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Pointed, an ETS domain transcription factor, negatively regulates the EGF receptor pathway in *Drosophila* oogenesis

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SUMMARY

Spatially regulated activation of the *Drosophila* epidermal growth factor (EGF) receptor by its ligand, Gurken, is required for establishment of the dorsal/ventral axis of the oocyte and embryo. During mid-oogenesis, Gurken is concentrated at the dorsal-anterior of the oocyte and is thought to activate the EGF receptor pathway in adjacent follicle cells. In response to this signal, dorsal follicle cell fate is determined. These cells further differentiate into either appendage-producing or midline cells, resulting in patterning in the dorsal follicle cell layer. We show here that Pointed, an ETS transcription factor, is required in dorsal follicle cells for this patterning. Loss of *pointed* results in the loss of midline cells and an excess of appendage-forming cells, a phenotype associated with overactivation of the EGF receptor pathway in the dorsal region. Overexpression of *pointed* leads to a phenotype similar to that generated by loss of the EGF receptor pathway. This suggests that Pointed normally down-regulates EGF receptor signaling in the midline to generate patterning in the dorsal region. Interestingly, *pointed* expression is induced by the EGF receptor pathway. These data indicate a novel antagonistic function for Pointed in oogenesis; in response to activation of the EGF receptor, *pointed* is expressed and negatively regulates the EGF receptor pathway, possibly by integrating information from a second pathway.

Key words: *pointed*, EGF receptor, cell fate, ETS protein, axis, oogenesis, *Drosophila*, transcription factor

INTRODUCTION

During *Drosophila* oogenesis, cell-cell signaling occurs between the germ-line-derived oocyte and the somatically-derived follicle cells that surround it. The Gurken(Grk)/Torpedo(Top) pathway mediates cell-cell signaling required for follicle cell fate decisions necessary to generate the dorsal/ventral (D/V) as well as the anterior/posterior (A/P) axes of the egg chamber and the future embryo (Schüpbach, 1987; Gonzalez-Reyes et al., 1995; Roth et al., 1995). *Top* encodes the *Drosophila* epidermal growth factor receptor (EGF receptor/Egfr/DER; Price et al., 1989; Schejter and Shilo, 1989) while *grk* encodes a transforming growth factor-alpha (TGF-α) homolog (Neuman-Silberberg and Schüpbach, 1993). Grk, acting in the oocyte, is thought to bind to the EGF receptor on adjacent follicle cells and activate the EGF receptor signaling pathway in these cells (reviewed in Anderson, 1995).

At least two follicle cell subpopulations are required for the establishment of these axes: posterior and dorsal follicle cells. The EGF receptor pathway is required for the determination of both of these subgroups; during early oogenesis, the EGF receptor and Notch pathways are required for proper posterior follicle cell fate determination (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Ruohola et al., 1991), while during mid-oogenesis, activated EGF receptor induces dorsal follicle cell fate (Neuman-Silberberg and Schüpbach, 1993; Roth and Schüpbach, 1994; for review, see Rongo and Lehmann, 1996). Furthermore, two different cell types are observed among the dorsal follicle cells: dorsolateral appendage-producing cells and dorsal midline cells (Spradling, 1993).

How activation of the EGF receptor pathway generates different follicle cell fates is not understood. Do common or unique downstream effectors and target genes determine whether activation of the EGF receptor pathway results in posterior versus dorsal-anterior follicle cells or appendage-producing versus midline cells in the dorsal region?

Some of the effectors and target genes of the EGF receptor pathway have already been identified. Ras 1, Raf and MAP kinase function downstream of the EGF receptor in follicle cells and affect dorsal follicle cell fate decisions during oogenesis (Schnorr and Berg, 1996; Brand and Perrimon, 1994; Lu et al., 1994). In addition, expression of both *rhomboid (rho)* and *nudel* in follicle cells is regulated by the EGF receptor signaling pathway (Ruohola-Baker et al., 1993; Neuman-Silberberg and Schüpbach, 1994; Hong and Hashimoto, 1995). Rho, a putative transmembrane protein, appears to be part of a positive feedback loop that strengthens EGF receptor signaling in the dorsal-anterior follicle cells, and overexpression of *rho* is sufficient to dorsalize egg chambers and embryos (Ruohola-Baker et al., 1993). Nudel, a putative

serine protease, is required for activation of the embryonic D/V pathway and its expression in dorsal follicle cells appears reduced in response to the EGF receptor pathway (Hong and Hashimoto, 1995). During embryogenesis, the EGF receptor also induces expression of Argos, an EGF-motif containing protein, which can inhibit activation of the EGF receptor in vitro (Schweitzer et al., 1995; Golembo et al., 1996).

To identify downstream components of the EGF receptor pathway that are required for follicle cell fate decisions in oogenesis, we have investigated the role of the ETS transcription factor, Pointed (Pnt). *pnt* encodes two proteins, PntP1 and PntP2, which each contain an ETS domain that mediates sequence-specific DNA binding through a novel helix-turn-helix structure (Klämbt, 1993; Scholz et al., 1993; Kodandapani et al., 1996). In addition, PntP2 contains a PNT domain, a motif conserved by a subset of ETS proteins, whose function is, as yet, unclear. In vitro studies have demonstrated that PntP2 transactivation activity can be stimulated by activated MAPK, while PntP1 appears constitutively active (O'Neill et al., 1994).

Pnt is required for proper photoreceptor cell determination during *Drosophila* eye development where it acts as a positive effector, downstream of the Sevenless receptor tyrosine kinase (RTK) pathway (Brunner et al., 1994; O'Neill et al., 1994). Pnt is also required for glial cell differentiation in *Drosophila* embryos, but its regulation is unknown (Klaes et al., 1994).

We show here that *pnt* is also required in *Drosophila* for proper follicle cell fate in the dorsal-anterior region of the egg chamber during oogenesis. Expression of *pnt* in both dorsal and posterior follicle cells is dependent on *grk* activity, indicating that *pnt* acts downstream of the EGF receptor pathway. In addition, overexpression of *pnt* generates a phenotype similar to that produced by loss of the EGF receptor. Therefore, whereas previous analyses in the eye have demonstrated a role for Pnt as a positive effector (Brunner et al., 1994; O'Neill et al., 1994), our data indicates a novel antagonistic function for *pnt* in oogenesis; in response to activation of the EGF receptor pathway, *pnt* is expressed and negatively regulates the EGF receptor pathway, possibly by integrating information from a second pathway.

MATERIALS AND METHODS

Stocks

Drosophila melanogaster stocks were raised on standard corn meal/yeast/agar media at 25°C. The following fly strains were used: *Oregon R*, *l(3)6D1* (*69D* enhancer trap line) (Bier et al., 1989); *fs(1) K10* (Wieschaus et al., 1978); top^{QY1}, grk^{DC29} (Schüpbach, 1987); grk^{2B6} (Neuman-Silberberg and Schüpbach, 1993); $pnt^{\Delta 88}$ (Scholz et al., 1993), $pnt^{\Delta 33}$, $pnt^{\Delta 78}$ (O'Neill et al., 1994); *UASpntP1*, *UASpntP2* (Klaes et al., 1994); *UASlacZ* (Brand and Perrimon, 1993); *KL32* (Clark et al., 1994); $D\text{-}raf^{F179}$, GAL 4 line *55B* (Brand and Perrimon, 1994); GAL 4 line *c324a* (on the second chromosome) was a kind gift from L. Manseau; *E3-26 (P[y⁺tubulin FRTlacZ, w⁺])* (Harrison

