. Thus, if Grk signaling is absent, all terminal cells express *decapentaplegic* (*dpp*), an anterior fate marker, which has been implicated in the formation of anterior chorion structures. However, in the presence of Grk *dpp* is suppressed specifically in posterior terminal follicle cells.

Between the two terminal populations are the main-body follicle cells, which can be induced to adopt dorsal rather than

ventral fates. Thus, within this cell population late Grk signaling restricts *pipe* (*pip*) expression to a ventral stripe and activates genes required for dorsal appendage formation at the dorsal side (Sen et al., 1998; Deng and Bownes, 1997; Dobens et al., 1997; Schüpbach, 1987). The dorsal appendages (DAs) are specialisations of the anterior-dorsal chorion that are derived from two dorsal patches of Broad-Complex (BR-C) expressing follicle cells. The DAs pattern is established by a signal amplification process initiated when Grk activates *rhomboid* (*rho*), which in turn leads to the processing of a second DER ligand, Spitz (Wasserman and Freeman, 1998). Although Grk signaling acts on all dorsal main-body follicle cells with regard to *pip* repression, the specification of dorsal chorion structures accompanied by signal amplification is restricted to the anterior region of the egg. It has been suggested that this local restriction derives from signals emanating from anterior-terminal follicle cells (Queenan et al., 1997; Sapir et al., 1998).

In this paper, we have investigated aspects of follicle cell patterning and competence in responding to Grk signaling by supplying Grk ectopically and at different time points during oogenesis. We show that even if terminal and main-body follicle cells receive the Grk signal simultaneously during mid-oogenesis one reacts by producing posterior and the other by producing dorsal cell types. However, anterior-dorsal cell types can only be induced in the main-body follicle cells if the terminal follicle cells maintain at least residual anterior characteristics and express residual amounts of *dpp*. By activating Grk signaling together with *dpp* at the posterior pole we show that the specification of the dorsal appendages and their correct positioning along the AP axis of the egg results from the intersection of DER signaling and a presumed AP gradient of Dpp. We confirm the positive requirement of Dpp signaling in that process by clonal analysis with the essential Dpp pathway component, *Mothers against dpp* (*Mad*; Sekelsky et al., 1995; Das et al., 1998). In addition, we show that the intersection of Grk and Dpp signaling is required for *rho* expression and thus controls the fine patterning of the dorsal appendages along DV axis.

MATERIALS AND METHODS

Fly stocks

The following *Drosophila melanogaster* strains were used: *Oregon* R; cni^{AR55}, cni^{AA12}; *Df(2L)H60* (Roth et al., 1995); *UAS-rho* (Sapir et al., 1998); *UAS-dpp* (Nellen et al., 1996); *E4-Gal4* and *CY2-Gal4* (Queenan et al., 1997); w, Mad^{12}FRT40A (Mad^{12} is a null allele; Raftery et al., 1995; Das et al., 1998); yw hs-Flp, FRT40A hs-N-Myc (Xu and Rubin, 1993).

Transgenic lines carrying *P[hs-cni, w+]*

The *cni* cDNA (0.9 kb) was cloned into the Hsp70-pCaSpeR (Pirrotta, 1988). *P[hs-cni, w+]*, cni^{AR55}/+, cni^{AR55} females were used to perform the heat-shock experiment.

Expression of *cni* in the follicular epithelium

The *cni* cDNA was cloned into pUAST (Brand and Perrimon, 1993) and injected into embryos from a *w*; cni^{AR55}/*CyO* stock (Rubin and Spradling, 1982). *UAS-cni*, cni^{AR55}/*CY2-Gal4*, cni^{AR55} females were generated. *CY2-Gal4* drove ubiquitous expression of *cni* in the follicular epithelium, which was confirmed by in situ hybridisation (data not shown). This did not alter the *cni* mutant phenotype, confirming that any effects observed in *hs-cni* experiments are due to Cni activating Grk signaling in the germ line.

Heat-shock treatment, ovary dissections and egg collections

3- to 5-day-old flies were incubated in a 39°C water bath for 10 minutes. They were kept at room temperature (25°C) in vials with food and dry yeast and subsequently used for timed egg collections or for ovary dissections in 1 or 2 hour intervals for a maximum period of 28 hours after heat shock. For each time point, on average, 50 stage-9 egg chambers were scored for the position of the oocyte nucleus and for *pip* or *kek* expression. Likewise, approximately 50 stage-10 egg chambers were scored for BR-C expression. Staging was according to King (1970) and to Spradling (1993). Molecular marker gene expression and/or oocyte nucleus position in egg chambers of known dissection time were correlated with an oogenesis timetable for flies kept at room temperature (Lin and Spradling, 1993).

Application of colchicine

Colchicine was mixed with fresh yeast and used at 25 μg/ml to destabilize microtubules as described in Theurkauf et al. (1993). 4-day-old females of the right genotype were fed with the yeast-colchicine mix. Ovaries dissected 1 day after treatment show a high frequency of posteriorly localised oocyte nuclei.

RNA in situ hybridisations

The *Broad-Complex* core domain (Deng and Bownes, 1997), *rho* (Bier et al., 1990), *kek* (Ghiglione et al., 1999), *pip* (Sen et al., 1998) and *dpp* (St Johnston and Gelbart, 1987) transcripts were detected by in situ hybridisation with digoxigenin-labeled antisense RNA probes. The hybridisation procedure was a modification of the protocol of Tautz and Pfeifle (1989).

Genetic mosaics in the follicular epithelium

Mad^{12} loss-of-function clones, genetically marked by the absence of N-Myc, were generated by the FRT/FLP recombination technique (Xu and Rubin, 1993). Adult females were heat shocked for 1 hour at 37°C and, 4-5 days later, for an additional hour in order to activate the N-Myc expression. Myc expression was detected by antibody staining under RNAse-free conditions. Subsequently, BR-C expression was detected by in situ hybridisation, as described above.

