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fringe and *Notch* specify polar cell fate during *Drosophila* oogenesis

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SUMMARY

***fringe* encodes a glycosyltransferase that modulates the ability of the Notch receptor to be activated by its ligands. We describe studies of *fringe* function during early stages of *Drosophila* oogenesis. Animals mutant for hypomorphic alleles of *fringe* contain follicles with an incorrect number of germline cells, which are separated by abnormally long and disorganized stalks. Analysis of clones of somatic cells mutant for a null allele of *fringe* localizes the requirement for *fringe* in follicle formation to the polar cells, and demonstrates that *fringe* is required for polar cell fate. Clones of cells mutant for *Notch* also lack polar cells and the requirement for *Notch* in follicle formation appears to map to the polar cells. Ectopic expression of *fringe* or of an activated form of Notch can generate an extra polar cell. Our results indicate that *fringe* plays a key role in positioning Notch activation during early oogenesis, and establish a function for the polar cells in separating germline cysts into individual follicles.**

Key words: *fringe*, *Notch*, Polar cells, Oogenesis, Follicle cells, Egg chamber, *Drosophila*

INTRODUCTION

Oogenesis in *Drosophila* involves collaboration between two adjacent cell populations, the germline cells, and the somatic follicle cells, which surround them. Throughout oogenesis, many follicle cells adopt specialized fates and perform crucial functions in egg chamber morphogenesis, follicle cell patterning, or signaling between somatic and germline cells (reviewed in Dobens and Raftery, 2000). The polar cells are pairs of cells at the anterior and posterior tips of each follicle. Although they are among the first somatic cells to adopt a distinct fate, the molecular basis for establishment of polar cell fate has not previously been identified.

Both germline and somatic cells derive from stem cells that are located in the germarium. Germline stem cells give rise to cystoblasts, which then undergo four rounds of division with incomplete cytokinesis to produce a cyst of 16 interconnected cells. One of these cells will become the oocyte, and the remainder develop into nurse cells. Each germline cyst becomes surrounded by approximately 16 somatic follicle cells, forming an individual follicle, or egg chamber, which then buds off from the germarium (Fig. 1). Follicles remain connected to each other by short stalks of somatic cells (stalk cells), and a chain of follicles at different developmental stages, together with the germarium, comprise an ovariole (Fig. 1; reviewed by King, 1970; Spradling, 1993). Several genes that are required for follicle formation have been identified, one of these is *Notch*.

Notch is a transmembrane receptor protein that is involved in a wide range of cell fate decisions during animal development (reviewed by Artavanis-Tsakonas et al., 1999). In *Drosophila*, two ligands are known that regulate its activity, Delta and Serrate. In imaginal discs, the ability of these ligands to activate Notch is modulated by Fringe (FNG) (reviewed by Irvine, 1999; Panin and Irvine, 1998). Expression of FNG inhibits the ability of cells to respond to Serrate, and potentiates the ability of cells to respond to Delta. In wings, eyes and legs, these effects of FNG, together with the distribution and regulation of Notch ligands, result in the establishment of stripes of Notch activation along borders of *fringe* expression (reviewed by Irvine, 1999). FNG is a glycosyltransferase that can directly modify Notch, and this modification of Notch presumably influences its interactions with ligands (Bruckner et al., 2000; Moloney et al., 2000).

Requirements for Notch signaling during oogenesis have been analyzed previously using conditional alleles of *Notch* and *Delta*, and by ectopic expression of Delta or constitutively activated forms of Notch. *Notch* and *Delta* are required for the pinching-off of individual follicles from the germarium, and for the generation of stalks between adjacent follicles (Bender et al., 1993; Xu et al., 1992). Overexpression of Delta or activated Notch generates abnormally long interfollicular stalks, and the cells in these long stalks do not exhibit a normal stalk fate (Keller Larkin et al., 1999; Larkin et al., 1996). Reduction of *Delta* or *Notch* function also results in a hyperplasia and loss of epithelial character among posterior follicle cells (Goode et al., 1996; Ruohola et al., 1991). Notch function is also required for the correct establishment of terminal follicle cell fates at both the anterior and posterior ends of the follicle (Gonzalez-Reyes and St Johnston, 1998; Keller Larkin et al., 1999), and for the establishment of developmental axes of the oocyte (Jordan et al., 2000).