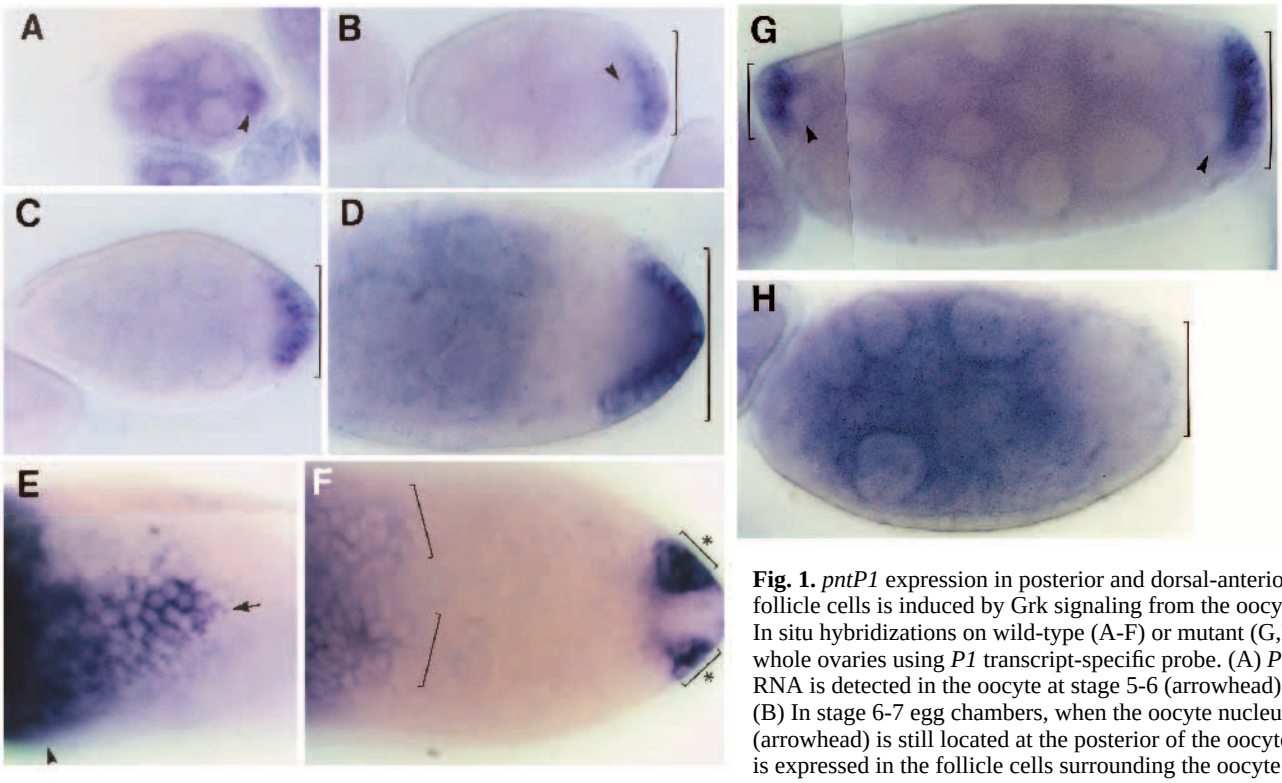

Fig. 1. *pntP1* expression in posterior and dorsal-anterior follicle cells is induced by Grk signaling from the oocyte. In situ hybridizations on wild-type (A-F) or mutant (G,H) whole ovaries using *P1* transcript-specific probe. (A) *P1* RNA is detected in the oocyte at stage 5-6 (arrowhead). (B) In stage 6-7 egg chambers, when the oocyte nucleus (arrowhead) is still located at the posterior of the oocyte, *P1* is expressed in the follicle cells surrounding the oocyte (bracket). (C,D) By stage 8, *P1* expression is restricted to posterior follicle cells (bracket). (E) In stage 9-10 egg chambers, *P1* RNA is detected in dorsal-anterior follicle cells (arrow indicates dorsal midline follicle cells, arrowhead indicates the nurse cell-oocyte boundary; dorsal view). (F) In late stage 10-11 egg chambers, *P1* expression in dorsal-anterior (brackets) and posterior follicle cells (brackets with stars) refines into two regions. (G) In a compound egg chamber (a low penetrance phenotype in top^{QY1}) where the oocyte is detected at both the anterior and the posterior ends of an egg chamber, *P1* expression is detected in the follicle cells adjacent to the anteriorly and posteriorly located oocyte (brackets). Arrowheads indicate the location of the oocyte nuclei. (H) *P1* RNA is not expressed in posterior follicle cells in *grk* null (grk^{2B6}/grk^{DC29}) mutant egg chambers (bracket). Anterior is to the left.

and Perrimon, 1993; D. Harrison, personal communication); *82-w*$^{+}$ (*FRT(82B), w*$^{+)}$ and *w*$^{-}$, *hsFLP1*; *FRT(82B)* (Xu and Perrimon, 1993); *FRT(82B), ovo*^{D1} (Chou et al., 1993; Bloomington Stock Center). Crosses producing flies ectopically expressing or overexpressing *UASpntP1* were carried out at 18°C.

Staining procedures, in situ hybridization and other methods

In situ hybridization and X-GAL staining procedures were performed as described previously (Ruohola et al., 1991) with the following modifications. Ovaries were hand dissected in phosphate-buffered saline. For in situ hybridizations, ovaries were dissected fully into ovarioles before probe was added and all solutions were treated with 0.1% DEPC. In addition, ovaries were not stored for longer than one week at −20°C before use. *pointed* transcript-specific DNA probes were generated from *Eco*RI-*Bam*HI digests of the plasmids pRSET P1 and pRSET P2 (O'Neill 1994 thesis, UC Berkeley). The probes correspond to nucleotides #1050-1733(P1) and #779-1746(P2) according to the numbering used in Klämbt (1993). For microscopic examination of chorion structures, eggs were mounted in lactic acid:Hoyers (1:1) as described previously (Wieschaus and Nüsslein-Volhard, 1986).

Generation of FRT/FLP-induced follicle cell clones

To obtain FLP-FRT-generated mosaics, recombinants between *pnt*$^{\Delta 88}$, *pnt*$^{\Delta 33}$, *pnt*$^{\Delta 78}$ and *w; P[ry*$^{+}$*, hs-neo, FRT]82B, P[ry*$^{+}$*, w*$^{+}$*]90E*, hereafter referred to as *FRT(82B)w*$^{+}$, were generated to produce *FRT(82B), pnt/TM3, Sb*. *FRT(82B), pnt/TM3, Sb* were crossed with *w*$^{-}$*, FLP1; FRT(82B)w*$^{+}$ and third instar larval progeny were heat shocked for 30 minutes in a 39˚C water bath on two consecutive days to induce FLP1 activity. Female offspring of the genotype *w, FLP1/w; FRT(82B), pnt/FRT(82B)* were fed yeast for two days and eggs laid thereafter were analyzed.

To mark induced clones, a tubulin promoter *FRTlacZ* construct inserted around 84 (E3-26, Harrison and Perrimon, 1993 and D. Harrison pers. comm.) was recombined with the *FRT(82B)w*$^{+}$ chromosome. *FRT(82B), tubulinFRTlacZ(84)/TM3, Sb* males were crossed to *w*$^{-}$*, FLP1; FRT(82B)w*$^{+}$ females to produce *w*$^{-}$*, FLP1/Y; FRT(82B), tubulinFRTlacZ(85D)/ FRT(82B)w*$^{+}$ males. These males were crossed with *FRT(82B), pnt*$^{\Delta 88}$*, w*$^{+}$*/TM3, Sb* females and mosaic clones were induced in their progeny as described above. *w*$^{-}$*, FLP1/w; FRT(82B), pnt*$^{\Delta 88}$*, w*$^{+}$*/FRT(82B), tubulinFRTlacZ(84)* females were distinguished by their dark red eye phenotype *[FRT(82B), pnt*$^{\Delta 88}$*, w*$^{+}$*]* and by X-GAL staining of their ovaries. As the *tubulin FRTlacZ* construct E3-26 contains a FRT, we checked the resultant *w, FLP1/w; FRT(82B), tubulinFRTlacZ(84)/TM3, Sb* progeny of the above cross for mosaic patches in their ovaries. Out of 100 dissected females, no mosaic patches were found in these control animals.