RESULTS

Egg phenotypes produced by activating Gurken at different stages of oogenesis

In order to investigate spatio-temporal changes in the competence of the follicular epithelium to respond to the Gurken (Grk) signal, we generated transgenic lines carrying a construct that places *cni* under the control of a heat-shock-inducible promoter in a *cni* minus background (Roth et al., 1995; for brevity we will refer to homozygous *cni* mutant flies carrying one copy of the heat-shock *cni* construct as *hs-cni* flies). Cni protein is required for normal Grk signaling by controlling the transport of Grk protein to the plasma membrane of the oocyte (Bökel et al., unpublished). Therefore, the absence of functional Cni prevents Grk signaling and consequently leads to eggs lacking both AP and DV polarity. *hs-cni* flies were heat shocked in order to induce the expression of *cni* and thus activate Grk signaling. Transgenic females, which had previously produced apolar eggs, deposited eggs with different morphologies after heat shock. The temporal sequence of egg phenotypes was reproducible and was apparently caused by the activation of the Grk signaling in egg chambers of different developmental stages. An estimation of the stage of oogenesis at which egg chambers giving rise to a certain phenotype

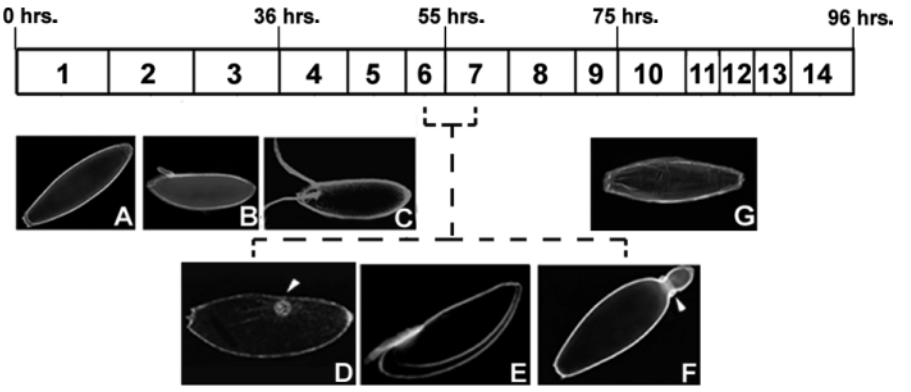

Fig. 1. Activation of Gurken signaling at different stages of oogenesis leads to distinct egg phenotypes. The upper panel shows a timetable of oogenesis according to Lin and Spradling (1993). The approximate time is indicated in hours. (A-G) Darkfield micrographs of eggs produced by *P[hs-cni, w^{+}], cni^{AR55}/+, cni^{AR55}* females at different intervals after heat shock (see Materials and Methods). Anterior is to the left. The micrographs in D-F have slightly higher magnification than those in A,B,C,G. (A,B) Heat shock *cni* during early oogenesis (stage 1-3) leads to ventralised eggshells. The egg shown in A has AP, but no DV polarity, while that shown in B has in addition a weak rescue of the DV axis. (C) Signaling between stage 3 and stage 6 leads to a complete rescue of the *cni* phenotype. (D-F) Eggs produced by a partial rescue of the *cni* phenotype at midoogenesis. (D) Egg with normal AP polarity and one patch of DA material in an intermediate between the egg poles (arrowhead). (E) Egg with a rescued dorsal-ventral polarity exhibiting two DAs, but lacking AP polarity as a micropyle is present at both poles. (F) Egg with a ring of DA material at the posterior pole (arrowhead). (G) Egg lacking both AP and DV polarity. The estimation of the developmental stages given in Fig. 1 is only approximate since abnormal eggs are often retained in the ovaries and laid less efficiently than wild-type eggs.

received the heat-shock pulse was obtained by correlating the time of egg deposition with a time scale of oogenesis at 25°C (Fig. 1; Lin and Spradling, 1993). Thus, the apolar eggs laid on the first day after heat shock show that Cni provided at late stages of oogenesis (stages 8-14) is unable to revert the mutant phenotype (Fig. 1G). However, Cni provided at mid-oogenesis (between stages 4 and 6) rescues the mutant phenotype completely and leads to wild-type eggs able to undergo normal embryonic development (Fig. 1C). Activation at earlier stages results in eggs with different degrees of ventralisation, likely due to progressive reduction of the dorsalising Grk activity caused by decreasing amounts of Cni protein (Fig. 1A,B). We confirmed that the different phenotypes observed in this experiment depend on germ line expression of *cni* as ectopic expression of *cni* in the follicular epithelium has no phenotypic consequences (see Materials and Methods).

We were most interested in the eggs resulting from a partial rescue of the mutant phenotype when *cni* is provided at mid-oogenesis (Fig. 1D-F). Three egg phenotypes can be distinguished. The first shows normal AP polarity associated with a spot of DA material located at different positions along the AP axis of the egg (Fig. 1D). The second lacks AP polarity having a micropyle at both ends (Fig. 1E), but shows a rescue of the dorsoventral chorion pattern. The third is a novel phenotype showing a posterior ring of DA material accompanied by a narrowing in the terminal portion of the egg (Figs 1F, 4A-F). The first and third classes are likely to be derived from egg chambers in which the oocyte nucleus remained posteriorly or in an intermediate position due to the inability of Grk signaling to induce normal nuclear migration. We used our experimental approach to determine more precisely the latest stage of oogenesis at which Grk signaling can induce correct nuclear migration.

Gurken signaling as late as stage 6 can promote normal oocyte nucleus migration

During wild-type egg chamber development Grk is already present in the oocyte when egg chambers emerge in the germarium (Neuman-Silberberg and Schüpbach, 1996). However, it is not known when Gurken is first required to specify the posterior terminal cells able to promote nuclear migration at stage 8 of oogenesis. To address this question, ovaries of *hs-cni* flies were dissected at different time points after heat shock. We looked at the position of the oocyte nucleus in stage-9 egg chambers where it can easily be recognized in an anterior-dorsal location in wild-type ovaries. The egg chambers were grouped into three classes depending on whether the nucleus had an anterior, posterior or intermediate position (Fig. 2). Heat shock after stage 7 does not rescue the *cni* phenotype with regard to nuclear migration: 70% of the egg chambers show a posterior localisation of the nucleus, a phenotype identical to that of null alleles of *cni* or *grk* (Roth et al., 1995). On the other hand, heat shock earlier than or during stage 6 leads to normal dorsal-anterior positioning of the nucleus in all egg chambers. Heat shock between late stage 6 and stage 7 leads to a large fraction of egg chambers with nuclei at intermediate positions. Thus during this period events take place that render Grk signaling ineffective in promoting nuclear movement.