However, these studies have not determined in which follicle cells *Notch* is actually required for any of these steps, and they

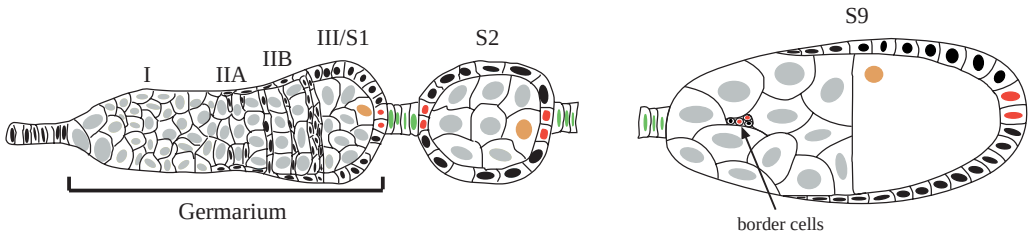

Fig. 1. Schematic of wild-type ovariole. The arrangement and fates of different cells in the germarium and in stage 2 (S2) and stage 9 (S9) follicles are indicated: oocyte nucleus (brown) other germline cell nuclei (gray), main-body follicle cell nuclei (black), polar cell nuclei (red) and stalk cell nuclei (green). The border cells include the anterior polar cells and several main-body follicle cells, and migrate between the nurse cells at stage 9. Roman numerals indicate different regions of the germarium. In all figures, anterior is towards the left.

have all been conducted with conditional alleles that do not completely eliminate *Notch* signaling. In imaginal discs, analysis of the requirements for *fng* have proved invaluable for elucidating the function and regulation of *Notch* signaling (reviewed by Irvine, 1999). In this work, we describe an analysis of the expression and requirements for *fng* during early stages of *Drosophila* oogenesis. Our results show that *fng* is required to specify polar cell fate. Analysis of clones of cells mutant for a null allele of *Notch*, or of clones that express a constitutively activated form of Notch, imply that this influence of *fng* reflects its modulation of *Notch* signaling, and demonstrate that *Notch* too is required for polar cell fate. Our studies further show that the specification of polar cells in the germarium is necessary for follicle formation.

MATERIALS AND METHODS

Drosophila stocks and crosses

fng^{2} and *fng*^{52} are hypomorphic alleles, *fng*^{13} and *fng*^{80} appear to be null alleles (Irvine and Wieschaus, 1994). *fng*^{M69} is a temperature-sensitive allele that behaves as a strong allele at 29°C (V. Papayannopoulos and K. D. I., unpublished), and was originally isolated by J. Jiang and G. Struhl. Flies carrying *fng*^{M69} were raised at 18°C and then switched to 29°C after eclosion, all other crosses were conducted at 25°C. *fng-lacZ*^{RF584} is an enhancer trap line isolated by U. Gaul, and confirmed as an insertion upstream of the *fng* ORF by inverse PCR and DNA sequencing (X. Wang and K. D. I., unpublished). *N*^{55e11} is a null allele (Heitzler and Simpson, 1991). The following fly strains were also used: Oregon-R, *93F/TM3* (Ruohola et al., 1991), *A101/TM3* (Bier et al., 1989), *EQ1Gal4/TM3*, *T155Gal4*, *E4Gal4*, *55BGal4* and *e22cGal4* (Queenan et al., 1997), *E(spl)mβ-lacZ* (Cooper et al., 2000).

fng^{13} or *N*^{55e11} clones were generated by Flipase-mediated mitotic recombination on FRT80-3 or FRT18A chromosomes, respectively, and marked using π-*Myc* or *arm-lacZ* transgenes (Xu and Rubin, 1993). Ectopic expression of *fng* and activated Notch was performed by generating Flip-out Gal4 clones in animals carrying UAS*fng*27 (Kim et al., 1995) or UAS-ΔN34a (Doherty et al., 1996) and *AyGal4 UASGFP* (Ito et al., 1997) transgenes. Flipase expression was induced by heat shocking 2-day-old females at 38°C for 1 hour to generate mutant clones, and at 32.5°C for 30 minutes to generate Flip-out clones.