Generation of homozygous mutant germ-line clones

Germ-line clones of *pnt*$^{\Delta 88}$ were generated using *FRT(82B), ovo*^{D1} (Chou et al., 1993). *w; FRT(82B), pnt*$^{\Delta 88}$*, w*$^{+}$*/TM3, Sb* females were crossed with *w, FLP1/Y; TM3, Sb/+* to obtain *w, FLP1/w; FRT(82B), pnt*$^{\Delta 88}$*, w*$^{+}$*/TM3, Sb* females, which were crossed to *w; FRT(82B), ovo*^{D1}*/TM3, Sb* males, and mosaic clones were induced in the progeny as before. Females of the genotype *FRT(82B), pnt*$^{\Delta 88}$*, w*$^{+}$*/FRT(82B), ovo*^{D1} and either *w, FLP1/w* or *w/w* were selected and their ability to lay eggs was scored.

RESULTS

pnt is expressed in posterior and dorsal-anterior follicle cells during oogenesis

The *pointed* locus encodes two proteins, PntP1 and PntP2, which share a region homologous to the ETS family of tran-

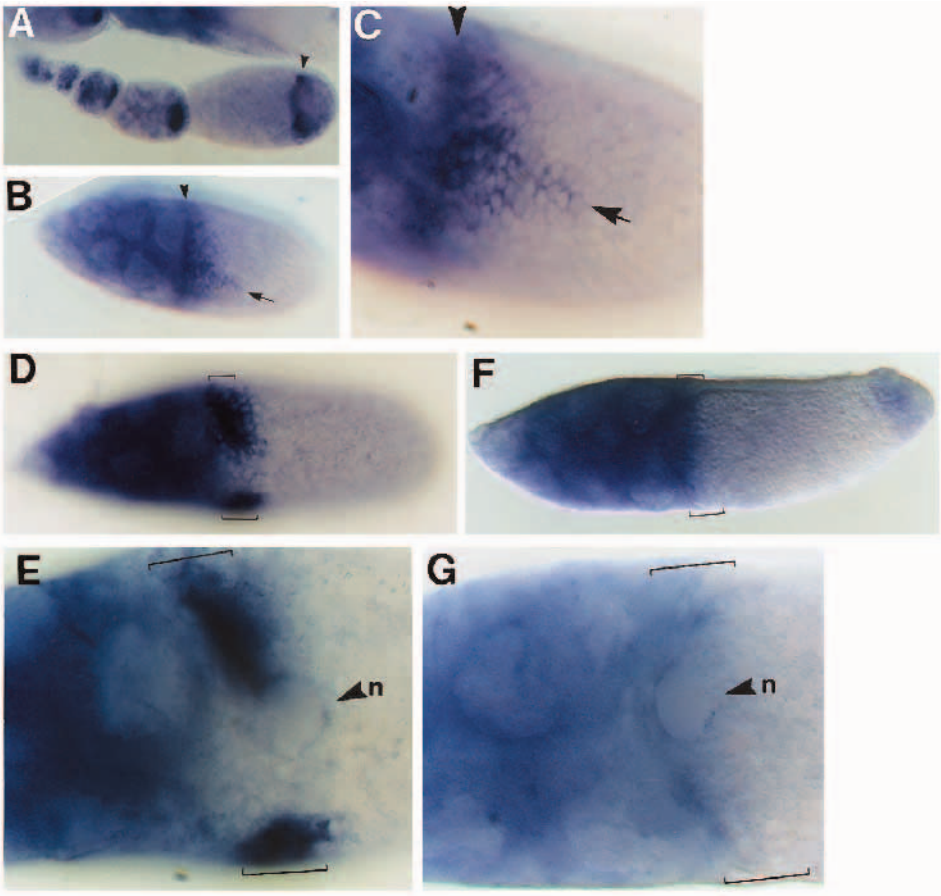

Fig. 2. *pntP2* expression in dorsal-anterior follicle cells is induced by Grk. In situ hybridizations on wild-type (A-E) or mutant (F,G) whole ovaries using *P2* transcript-specific probe. (A) Germarium to stage 8 egg chambers. *P2* RNA is expressed in the germarium and is also detected in the oocyte in stage 2-6 egg chambers. In stage 8 egg chambers, *P2* RNA is concentrated at the anterior region of the oocyte (arrowhead). (B,C) Expression of *P2* RNA in dorsal follicle cells is first detected in stage 9-10 egg chambers (arrow indicates dorsal midline follicle cells, arrowhead indicates the nurse cell-oocyte boundary, dorsal view). (D,E) A later pattern of *P2* RNA expression in follicle cells is detected in late stage 10-11 egg chambers. Two groups of dorsal-anterior follicle cells that flank the oocyte nucleus express *P2* RNA (brackets; dorsal view). No follicle cell expression is detected at the dorsal midline or in ventral follicle cells. (F,G) *P2* RNA expression in dorsal-anterior follicle cells is no longer detected in *grk* null mutant egg chambers (*grk*^{2B6}*/grk*^{DC29}; brackets; dorsal view). (A,B,D,F) 40× magnification and (C,E,G) 100× magnifications of the dorsal region of the middle of the egg chambers in pictures B, D and F, respectively. n is oocyte nucleus.

scription factors (Klämbt, 1993). The *P1* and *P2* transcripts also contain mutually exclusive exons that allow the generation of transcript-specific probes to distinguish the expression of *P1* and *P2* RNA during oogenesis by in situ hybridization (see Materials and Methods for details).

In wild-type *Drosophila* ovaries, the *P1* transcript is expressed in the germ line and in posterior and dorsal-anterior follicle cells. Weak expression of *P1* RNA is detected in the germarium (data not shown). Accumulation of the transcript in the oocyte is detected around stage 5-6 (Fig. 1A). At stage 6-7, *P1* is expressed in the follicle cells that surround the oocyte at a time when the oocyte nucleus is still located at the posterior of the oocyte (Fig. 1B). By stage 8, expression is restricted to posterior follicle cells (Fig. 1C,D). During stage 9-10, *P1* is detected in dorsal-anterior (Fig. 1E, 'early pattern') and posterior follicle cells (comparable to Fig. 1D). During later stages, both of these expression patterns refine into two areas; two dorsal patches and two posterior half-circles (Fig. 1F, 'late pattern').