Gurken activation at stage 7 of oogenesis leads to eggs with posterior dorsal appendages

A fraction of the eggs laid by *hs-cni* flies shows a novel phenotype in which a ring of DA material can be seen at the posterior of the egg (Figs 1F, 4A-F). To study the early fate shifts in the follicular epithelium which lead to this phenotype and to determine the stage at which it was induced, we analysed the expression of genes implicated in follicle cell patterning. We used *kekkon* (*kek*), a primary target of Grk signaling, induced early in posterior and later in dorsal follicle cells, *pip* which is normally repressed by Grk at the dorsal side, and *BR-C*, expressed at stage 10 of oogenesis in two groups of dorsolaterally located cells that give rise to DAs (Fig. 3A,E,I). In the absence of Grk signaling *kek* is never induced, *pip* is uniformly expressed in all follicle cells and *BR-C* shows a weak expression in anterior terminal follicle cells (Fig. 3B,F,L; Deng and Bownes, 1997; Ghiglione et al., 1999; Peri et al., 1999; Sen et al., 1998).

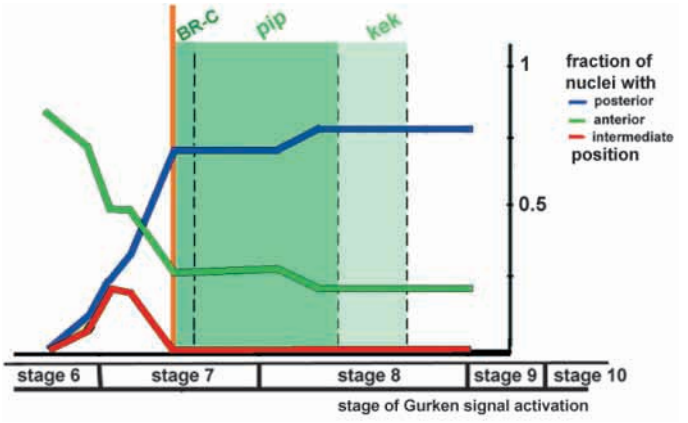

Fig. 2. The effects of delayed Gurken signaling on nuclear migration and follicle cell gene expression. The position of the oocyte nucleus and the expression of target genes of Grk signaling has been determined in stage 9 (or stage 10) egg chambers at different intervals after heat shock (see Materials and Methods). The frequencies of anterior (green line), intermediate (red line) and posterior (blue line) nuclear positions are shown as a function of the developmental time, depicted as stages at which the heat shock occurred. Grk signaling at the posterior has to occur before mid-stage 6 in order to efficiently promote the movement of the oocyte nucleus. Activation after mid-stage 7 leads to signaling of the posteriorly localised nucleus to the overlaying follicular epithelium. *BR-C* expression (dark green) can be induced during a small time window at stage 7. *pip* repression (medium green) is achieved by signaling between stages 7 and 8. *kek* expression (light green) is induced by signaling between stages 7 and 9. For each time point an average of 50 egg chambers were analysed.

The analysis shows that heat shock at stage 7 leads to egg chambers in which the nucleus remains posteriorly, but still signals to the overlying follicle cells. The posterior nucleus is associated with a small symmetric zone of *kek* expression and a broad posterior region of *pip* repression (Fig. 3C,G). As in wild type, the threshold responses for activation and repression are preserved and a normal set of DV cell types can be generated along the AP axis (compare Fig. 3A,E with C,G). *BR-C*, the marker for the DAs, is strongly expressed in a posterior ring of cells anterior to the nucleus (Fig. 3M). These *BR-C* expressing cells most likely give rise to the ring of DA material seen in deposited eggs (Fig. 4A-F).

Posterior *BR-C* expression can be induced by heat shock only in a very narrow time window succeeding the stage when Grk signaling has become insufficient to promote nuclear movement (Fig. 2). *pip* repression and *kek* activation, on the other hand, can also be induced at later stages. This might suggest that *BR-C* expression requires other signaling inputs. Furthermore, *BR-C* expression cannot be induced in the posteriormost cells in accordance with the phenotype of the deposited eggs, which never showed DA material in posteriormost positions (Fig. 4). This indicates that the terminal follicle cells cannot be induced to adopt dorsal fates even if they receive the Grk signal late and at a stage where main-body follicle cells become dorsalised. In cases where partial movement was observed (Fig. 3D,H,N) *kek* expression, *pip* repression and BR-C activation were found in intermediate positions dependent on the location of the nucleus. These egg chambers probably give rise to the first class of eggs with a spot of DA material at different positions along the AP axis (Fig. 1D).

The behavior of terminal follicle cells upon late Grk signaling

The eggs with a posterior ring of DA material can be divided into two subgroups based on the type of structures induced at the posterior tip. Eggs of the first group show posterior chorion structures resembling an aeropyle (Fig. 4A-C). Eggs of the other group show a posterior tip with anterior structures such as a micropyle (Fig. 4D-F). No significant differences can be observed between the ring of posteriorly localised DA material present in the two egg types. This indicates that Grk signaling at stage 7 can induce dorsal fates in main-body follicle cells and simultaneously can promote posterior fates in terminal follicle cells. However, using *dpp* as molecular marker we can show that the posteriorisation is only partial and not accompanied by complete suppression of anterior characteristics (see schematic drawing in Fig. 5A).