Follicle staining

β-galactosidase activity detection and Hoechst staining were carried out as described previously (Grammont et al., 1997). F-actin was labeled with Texas Red X-phalloidin (1:20 Molecular Probes). Whole-mount in situ hybridization to follicles and antibody staining was performed as previously described (Sahut-Barnola et al., 1995), using goat anti-β-galactosidase (1:1000, Biogenesis), rabbit anti-Myc (1:100, Santa Cruz), mouse anti-Fasciclin III (1:25, Patel et al., 1987), mouse anti-Bicaudal D (1:40, Suter and Steward, 1991), rat anti-E-Cadherin (1:50, Oda et al., 1993) and rat anti-Serrate (1:1000, Papayannopoulos et al., 1998).

RESULTS

fng mutant ovaries display defects at several steps of oogenesis

To assess the requirements for *fng* during oogenesis, we examined the phenotypes of three hypomorphic alleles: *fng*^{2}, *fng*^{52} and *fng*^{M69}. Null alleles of *fng* die as first instar larvae, but these alleles are viable. Examination of the ovarian morphology of young (<5 days old) *fng* mutant females revealed three consistent defects: compound follicles, long and abnormal interfollicular stalks, and loss of epithelial structure at the posterior of the follicle. The frequency and severity of these defects ranged among the genotypes examined, and the mutations appear to form an allelic series comparable with that derived from studies of wing phenotypes (Table 1 and data not shown; Irvine and Wieschaus, 1994).

Compound follicles

In wild-type ovaries (Fig. 2A,B), each follicle contains 16 germline cells. In stronger *fng* mutants, follicles often contain an abnormal number of germline cells (Table 1, Fig. 2C,D). In most cases (31/36), these abnormal follicles include more than 16 germline cells (Fig. 2C), although we also found occasional examples (5/36) of follicles containing less than 16 germline cells (Fig. 2D). Follicles with multiples of 16 germline cells contain extra oocytes (Fig. 2C). Follicles that contain less than 16 germline cells are always adjacent to follicles that contain more than 16 germline cells, with the

Table 1. Frequency of defects observed in *fng* mutant ovaries

	% Compound follicles	% Long stalks	% Follicles with abnormal epithelium
fng^{2}/*fng*^{2} (*n*=100)	0	0	9
fng^{2}/*fng*^{80} (*n*=100)	0	26	48
fng^{2}/*fng*^{M69} (*n*=50)	0	11	37
fng^{2}/*fng*^{13} (*n*=50)	0	12	42
fng^{52}/*fng*^{52} (*n*=60)	43	18	82
fng^{52}/*fng*^{M69} (*n*=30)	33	ND	22

Follicles containing more than 16 germline cells were scored as compound. Stalks were scored as long when the number of interfollicular cells exceeded eight. The epithelium was scored as abnormal when the posterior epithelium of stage 5 or older follicles was no longer a monolayer. ND, not determined.