Similar to *P1*, *P2* expression is detected in the germ line and follicle cells in wild-type ovaries. *P2* is present in the germarium and in the oocyte from stages 2-8 (Fig. 2A). During stage 8, the *P2* transcript is localized at the anterior of the oocyte (Fig. 2A). Follicle cell expression is observed in stage 9-10 egg chambers in a pattern resembling the early *P1* expression pattern in dorsal-anterior follicle cells (Fig. 2B,C, 'early pattern'). During later stages, *P2* expression is restricted to two groups of anterior dorsolateral follicle cells that flank the oocyte nucleus (Fig. 2D,E, 'late pattern').

pnt expression in posterior and dorsal-anterior follicle cells is induced by *grk*

The expression of the *P1* and *P2* transcripts specifically in posterior and dorsal-anterior follicle cells (Figs 1, 2) suggested that expression of *pnt* may be regulated by the signaling cascade that determines these follicle cells' fates, namely, the EGF receptor pathway. As activation of the EGF receptor pathway is dependent upon a signal from the oocyte (Schüpbach, 1987; Neuman-Silberberg and Schüpbach, 1993), we first examined whether *P1* expression in follicle cells is induced by the oocyte. In some mutants that cause female sterility, compound egg chambers are generated in which an oocyte can be found at both the anterior and posterior end of the egg chamber (King, 1970; Neuman-Silberberg and Schüpbach, 1993). In situ hybridization of compound egg chambers produced by top^{QY1} mutants with a *P1*-specific probe indicated that *P1* is specifically expressed in follicle cells at both ends of the egg chamber, adjacent to the oocytes (Fig. 1G). These data show that anterior follicle cells can express *P1* if the oocyte is in close proximity and suggests that signaling from the oocyte induces *P1* expression in posterior follicle cells in wild-type egg chambers. To determine whether *grk*-mediated signaling from the oocyte is required for *P1* expression in posterior follicle cells, *P1* expression was examined in grk^{2B6}/grk^{DC29} null mutant egg chambers. Whereas control egg chambers exhibit strong posterior follicle cell staining (Fig. 1D), no *P1* RNA is detected in posterior follicle cells in grk^{2B6}/grk^{DC29} egg chambers (Fig. 1H). In addition, *P1* expression in dorsal follicle cells was not observed in these mutant egg chambers (data not shown), however germ-line expression was unaltered. These results demonstrate that *P1* RNA expression in the follicle cells is dependent upon *grk* and suggest that PntP1 expression in posterior and dorsal follicle cells is controlled by the EGF receptor signaling pathway.

To test whether *P2* expression is also dependent on the Grk/Top signaling pathway, *P2* RNA expression was examined in grk^{2B6}/grk^{DC29} egg chambers by in situ hybridization with a *P2* transcript-specific probe. In contrast to the dorsal-anterior follicle cell expression observed in wild-type egg chambers (Fig. 2B-E), *P2* is not detected in follicle cells in these *grk* null mutant egg chambers (Fig. 2F,G). Germ-line expression of *P2*, however, was unaffected by the loss of *grk* activity (compare Fig. 2D to F). Mutations in genes involved in the Grk/Top signaling pathway, such as *top* and *fs(1)K10* (Wieschaus et al., 1978) also affect *pnt* expression. In non-compound egg chambers generated by top^{QY1} mutants, the region of *P2* expression is reduced, while in *fs(1)K10* mutants, *P2* expression is expanded (data not shown). These alterations in *pnt* expression correlate with the changes in dorsal follicle cell fate previously observed in *top* or *fs(1)K10* mutants (Schüpbach, 1987; Wieschaus et al., 1978). These results indicate that *P1* and *P2* expression in posterior and dorsal follicle cells is dependent on Grk/Top signaling.

pnt function is required for oogenesis

The induction of *pnt* expression by the Grk/Top pathway suggested a functional requirement for *pnt* in follicle cell determination. To investigate this possibility, the phenotype of *pnt* null mutants in oogenesis was examined. As null alleles of *pnt* cause embryonic lethality (Mayer and Nüsslein-Volhard, 1988), mosaic animals were generated using the FLP/FRT recombination system (Golic, 1991) and the allele, $pnt^{\Delta 88}$, which is null for both *P1* and *P2* (Scholz et al., 1993; Brunner et al., 1994). The fate of the follicle cells was examined by analyzing the outer layer of the egg shell, the chorion, which is secreted by follicle cells and therefore serves as a 'footprint' for these cells. The dorsal appendages, located at the dorsal-anterior end of the egg shell, are part of the chorion and serve as a marker for the fate of dorsal-anterior follicle cells. Whereas two dorsolateral appendages are detected in control eggs (Fig. 3A), the eggs laid by mosaic animals exhibit a single broad appendage. This mutant appendage is four times wider than a single wild-type appendage, suggesting that the phenotype does not result from two appendages fusing together, but rather cells in the middle region taking on an appendage-producing cell fate (Fig. 3B). Importantly, a similar phenotype (comparable to Fig. 3B) was detected when either $P1(pnt^{\Delta 33})$ or $P2(pnt^{\Delta 78})$ specific mutant alleles were used, suggesting that both *P1* and *P2* are required in the same process during oogenesis. $pnt^{\Delta 88}$, the allele null for both *P1* and *P2* (Scholz et al., 1993) was used in subsequent experiments.

To determine which cells require *pnt* function, marked mosaic clones were generated (for details see Materials and Methods). Briefly, the wild-type chromosome was marked with the *lacZ* gene controlled by a *β-tubulin* promoter (Harrison and Perrimon, 1993) which directs expression in both follicle cells and the germ line. The chromosome bearing the $pnt^{\Delta 88}$ null allele does not carry the *lacZ* marker, thus $pnt^{\Delta 88}$ homozygous mutant cells can be detected due to a lack of β-galactosidase (β-gal) activity (Fig. 4B-D; Table 1B). X-gal staining of ovaries from potentially mosaic females indicated

that eggs lacking a mosaic patch had wild-type dorsal appendages (Fig. 4A; Table 1B, *n*=740). In addition, a complete lack of *pnt* in the germ line had no effect on dorsal appendage formation (Fig. 4B, *n*=48). However, the mutant broad appendage phenotype was detected in all egg chambers that had a β-gal-negative patch of follicle cells in the dorsal-anterior region (Fig. 4C,D; Table 1B, *n*=17/17). Lastly, in eggs in which the entire follicle cell layer was mutant for $pnt^{\Delta 88}$, no stronger phenotypes in the dorsal-anterior region were detected (Fig. 4C) indicating that the broad appendage is the strongest phenotype generated by the lack of *pnt*.

The appendage phenotype generated by the loss of *pnt* indicated that *pnt* affects patterning of the dorsal-anterior region of the chorion. Mutations in *grk* and *top* also affect the dorsal region of the egg shell (Schüpbach, 1987). However, these mutants affect the entire dorsal region whereas loss of *pnt* specifically affects the midline region (Figs 3B, 4C-D). *pnt* may also have a function in posterior follicle cells since loss of *pnt* function in these cells leads to mislocalization of an A/P axis marker, Kinesin-β-galactosidase (Clark et al., 1994; K. T. and H. R.-B., unpublished observations).

As the Grk/Top pathway affects the D/V axis of the embryo as well as the egg shell (Schüpbach, 1987), we next tested whether loss of *pnt* in the follicle cells also alters embryonic patterning. Eggs exhibiting the broad appendage phenotype were isolated and allowed to develop. The majority of these eggs hatched and developed into fertile adults. Therefore loss of *pnt* in the dorsal region does not lead to severe *grk*- or *top*-like D/V axis defects in the embryo. This was expected as the loss of *pnt* only affects a subgroup of the dorsal follicle cells affected by mutations in *grk* or *top*. However, since the percentage of eggs without early embryonic development was higher than that detected in the control group (data not shown), we cannot rule out the possibility that the loss of *pnt* in follicle cells may cause some embryonic defects.