During normal oogenesis *dpp* is expressed in anterior follicle cells and is known to be involved in the specification of an anterior chorion structure of the mature egg, the operculum (Fig. 6A; Twombly et al., 1996). *dpp* is present at both ends in egg chambers lacking Grk signaling since the terminal follicle

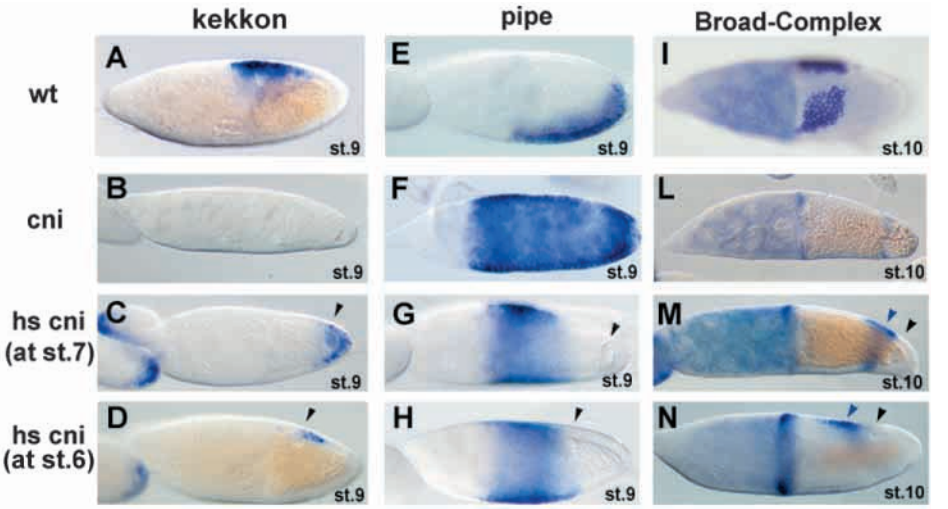

Fig. 3. Follicle cell patterning in egg chambers with mislocalised oocyte nucleus. (A,E,I) Egg chambers from wild-type females. (B,F,L) Egg chambers from cni^{AR55}/cni^{AR55} mutant females. (C,G,M) Egg chambers from *P[hs-cni, w⁺], cni^{AR55}/+, cni^{AR55}* females heat shocked at stage 7. (D,H,N) Egg chambers from *P[hs-cni, w⁺], cni^{AR55}/+, cni^{AR55}* females heat shocked at stage 6. (A-D) *kekkon (kek)* mRNA distribution in stage-9 egg chambers. (E-H) *pipe (pip)* mRNA distribution stage-9 egg chambers. (I-N) *Broad-Complex (BR-C)* mRNA distribution in stage-10 egg chambers. The black arrowheads mark the position of the oocyte nucleus. The blue arrowheads mark the ectopic BR-C expression.

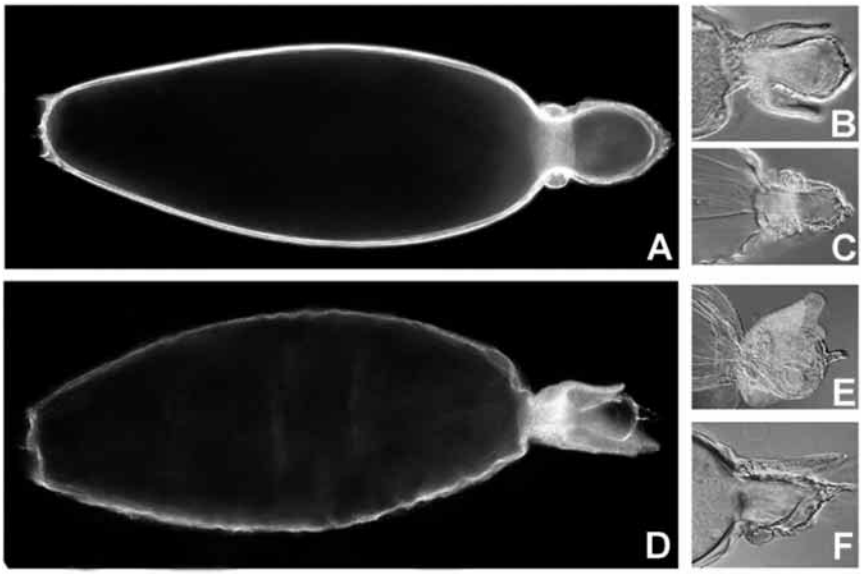

Fig. 4. Gurken signaling from posteriorly localised nuclei leads to eggs with posterior dorsal appendages. (A,D) Dark field micrographs of eggs with dorsal appendage (DA) material at the posterior. (B,C; E,F) Phase-contrast micrographs showing details of the posterior tip of eggs displaying posterior rings of DA material. (A-C) Posterior DA material is associated with an aeropyle. (D-F) Posterior DA material is associated with a micropyle.