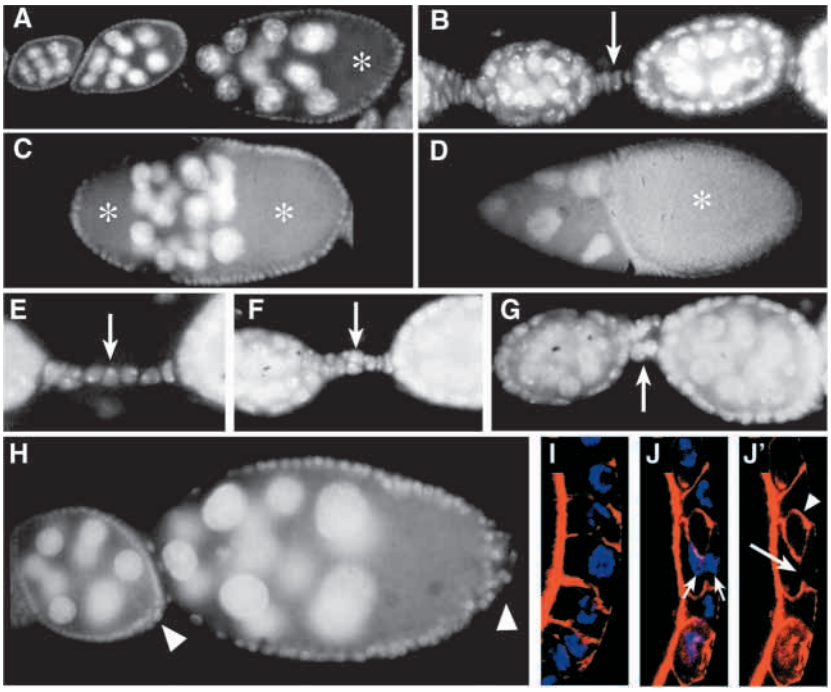

Fig. 2. Oogenesis defects in *fng* hypomorphs. (A-H) Follicles with nuclei stained (Hoechst) and oocytes (asterisks) and interfollicular stalks (arrows) are indicated. (I,J) Follicles stained with Phalloidin (red) and Hoechst (blue). (A,B) wild type. (C) fng^{52}/fng^{M69} follicle with two 16-cell cysts. (D) fng^{52} follicle with six nurse cells. (E) fng^{2}/fng^{80} stalk with nine cells. (F) fng^{52} stalk with a cluster of cells. (G) fng^{2}/fng^{M69} double stalk. (H) fng^{52} ovariole with an abnormal epithelium at the posterior of stage 5 and stage 9 follicles (arrowheads). (I) Posterior of a wild type stage 7 follicle with follicular cells in a monolayer. (J,J′) Posterior of a fng^{52} stage 7 follicle with follicular cells that do not form a monolayered epithelium (large arrow). Nuclei of two adjacent cells (small arrows) and a round cell that does not contact the germline (arrowhead) are indicated.