pnt is not required in the germ line for oogenesis

Previous studies indicated that *pnt* is not required in the germ line during oogenesis; mosaic females with a germ line homozygous for the pnt^{9J31} or pnt^{B74} mutation did not exhibit any apparent defects in oogenesis (Mayer and Nüsslein-Volhard, 1988). As the possibility that pnt^{9J31} *and* pnt^{8B74} are not null mutations could not be ruled out, the function of *pnt* in the germ line was reinvestigated. Using the mutant allele, $pnt^{\Delta 88}$, that is null for both *P1* and *P2* (Scholz et al., 1993), we generated germ-line clones by both ovo^{D1} (Chou et al., 1993) and FLP/FRT-methods (Golic, 1991). Similar to the results found with pnt^{9J31} and pnt^{8B74}, eggs from mosaic females with a germ line homozygous for the $pnt^{\Delta 88}$ mutation did not exhibit any defects in the dorsal appendages or any discernible embryonic phenotype (Fig. 4B; Table 1). Thus, in the germ line, *pnt* does not appear to play an important role in oogenesis. These observations indicate that the phenotype detected in the $pnt^{\Delta 88}$ mosaics is due to a lack of *pnt* in follicle cells. The following analyses therefore focus on the function of *pnt* in follicle cells. Furthermore, within the follicle cell layer, Pnt function appears to be required in midline follicle cells for proper formation of that region. In marked mosaic clones, the presence of cells wild type for *pnt* in the midline region but not in the dorsolateral region correlated with wild-type midline structures (Table 1B). This observation indicates that *pnt* expression is required in the midline region to elicit proper dorsal follicle cell patterning and suggests that the early pattern of *pnt* expression with high concentration in the midline region (Figs 1E, 2B,C) relates functionally to the above observed phenotypes.

Table 1. *pointed* function is required in the follicle cells for oogenesis

(A) FLP/FRTovoD1-induced germ-line clones

Genotype	*n*	unfert.	% fert., not hatched
(a) $FRT,pnt^{\Delta 88}/FRT,ovo^{D1} \times pnt^{\Delta 88}/TM3Sb$	17	15	52 (102/211)
(b) $FRT,pnt^{\Delta 88}/FRT,ovo^{D1} \times yw/Y$	16	12	3 (6/191)
(c) $FRT,ovo^{D1}/TM3Sb \times yw/y$	19	na	na

[n]Females carrying potential germ-line clones were crossed with $pnt^{\Delta 88}$ (a) or wild-type (b) males and eggs were allowed to develop at 25°C for 2 days. Only 3% of fertilized eggs did not hatch when females carrying potential clones were backcrossed with wild type (b), but 52% did not hatch when backcrossed with $pnt^{\Delta 88}/TM3, Sb$. (a) These remaining eggs exhibit the same phenotype as $pnt^{\Delta 88}$ as judged by their cuticle. As a control, the ability of FRT, ovo^{D1} females to lay eggs was assessed. No eggs were laid, indicating the presence of the ovo^{D1} mutation.

(B) FLP/FRT-induced marked mosaics

Type of clone	*n*	Wild-type appendages	Broad appendage
Germ line	48	48	–
Somatic, in appendage sheath*	17	–	17
No clones in appendage sheath	740	740	–

*A clone can be identified in the appendage sheath. Approximately 160 follicle cells were associated with wild-type appendages. The size of mosaic patches varied from 75% to 100% of this area (wild-type cells detected in the mosaics with appendage phenotype: *n*=0-40, average=10). A single broad appendage was detected when the wild-type cells were located in the dorsolateral region (6/14). However, wild-type cells located in the midline region correlated with partial restoration of wild-type midline structures (e.g. holes in a broad appendage, 8/14). In flies of the same genotype without obvious clones, an average of 142 β-gal-positive cells was detected (*n*=7).

Targeted expression of *pnt P1* and *P2* in follicle cells

The lack of *pnt* expression in *grk* null mutant egg chambers (Figs 1H, 2F,G) and the phenotype associated with null alleles of *pnt* (Figs 3B, 4C,D) suggest that *pnt* acts downstream of the Grk/Top pathway and affects dorsal follicle cell patterning. To further examine the role of *pnt* in dorsal follicle cells, we overexpressed it in subsets of follicle cells by using the GAL4/UAS system (UAS=upstream activation sequence for GAL 4; Brand and Perrimon, 1993). Two different GAL4 insertion lines, *55B* (Brand and Perrimon, 1994) and *c324a* (a gift from L. Manseau) were used (see Materials and Methods). The GAL4 expression pattern for these lines was determined by crossing them to a line carrying a *UAS-lacZ* gene and staining their progeny for β-gal activity. *55B* activates β-gal expression in an approximately 8-cell-wide band of follicle cells at the anterior of the oocyte in stage 8-9 egg chambers (Brand and Perrimon, 1994). Line *c324a* activates β-gal expression in a patchy manner in anterior follicle cells with higher (Fig. 5I; 50% of the egg chambers) or equivalent expression in dorsal versus ventral follicle cells around stage 9-10. Thus, the *55B* line will induce expression of a *UAS*-target gene in the anterior follicle cells at the ventral and dorsal sides, and *c324a* will

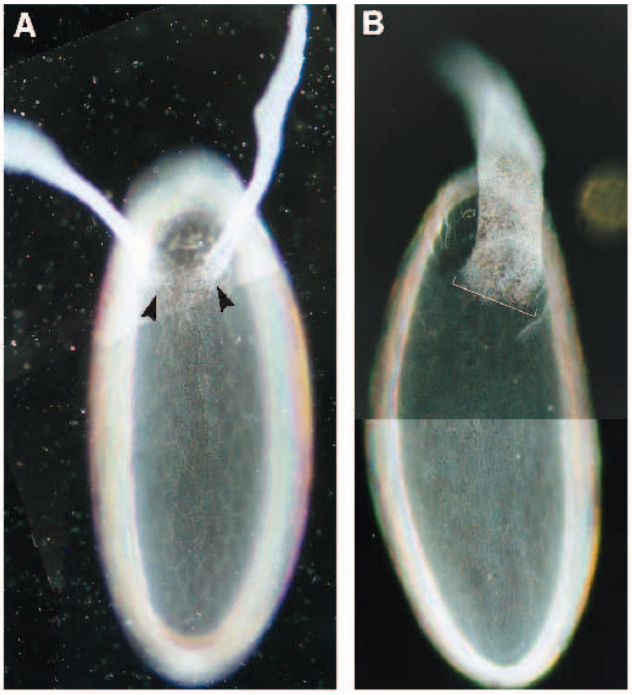

Fig. 3. Both *pntP1* and *pntP2* are required for proper patterning of the dorsal region of the egg shell. Chorion phenotypes of wild-type (A) and $pnt^{\Delta 88}$ mosaics (*w,FLP/w; FRTpnt*$^{\Delta 88}$*/FRT*; B). (A) A wild-type egg shell produces two respiratory dorsal appendages (arrowheads) that flank the dorsal midline. (B) Ovaries mosaic for *pnt* loss-of-function mutations (*Δ88 [P1 and P2]*; *Δ33[P1]* and *Δ78[P2]* null alleles) produce egg shells in which one broad appendage is detected in the dorsal region (bracket) in 5-10% of the total population of eggs laid by animals in which FLP activity was induced. The broad appendage (bracket, B) is approximately four times wider than one wild-type appendage (arrowhead, A) suggesting that the phenotype is not a result of two appendages fusing together, but rather cells in the middle region taking an appendage-producing cell fate.

induce expression either in dorsal (50% of the egg chambers) or in dorsal- and ventral-anterior follicle cells. To create GAL4-responsive *pnt P1* and *P2* genes, the coding sequences for *pntP1* and *pntP2* were cloned downstream of a tandem array of five optimized GAL4-binding sites (a generous gift from C. Klämbt; Klaes et al., 1994).