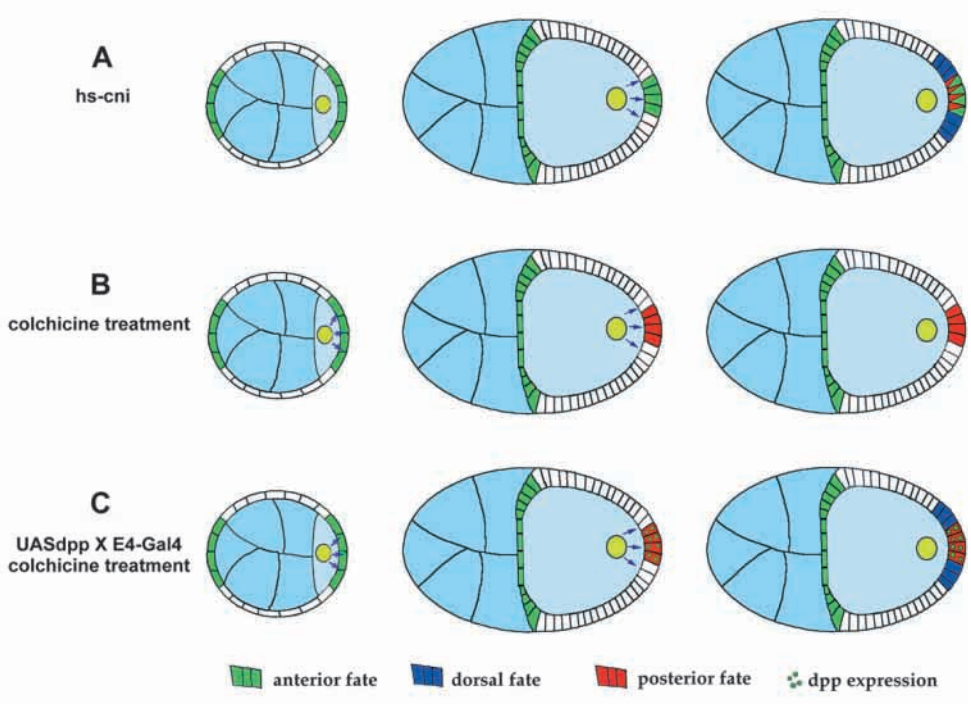

Fig. 5. Fate changes in the follicular epithelium induced by posteriorly localized oocyte nuclei in different experimental contexts. Schematized egg chambers showing early (left) and late Grk signaling (middle) and its consequences for follicle cell patterning (right). (A) Late Grk signaling in the *hs-cni* experiment may induce partial posteriorisation of terminal follicle cells (mixed green and red colours) and dorsal appendage fates in neighbouring mainbody follicle cells (blue). (B). In *mago* mutant or colchicine-treated egg chambers continuous Grk signaling induces posterior terminal fates (red) with no induction of dorsal appendage fate in main-body follicle cells. (C) Posterior missexpression of *dpp* (green dots) in colchicine-treated egg chambers leads to the induction of dorsal appendage fates in neighbouring main-body follicle cells (blue).

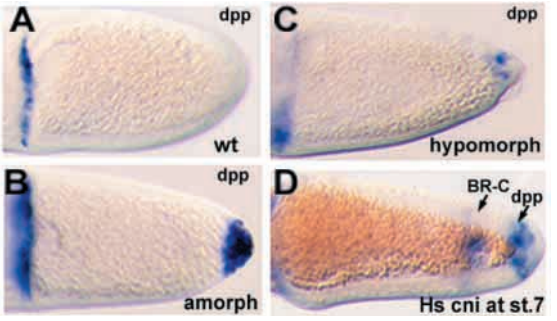

Fig. 6. Posterior expression of Dpp in egg chambers with partially posteriorised terminal follicle cells. (A-C) *dpp* mRNA distribution in stage-10 egg chambers. (A) Wild type. (B) Complete loss-of-function: $cni^{AR55}/Df(2L)H60$. (C) Partial loss-of-function: cni^{AR55}/cni^{AA12}. (D) *dpp* mRNA and *BR-C* mRNA distribution in stage-10 egg chambers from *P[hs-cni, w+], cni^{AR55}/+,cni^{AR55}* females heat shocked at stage 7. Arrows indicate the *BR-C* and *dpp* domains. The level of *dpp* expression in D is lower than that in B and similar to that seen in the *cni* hypomorph (C).

cells at the posterior pole acquire anterior fates by default (Fig. 6B; Twombly et al., 1996). We examined the expression of *dpp* in stage-10 egg chambers giving rise to eggs with posteriorly localised DAs. In all egg chambers *dpp* could be seen weakly expressed in posterior terminal follicle cells adjacent to the ring of BR-C expression (Fig. 6D). We therefore assume that deposited eggs with a posterior ring of DAs combined with an aeropyle are also derived from egg chambers expressing *dpp* at the posterior end. Apparently, the expression of *dpp* is not always linked to the formation of anterior chorion structures, like a micropyle.

In agreement with this assumption *dpp* expression can be found at the posterior of egg chambers from strong hypomorphic *grk* and *cni* allele combinations even though they display a posterior aeropyle. For example, cni^{AR55}/cni^{AA12} females lay ventralised eggs with apparently normal AP polarity. However, all egg chambers show some posterior *dpp* expression (Fig. 6C). Thus, in this case also, weak *dpp* expression at the posterior is not linked to the formation of anterior chorion structures in the mature egg. In variance with *hs-cni* egg chambers, however, the oocyte nucleus is localized normally in egg chambers from cni^{AR55}/cni^{AA12} females, indicating that back signaling followed by cytoskeletal repolarisation has occurred. The difference between the two phenotypes might result from the fact that late Grk activation in the *hs-cni* experiment does not allow enough time for back signaling to induce the cytoskeletal rearrangements necessary for oocyte nucleus movement.

Both Grk and Dpp signaling are required for dorsal appendage formation

We have shown that during stage-7 Grk signaling towards the terminal portion of *cni* mutant egg chambers induces dorsal fates in a ring of main-body follicle cells resulting in the production of DA material at the posterior of the mature egg (Figs 1F, 4A-F). However, mutants have been described in which late Grk signaling at the posterior does not lead to DA formation. In *mago nashi (mago)* mutant egg chambers a delay in the repolarisation of the microtubule network prevents oocyte nucleus movement and leads to Grk signaling to posterior cells until stage 9 (Micklem et al., 1997; Newmark et al., 1997). Nevertheless, *mago* mutant eggs do not form DAs at the posterior. Posterior localisation of the oocyte nucleus during late stages of oogenesis can also be obtained by feeding flies with colchicine, a drug that disrupts the microtubule network of the oocyte (Koch and Spitzer, 1983; Saunders and Cohen, 1999). Colchicine-treated females lay eggs with a *mago*-like phenotype (Fig. 8A). Both in *mago* mutants and after colchicine treatment, signaling to posterior follicle cells starts at early stages and therefore most likely leads to a complete suppression of anterior fates. Thus, the anterior characteristics of the terminal follicle cells observed in *hs cni* egg chambers with a posterior nucleus, seem to distinguish the heat-shock experiment from the loss of *mago* or from the colchicine treatment (Fig. 5, compare A and B).

We tried to identify the anterior molecular components that, together with Grk signaling, promote the formation of DAs. In addition to *dpp*, *rho* is also expressed in anterior follicle cells independently from Grk signaling (Ruohola-Baker et al., 1993; Sapir et al., 1998; Wasserman and Freeman, 1998). In order to investigate a possible function of these two genes in specifying the DA anlagen, *UAS-rho* and *UAS-dpp* flies were independently crossed with the E4-Gal4 line that drives expression at the posterior of the follicular epithelium (Brand and Perrimon, 1993; Queenan et al., 1997). In a wild-type background the ectopic posterior expression of these genes has no visible effect on the chorion structure (data not shown). *rho* dependent DER activation leads, however, to a posterior repression of *pip* (Fig. 7C) and consequently dorsalises the embryo at the posterior end (Fig. 7D). In order to combine the expression of these two genes at the posterior with Grk signaling we fed colchicine to *UAS-rho/E4-Gal4* and *UAS-dpp; E4-Gal4* flies. Under these circumstances *UAS-rho/E4-Gal4* females produced eggs with the above-described *mago*-like phenotype, indicating that even in combination with Grk signaling *rho* cannot induce dorsal anterior fates (data not shown). Interestingly, colchicine-treated *UAS-dpp; E4-Gal4* females produce eggs with DA material posteriorly (Fig. 8D). Egg chambers from such females express BR-C in a posterior ring (Fig. 8F). Thus, ectopic posterior expression of *dpp* in combination with DER activation by Grk induces dorsal anterior fates in main-body follicle cells. This indicates that a positive interaction between the EGF and the TGFβ pathways is required to specify these cell fates. To confirm the requirement of Dpp signaling for BR-C expression we induced follicle cell clones mutant for *Mad*, an essential cytoplasmic signal transducer of the Dpp pathway (Sekelsky et al., 1995). In *Mad* mutant clones located in anterior-dorsal regions of wild-type egg chambers *BR-C* expression is abolished (Fig. 8, compare the BR-C wild-type expression patterns in G and I with the expression patterns in H and L). We conclude that *dpp* not only is essential for the formation of the operculum (Twombly et al., 1996), but also for that of the DAs.

Dpp signaling controls the positive feed back loop of DER activation

One of the distinctive features of Grk signaling to terminal follicle cells as compared to main-body follicle cells is that Grk induces *rho* expression only in the latter case and thereby initiates a positive feed-back loop of DER activation (Wasserman and Freeman, 1998). Since this mechanism is required to pattern the DA anlagen, which we have shown to be specified by an interaction of Dpp and Grk signaling, we wondered whether it itself depends on *dpp*.

In wild-type egg chambers, treated with colchicine, the posteriorly localized oocyte nucleus leads to DER activation in the overlying follicle cells, as can be seen from the posterior expression of *kek*. However, the transcription of *rho* is not induced, even though *rho* has been described previously as a DER target gene in the follicular epithelium (Fig. 8B,C; Ruohola-Baker et al., 1993; Sapir et al., 1998; Wasserman and Freeman, 1998). Surprisingly, *rho* expression is observed at the posterior of *UAS-dpp; E4-Gal4* flies treated with colchicine (Fig. 8E). We conclude that *rho* expression in main-body follicle cells occurs only where Grk and Dpp signaling coincide, i.e. at the dorsal anterior corner of wild-type egg chambers. Thus, the interaction between the EGF and TGFβ pathways controls the specification of the dorsal cell fate leading to DA production, their positioning along the AP axis and the mechanism that patterns them along the DV axis. Since Spitz, the DER ligand activated by *rho* is ubiquitously expressed in all follicle cells (Wasserman and Freeman, 1998),

anterior restriction of *rho* expression in wild type might be essential to prevent the spreading of DER activation throughout the main-body follicle cells.

DISCUSSION

The induction of oocyte nucleus movement and the creation of the DV axis

Nuclear movement is essentially a self-induced event. Grk localized with the nucleus activates DER in posterior-terminal cells. Consequently, these cells acquire the property to signal back to the oocyte, reorganize the oocyte cytoskeleton and thus initiate nuclear movement to the anterior-dorsal pole where Grk signals again (González-Reyes et al., 1995; Roth et al., 1995). The timing of posterior fate induction and back signaling is still elusive since the molecular nature of these processes has not been analysed. Our findings show that Grk signaling as late as midstage 6 leads to normal anterior localisation of the nucleus (Fig. 2). This means that posterior-fate induction followed by back signaling can occur close to the time when nuclear movement normally takes place.

Starting from midstage 6 Grk signaling becomes insufficient to promote nuclear movement although up to midstage 7 an influence on nuclear movement can be detected (Fig. 2). This transition is probably caused by changes both in the follicular epithelium and in the oocyte. During this period the terminal follicle cells appear to lose their competence to respond to Grk signaling since they start to show mixed anterior/posterior terminal fates, which might result in a lack of back signaling (Figs 4, 5A, 6D). However, egg chambers mutant for hypomorphic *grk* or *cni* alleles with partial posteriorisation show normal nuclear behaviour, indicating that back signaling has caused correct cytoskeletal rearrangements (Fig. 6C). We therefore conclude that in the case of late Grk signaling the response of the oocyte to back signaling is also impaired so that cytoskeletal rearrangements necessary for nuclear movement are incomplete.

If Grk activation occurs too late to induce nuclear movement, or if the movement is incomplete so that the nucleus resides in an intermediate position, Grk remains localised with the nucleus and signals from there to the nearby follicular epithelium. The resulting patterns of *kek*, *BR-C* and *pip* expression are dictated by the nuclear position and suggest that there is no intrinsic DV polarity in egg chambers. This is especially obvious when completely symmetric rings of DA material are induced from a posteriorly localised nucleus (Fig. 4). In this situation, a set of marker genes corresponding to the entire DV axis of the follicular epithelium is expressed along the AP axis indicating that there is, with one exception discussed below, no principal bias to the way these genes are activated or repressed in main-body follicle cells. Similar conclusions had been drawn from the study of *mago* mutants, although in this case only the expression of the primary DER target *kek*, which is not a dedicated DV patterning gene, had been analysed (Micklem et al., 1997; Newmark et al., 1997). Together these observations, along with a previous analysis of egg chambers with two oocyte nuclei, demonstrate that the movement of the nucleus is the sole determinant of the orthogonal orientation of the body axes and that it stochastically determines the position of the dorsal side of the egg (Roth et al., 1999).