When either *55B* or *c324a* was crossed to flies carrying *UASpntP2*, the female progeny laid wild-type eggs which developed normally (data not shown). However, when *55B* or *c324a* was crossed to flies carrying the *UASpntP1* construct, the females were sterile or semi-sterile, respectively, and produced eggs that had dorsal defects as judged by their chorion phenotypes. Instead of two wild-type dorsal appendages (Fig. 5A), no appendages or small 'nubs' of appendage material were detected (Fig. 5B, big arrowheads). In addition, 'crumpled' chorion material was consistently observed (Fig. 5B, small arrowheads). However, no defects were detected on the ventral side of the egg shell. Ectopic expression or overexpression of *P1* but not *P2* can therefore cause a loss of dorsal appendages, a phenotype associated with loss of Grk/Top signaling. In contrast, the loss-of-function studies (Figs 3, 4) indicated a functional requirement for both *pntP1* and *pntP2* for proper dorsal patterning. As ectopic or overexpression of *pntP1* suffices to affect dorsal cell fates, whereas expression of *pntP2* does not, the results together suggest that *pntP1* may act in parallel or downstream of *pntP2*. For instance, the expression of *pntP1* may be regulated by *pntP2* which may in turn require post-translational modifications such as phosphorylation for full activity (O'Neill et al., 1994).

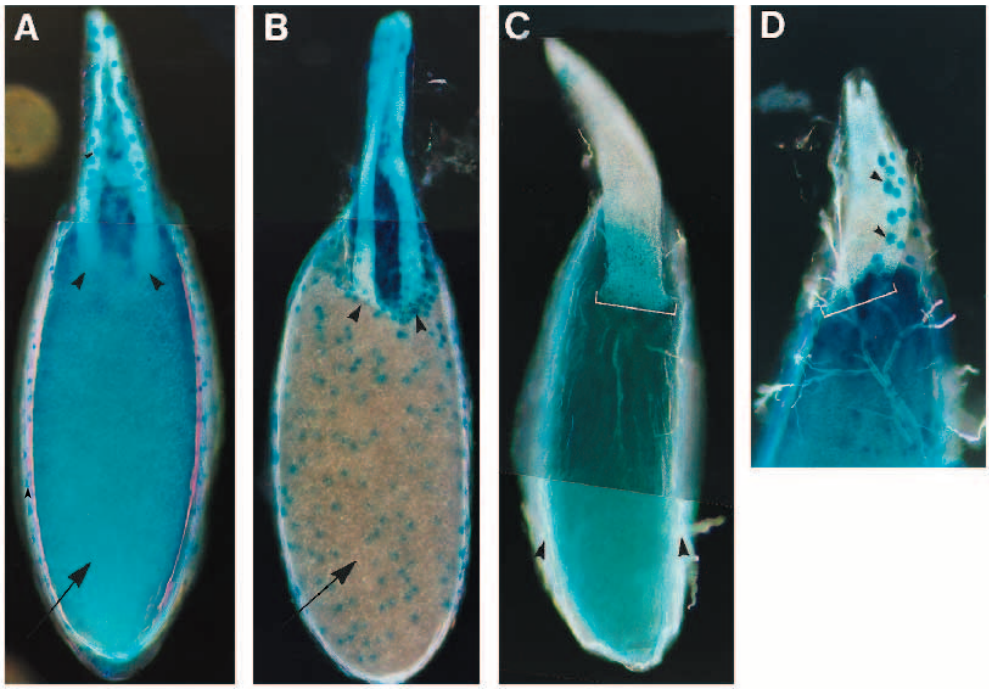

Fig. 4. *pnt* is required in dorsal-anterior follicle cells. Mosaic clones detected in eggs laid by *w,FLP/w; FRT,pnt*$^{\Delta 88}$*/FRT,E3-26(tublacZ)* females. Lack of β-galactosidase (β-gal) activity indicates a homozygous $pnt^{\Delta 88}$ mutant cell. (A) Wild-type appendages are detected in eggs in which no mosaic patches are observed (arrowheads). β-gal is detected in the nuclei of all the follicle cells (small arrowheads) and in the oocyte (arrow). (B) Eggs homozygous mutant for *pnt* in the germ line (the oocyte lacks β-gal activity, arrow) have no apparent egg shell phenotype (arrowheads indicate the wild-type appendages) suggesting that *pnt* is not required in the germ line for the broad appendage phenotype. (C) If the entire follicle cell layer is mutant for *pnt* (β-gal activity is not detected in the entire follicle cell layer, arrowheads) a broad appendage is detected (bracket) indicating that it is the strongest phenotype generated in the dorsal-anterior region. The blue color seen at the base of the broad appendage is from β-gal expression in the germ line. (D) Loss of *pnt* function in follicle cells in the dorsal-anterior region results in a broad appendage phenotype (bracket, Table 1B). The maximum amount of wild-type cells (arrowheads) detected in the mutant appendage was one fourth of the cells in the area (40/160).