The responsiveness of terminal and mainbody follicle cells towards Grk

In our experiments we have manipulated the spatial and temporal expression pattern of the endogenous Grk signal, to probe the competence of the follicular epithelium. If activation of Grk signaling occurs after midstage 7 the oocyte nucleus remains posteriorly and Grk signals to a region that includes both the terminal follicle cells and an abutting ring of main-body follicle cells. Simultaneous Grk signaling to both cell populations clearly shows their different developmental responses: the terminal cells form either anterior or posterior structures, while the encircling ring of cells forms dorsal cell types characterized at the molecular level by repression of *pip* and by the activation of BR-C. This definitely excludes the possibility that posterior and dorsal cell-fate specification is controlled by timing so that late Grk signaling always has a dorsalising effect, irrespective of the cell group receiving the signal. The observation confirms earlier studies, which showed that terminal and main body follicle cells have different default states in the absence of signaling and respond differently upon ectopic activation of Ras or DER (Lee and Montell, 1997; González-Reyes and St Johnston, 1998; Keller Larkin et al., 1999). It also demonstrates that the different responses of terminal and main-body follicle cells to Grk signaling are not strictly separated in time such that the former have entirely lost their ability to react when the latter are competent. We see posteriorisation occurring simultaneously with dorsal fate induction.

However, whenever dorsal fates, i.e. dorsal appendages were found together with posterior chorion structures, a closer inspection of these egg chambers revealed that the posteriorisation was incomplete and *dpp*, normally only found anteriorly, was still present at the posterior pole. The possibility of mixed populations consisting of anterior and posterior follicle cells, a situation also caused by certain hypomorphic *cni* and *grk* alleles (Fig. 6C), is probably linked to the fact that the terminal cells are not a homogenous cell group. They seem to be divided into three subgroups by Grk-independent patterning mechanisms (Lee and Montell, 1997; González-Reyes and St Johnston, 1998; Keller Larkin et al., 1999). These subgroups might have different sensitivities toward Grk signaling.

EGF and TGFβ signaling have to coincide to induce anterior-dorsal fates

After nuclear movement, Grk signaling has at least two effects on follicle cell patterning that are essential for later embryonic development and egg morphology. Firstly, it leads to *pipe* repression and thereby defines the region of the egg from which the embryonic DV axis emerges. Secondly, it induces the formation of such anterior-dorsal chorion specialisation, like the DAs. While the former action of Grk occurs along the entire AP axis, the latter is confined to approximately the anterior third of the mainbody follicle cells. How is this difference in range of Grk action achieved? According to a first model, timing might play a crucial role. *pipe* repression starts earlier than *BR-C* activation; in the intervening time the egg chamber grows and the follicle cells continue to migrate over the oocyte

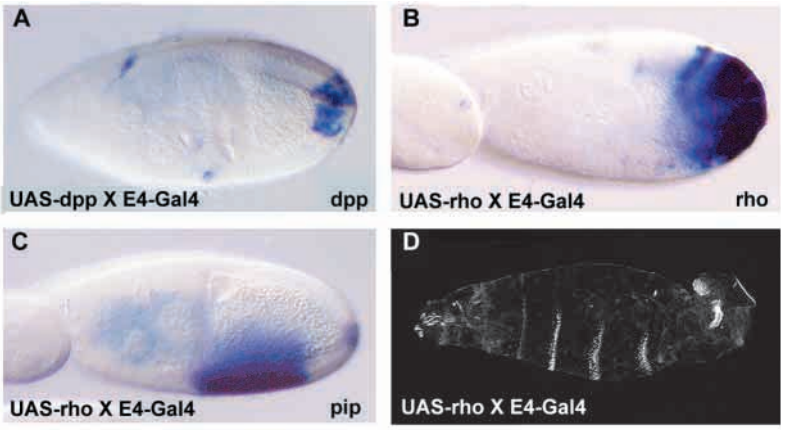

Fig. 7. Ectopic expression of *dpp* and *rhomboid* at the posterior of wild-type egg chambers. (A) *dpp* mRNA distribution in egg chambers from *UAS-dpp*; *E4-Gal4* females. *dpp* is expressed ectopically at the posterior of the egg chamber. The staining procedure had to be stopped before endogenous *dpp* transcripts in centripetal follicle cells (see Fig. 6A) were fully visible. (B-D) Egg chambers or embryo from *UAS-rho*/*E4-Gal4* females. (B) Ectopic *rho* mRNA at the posterior of a stage-9 egg chamber. The staining procedure had to be stopped before endogenous *rho* transcripts in centripetal follicle cells could be detected. Despite using the same Gal4 driver, *dpp* was always expressed in a narrower domain than *rho*. Probably, *rho* induces its own expression as a result of Spi-mediated DER activation. (C) *pip* expression in stage-10 egg chambers. *pip* is repressed at a narrow posterior domain corresponding to the region of ectopic *rho* expression. (D) Dark-field micrograph of a posteriorly dorsalised embryo derived from an egg chamber expressing high levels of *rho* at the posterior.