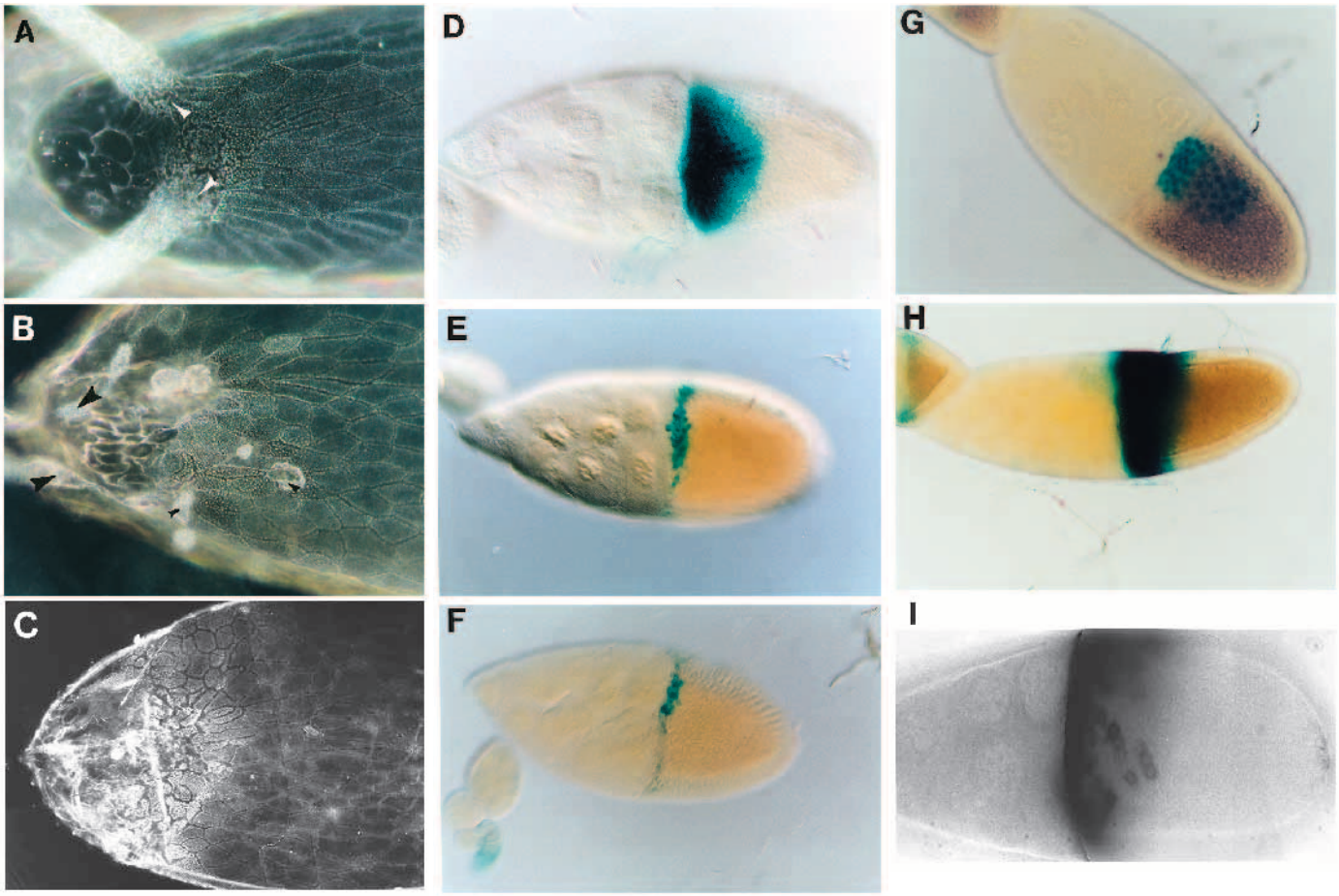

Fig. 5. Overexpression of *pntP1* results in a loss of dorsal follicle cells. (A-C) Dark-field pictures of wild-type and mutant egg shells (dorsal view of anterior end of egg). (A) Wild-type chorion produces two respiratory appendages that flank the dorsal midline (arrowheads). (B) Intermediate egg shell phenotype with small appendage 'nubs' (arrowheads) and 'crumpled' chorion material (small arrowheads) were generated by expression of *UASpnt P1* using the GAL 4 lines *55B* (46% of the eggs) and *c324a* (14% of the eggs). GAL 4-dependent expression is specifically turned on in a 6-8 cell wide band of anterior follicle cells in the *55B* line (Brand and Perrimon 1994). The *c324a* line directs UAS-dependent expression at equivalent levels in dorsal versus ventral cells or at a slightly higher level in dorsal versus ventral follicle cells. (C) No appendages are detected in the strongest egg shell phenotypes detected in *55B/UASpntP1* or *c324a/+;UASpntP1/+* eggs (34% and 13% of the eggs, respectively). In weaker phenotypes, the appendage 'nubs' were accompanied by nearly wild-type looking appendages. Overexpression of *pntP1* in dorsal-anterior follicle cells using the *c324a* line was verified by in situ hybridization using a *P1* transcript-specific probe (I) (dorsal view). (D-H) X-GAL staining of the enhancer trap line *69D* in wild-type and mutant ovaries. (D) The enhancer trap line, *69D*, specifically expresses β-gal in both appendage-producing and midline cells (dorsal view). (E,F) The expression of *69D* in dorsal-anterior follicle cells is reduced due to overexpression of *pntP1* directed by either the *55B* or *c324a* GAL4 line in 70% (n=35) egg chambers and 30% (n=74) of the egg chambers, respectively (dorsal view). In extreme phenotypes, a nearly complete loss of dorsal follicle cells expressing the marker was observed, indicating a loss of both the midline and appendage-producing cells. Intermediate cases were also observed in which follicle cells expressing the dorsal follicle cell marker were intermingled with follicle cells no longer expressing the marker. This uneven expression is probably due to the GAL 4 expression system as *UASpntP1* expression was activated in an uneven manner (Fig. 5I and data not shown). (G) Mutations in *top* (top^{QY1}) result in a reduction in the number of *69D*-expressing follicle cells (dorsal view). (H) Mutations in *fs(1)K10* generate an increase in the number of *69D*-expressing follicle cells (lateral view).

Ectopic expression or overexpression of *pnt P1* results in a lack of dorsal-anterior follicle cells

The loss of dorsal appendages caused by ectopic expression or overexpression of *P1* suggests that *pnt* acts in an opposite manner to the Grk/Top signal with respect to the chorion. This chorion defect, however, is a terminal phenotype which could be generated either by a transformation of appendage-producing cells into midline follicle cells or by loss of both follicle cell types. To analyze the follicle cell defect that leads to the chorion defect following overexpression of *P1*, a P element enhancer trap line, *69D* (Fig. 5D) was used as a cell fate marker. *69D* is downstream of the Top pathway and expression of this marker is expanded in dorsalized egg chambers overexpressing Rho (Ruohola-Baker et al., 1993), or a constitutively active form of Raf ($D\text{-}raf^{F179}$; data not shown) and in *fs(1)K10* mutant egg chambers (Fig. 5H), whereas expression is reduced in a top^{QY1}mutant background (Fig. 5G). Importantly, this line marks both the dorsal midline and the dorsolateral follicle cells during stages 9-14 in oogenesis Therefore, if overexpression of *pntP1* transforms dorsolateral,

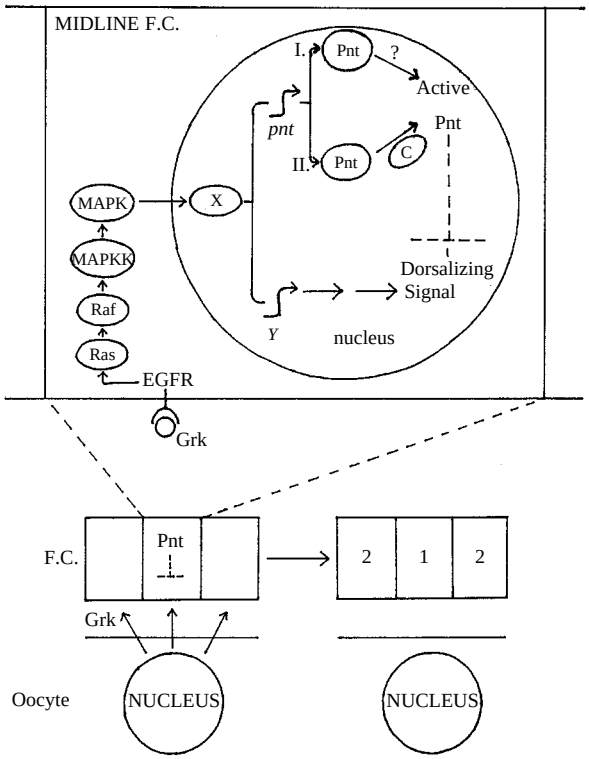

Fig. 6. A model for *pnt* function in dorsal follicle cell patterning. *grk* accumulates around the oocyte nucleus and activates the EGF receptor signaling pathway in the adjacent, dorsally located follicle cells (FC). *pnt* in the midline region partially antagonizes Grk signaling, preventing fate 2 ('appendage fate') and allowing the midline to take fate 1. The pattern 2, 1, 2 (appendage, midline, appendage) is generated in the dorsal region. The midline region has been enlarged to indicate the molecular basis of *pnt* function. Grk, produced by the oocyte, binds to the EGF receptor in the midline and dorsolateral follicle cells, activating the EGF receptor signaling pathway that includes Ras, Raf and the MAPKs. MAPK activates or inactivates (Hsu et al., 1996) a transcription factor(s), denoted X, in the follicle cell nucleus which culminates in the transcription of *pnt* and another target gene, denoted *Y*. *Y* transmits the EGF receptor 'dorsalizing' signal to these cells. An active form of Pnt in the midline may be generated by (I) a signal from an unknown inhibitory pathway or by (II) association with a cofactor (C) that is present in limited amounts. The active form of Pnt in the midline then negatively modulates the 'dorsalizing' signal transmitted by Y. This antagonism of the EGF receptor signal is required for the formation of midline region. Pnt does not act in the dorsolateral cells and thus allows a high level of EGF receptor signaling to occur, which results in appendage formation.

appendage-producing cells to midline cells, no change in *69D* pattern is expected. However, if both cell types are lost, loss of *69D* marker should be observed.

Overexpression of *pnt*, generated by GAL4 lines *55B* or *c324a*, leads to a severe reduction in the number of follicle cells expressing the *69D* marker (Fig. 5E,F). Using the *55B* line, the extreme phenotype detected in 34% of the egg chambers (n=35 egg chambers) was a loss of *69D* expression in all dorsal-anterior follicle cells except a row of cells between the nurse cells and the oocyte (Fig. 5E,F). The remaining ring of *69D*-positive follicle cells is thought to be independent of the D/V patterning system (Neuman-Silberberg and Schüpbach, 1994) suggesting that the extreme phenotype generated by overexpression of *pntP1* is a total loss of dorsal follicle cell fate. An intermediate phenotype was detected in 29% of the egg chambers in which a milder reduction of the *69D* pattern was observed (data not shown). These results demonstrate that ectopic or overexpression of *pntP1* results in an overall reduction in the number of both dorsolateral and dorsal midline follicle cells, a phenotype similar to that generated by the loss of *grk* or *top* (Schüpbach, 1987).

Preliminary results also indicate that overexpression of *pntP1* reduces expression of *rhomboid*, another dorsal follicle cell marker (data not shown). As *rhomboid* is required downstream of the EGF receptor for dorsal follicle cell determination (Ruohola et al., 1993; Neuman-Silberberg and Schüpbach, 1994), the results with both the *69D* and *rhomboid* markers indicate that overproduction of *pnt* results in a down-regulation of components of the EGF receptor pathway. These results suggest that Pnt antagonizes EGF receptor signaling by affecting components of this pathway.

DISCUSSION

We have examined the expression pattern and functional requirements for Pnt, an ETS transcription factor, during *Drosophila* oogenesis. We show here that *pnt* is required for proper dorsal follicle cell fate. Loss of *pointed* results in a transformation of dorsal midline follicle cells to appendage-producing follicle cells. Overexpresssion of *pntP1* results in a loss of dorsal-anterior follicle cells, a phenotype similar to that generated by loss of EGF receptor pathway. In addition, expression of *pointed* is induced by the EGF receptor pathway and overproduction of *pointed* down-regulates components of the EGF receptor pathway. These results indicate a novel antagonistic function for *pointed*; in response to activation of the EGF receptor pathway, *pointed* is expressed and negatively regulates the signal mediated by the EGF receptor pathway in dorsal follicle cells. As such, *pointed* may identify an inhibitory branch of the EGF receptor pathway or may integrate inhibitory information from another pathway.

A simple Gurken morphogen gradient cannot explain dorsal patterning

During mid-oogenesis, Grk induces dorsal follicle cell fate (Neuman-Silberberg and Schüpbach, 1993, 1994). In response to Grk/Top signaling, dorsolateral follicle cells secrete the dorsal appendage chorion structures, whereas the dorsal midline cells do not. Thus, the dorsal region of the egg shell exhibits a pattern arbitrarily designated 2-1-2 where 2 designates the appendage regions and 1 designates the dorsal midline (Fig. 6). Although *grk* is required to establish the entire dorsal region of the egg, it is currently unclear how these different dorsal cell fates are generated.

One possibility is that a simple concentration gradient of a morphogen induces these different dorsal follicle cell fates. For example, a gradient of nuclear localization of the morphogen Dorsal is sufficient to specify the expression of different sets of zygotic genes and, thus, different cell fates (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). During mid-oogenesis, *grk* is localized at the dorsal-anterior of the oocyte

and appears most concentrated at the dorsal midline in wild-type flies (Roth and Schüpbach, 1994). A simple concentration gradient model predicts that a low concentration of Gurken would induce the appendage-forming cell fate and a high concentration would induce the midline cell fate. The model also predicts that overactivation of the EGF receptor pathway would induce the midline fate. However, two sets of experiments indicate that overactivation of the EGF receptor pathway can result in the appendage-producing, not midline, cell fate. First, the most extreme phenotype generated by overexpression of *grk* is an excess of dorsal appendage material over the dorsal side (Fig. 1D in Neuman-Silberberg and Schüpbach, 1994). Second, flies bearing a gain-of-function mutation in Raf(*D-raf*^{F179}) lay eggs that exhibit a phenotype in which more cells take on an appendage-producing cell fate, including cells at the dorsal midline (Brand and Perrimon, 1994; this laboratory). It is worth pointing out that while the milder phenotype in both of these cases is expansion of the midline region, the extreme phenotype is the transformation of the midline cells to the appendage-producing cells. Therefore a simple morphogen concentration gradient model does not explain how wild-type patterning is generated in the dorsal region of the egg.

Does pnt induce the dorsal midline cell fate?

Alternatively, the EGF receptor pathway may have the potential to induce the entire dorsal region to take the follicle cell fate 2 and an additional mechanism produces the cell fate 1 at the dorsal midline. *pnt* could represent a component of an independent pathway that induces the dorsal midline cell fate. However, ectopic expression of *P1* in ventral follicle cells had no apparent effect. Also overexpression of *pnt* results in a loss of the *69D* marker in dorsal midline cells (Fig. 5E,F), indicating that overproduction of *pnt* reduces the number of dorsal follicle cells and that *pnt* does not directly induce the dorsal midline cell fate.

pnt negatively regulates the dorsal signal induced by the EGF receptor pathway

How then is patterning generated in the dorsal region of the egg? If the effective activity of EGF receptor signaling were reduced at the dorsal midline by an inhibitory factor, a pattern 2, 1, 2 could be generated. Our data suggest that *pnt* may encode this inhibitory activity. *pnt* is expressed in dorsal midline follicle cells ('early pattern', Figs 1E, 2B,C) and the loss of *pnt* in this region results in the midline cells taking on an appendage-producing cell fate, 2 (Figs 3B, 4C-D). This result suggests that Pnt reduces the effective activity of the EGF receptor pathway at the dorsal midline (Fig. 6). Loss of *pnt* may result in the dorsal region receiving a uniform level of the EGF receptor signal, resulting in a loss of patterning in the dorsal region. Preliminary results indicate that overexpression of *pntP1* reduces expression of another dorsal follicle cell marker, *rhomboid*. As *rhomboid* is required downstream of the EGF receptor for dorsal follicle cell determination (Ruohola et al., 1993; Neuman-Silberberg and Schüpbach, 1994), the results with both the *69D* and *rhomboid* markers argue that, when overproduced, *pnt* affects dorsal follicle cell determination and not necessarily later stages of differentiation. However, it is currently unclear whether *pnt* directly or indirectly affects the expression of these components of the EGF receptor pathway. For example, *pnt* could act indirectly by inducing the expression of a factor that creates a negative feedback loop. It is interesting to note that in *C. elegans*, Lin-1, an ETS-domain transcription factor defines an inhibitory branch for the EGF receptor pathway (Beitel et al., 1995).

Does *pnt* require a cofactor to antagonize EGF receptor signaling?

As *grk* induces expresssion of *pnt* (Figs 1H, 2F,G), high levels of *grk* should induce high levels of *pnt* expression. Our model predicts that when Grk is overexpressed, patterning in the dorsal region should be maintained, as high levels of *pnt* would negate the effects of high levels of the Grk/Top signal. However, the extreme phenotype generated by overexpression of Grk is dorsal midline cells taking on an appendage-producing cell fate, suggesting that high levels of Pnt alone are insufficient to inhibit high levels of Grk activity. As many ETS proteins require a protein partner to function (for review see Wasylyk et al., 1993) an additional factor may be required for Pnt to function. The expression of this cofactor would be predicted to be limiting and not induced by Grk (Fig. 6II). Alternatively, an as yet unidentified pathway may mediate cell-cell signaling that establishes the dorsal midline cell fate and *pnt* may function to integrate this pathway with the Top pathway (Fig. 6I).

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