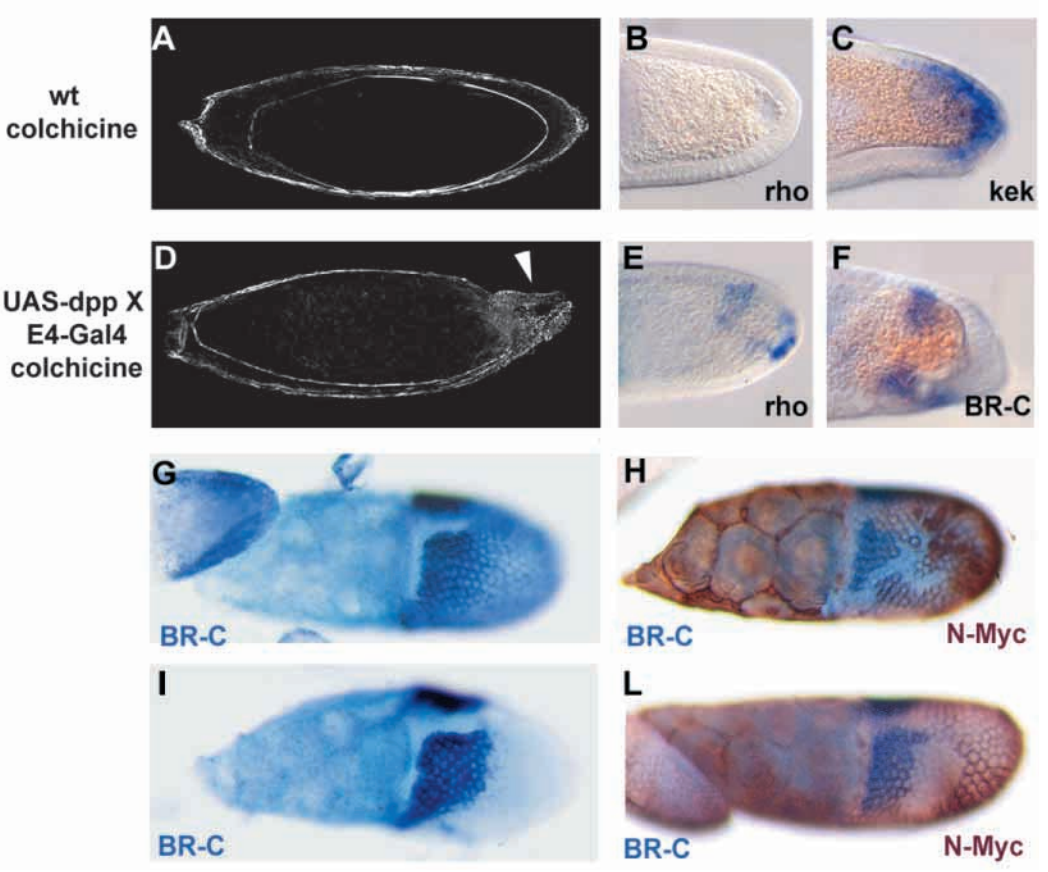

Fig. 8. TGFβ and EGF pathways collaborate to specify and pattern the dorsal appendages of the egg. (A-C) Egg and egg chambers derived from wild-type females fed with colchicine. (D-F) Egg and egg chambers derived from *UAS-dpp*; *E4-Gal4* females fed with colchicine. (A,D) Dark field micrographs of eggs. The eggs lack normal dorsal-ventral polarity due to a failure of oocyte nucleus movement. Expression of *dpp* at the posterior (D) leads to the formation of a ring of dorsal appendages (arrowhead). (B,C,E,F) Micrograph of posterior region of stage-10 egg chambers. (B,E) *rho* mRNA distribution. (C) *kek* mRNA distribution. (F) *BR-C* mRNA distribution. In wild-type egg chambers treated with colchicine the presence of the oocyte nucleus at the posterior leads to *kek* (C) but not to *rho* expression (B). However, in egg chambers from *UAS-dpp*; *E4-Gal4* females treated with colchicine *rho* (E) and *BR-C* (F), expressions can be detected at the posterior. (G-L) *BR-C* mRNA distribution in stage 10A (G,H) and 10B (I,L) egg chambers. *BR-C* expression gradually becomes confined to two lateral patches. (G,I) Wild type. (H,L) Egg chambers carrying *Mad* mutant clones, which are marked by the absence of N-Myc (brown). *BR-C* expression is abolished within the clones of both stage 10A and stage 10B egg chambers.

nucleus. Thus, different egg chamber geometries at the time of pattern induction might explain the differences in range of the signal. According to another model an anteriorposterior prepattern is established within the follicular epithelium, which allows Grk's induction of DA fates only in anterior main-body follicle cells. This model was first proposed in a study in which an activated version of DER was expressed uniformly in all main-body follicle cells (Queenan et al., 1997). Despite uniform activation of primary DER targets like *kek*, other target genes were only activated in proximity to anterior terminal follicle cells. This leads to the suggestion of a signal emanating from anterior terminal follicle cells, which modulates the response towards DER activation in the main-body follicle cells (Sapir et al., 1998). Our results clearly favor the second

model and identify Dpp as the actual signaling molecule that prepatterns the main body follicle cells. Residual *dpp* expression in posterior terminal cells explains the difference between the *hs-cni* and *mago* phenotypes, and most importantly, ectopic posterior *dpp* expression in egg chambers with posterior Grk signaling is sufficient to induce DA formation. Currently, we cannot assess the relative contribution of Dpp signaling to the specification of the DAs and operculum, respectively. Previous observations (Twombly et al., 1996; Deng and Bownes, 1997) suggest that high levels of Dpp repress DA and promote operculum formation. Our analysis of follicle cell clones lacking Mad function, however, demonstrates that Dpp signaling is required for *BR-C* expression and suggests that lower levels of Dpp, insufficient for operculum formation, are likely to specify DAs.

The finding that *rho* expression in the follicular epithelium cannot be induced by Grk alone, but also requires Dpp, shows that both cell-fate specification and cell-fate patterning are controlled by the intersection of the two pathways. Loss-of-function clones have been used to demonstrate that *rho* and *spi* are not required for DA formation per se but that they are necessary to separate the two appendages and to position them dorsolaterally (Wasserman and Freeman, 1998). Since this patterning mechanism involves the self-amplification of DER activation and includes the diffusible ligand Spi, the process must be under tight spatial control to prevent runaway activation in the follicular epithelium. While the localisation of Grk limits the process along the DV axis, we propose that a Dpp gradient emanating from anterior-terminal cells prevents its spreading along the AP axis of the main-body follicle cells.

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