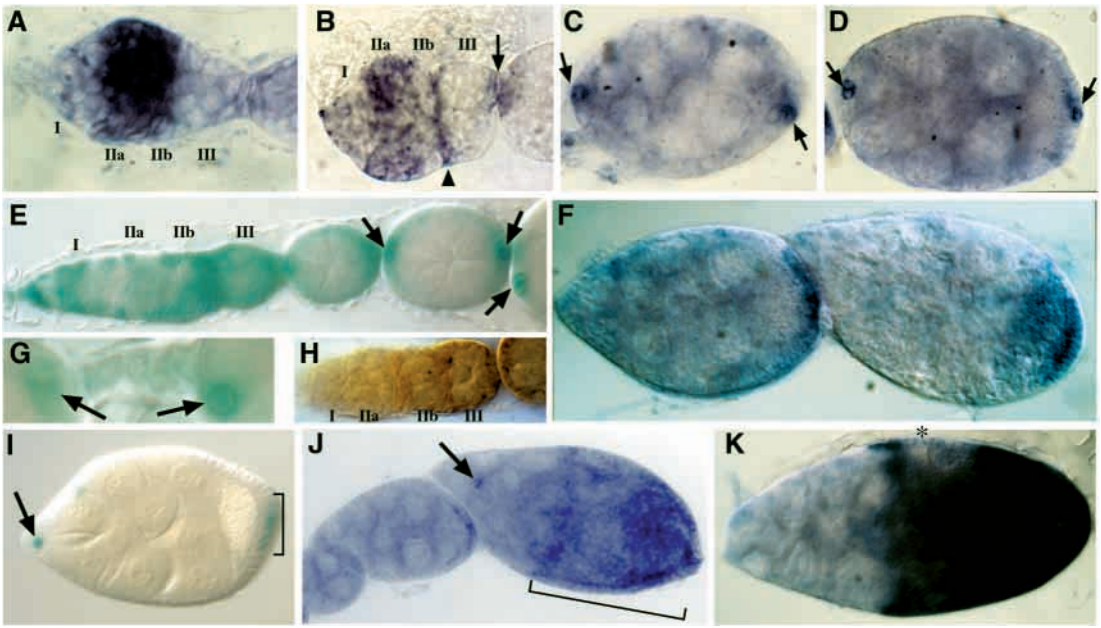

Fig. 3. *fng* expression. (A-D,F,J,K) *fng* expression shown by in situ hybridization. (E,G,I) X-gal staining in *fng-lacZ*^{RF584}. (H) Serrate expression shown by antibody staining. Arrows point to polar cells. (A,B,E) Germaria, with different regions, as defined by morphological criteria (King, 1970; Spradling, 1993) indicated by roman numerals. Arrowhead in B points to the IIb-III border. (C,D) Stage 3 (C) and stage 5 (D) follicles with *fng* expression restricted to polar cells. (E) Germarium, followed by stage 2 and 3 follicles. (F) Stage 7 and 8 follicles with *fng* expression in posterior cells that contact the oocyte. (G) Polar and stalk cells between stage 2 and 3 follicles express *fng*. (H) Serrate is expressed in all somatic cells during follicle formation. (I) Stage 7 follicle with *fng* expression in anterior and posterior polar cells. Expression in other posterior cells is present (bracket). No expression is detectable in germline cells. (J) Stages 7 and 9 follicles. At stage 9, *fng* is expressed in follicle cells that migrate towards the oocyte (bracket). Among the border cells, *fng* is expressed only in polar cells (arrow). (K) Stage 10b follicle; antero-dorsal follicle cells do not express *fng* (asterisk).

total number of germline cells in adjacent follicles being a multiple of 16. Along the length of the entire ovariole, the oocyte:nurse cell ratio is normal (1:15). Phalloidin staining reveals that all of the germline cells are connected by normal ring canals (not shown). Together, these observations indicate that the presence of abnormal numbers of germline cells derives from defects in the enclosure of germline cysts by somatic cells.

Long and disorganized stalks

In wild-type ovaries, a single column of four to six cells separates adjacent follicles (Fig. 2B). In *fng* mutant ovaries, long stalks can form that contain, on average, 10 cells (Fig. 2E). Thirteen of 58 stalks examined exhibit an additional defect, in which the stalk cells were not correctly organized. Instead of forming a single column of cells, they form clusters (Fig. 2F), or a double stalk (Fig. 2G). Thus, *fng* influences both the number and the organization of stalk cells.

Disorganized posterior epithelium

In wild type, follicle cells form an even, single-layered columnar epithelium around the germline cells (Fig. 2A,I). By contrast, in *fng* mutant ovaries the posterior follicular cells can form extra cell layers (Fig. 2H,J). This defect is most obvious in stage 9 and later follicles, but can also be detected earlier (Fig. 2H). Thus, *fng* is required to maintain a monolayer of epithelial cells around the oocyte.

fng is expressed in a complex and dynamic pattern in somatic follicle cells

Although some aspects of *fng* expression during oogenesis have been described recently (Jordan et al., 2000; Zhao et al., 2000), our analysis is more detailed, and has been performed using both in situ hybridization to mRNA and an enhancer trap insertion, *fng-lacZ*^{RF584}, that appears to mimic *fng* expression throughout development (K. D. I., unpublished). *fng* expression is first detected in the germarium. This staining appears in some germaria as a solid block in somatic cells throughout region II (Fig. 3A), while in others it appears as a broad stripe throughout region IIa, absence of staining in region IIb, and a thin stripe of staining between regions IIb and III (Fig. 3B). Oogenesis is a dynamic process in which germline cysts move towards the posterior of the germarium as they mature. The variability in *fng* expression presumably reflects this dynamism, and derives from individual germaria having been frozen by fixation at different points of follicle development. *fng* expression along the IIb-III border corresponds to the somatic cells that invaginate between and separate adjacent cysts. At the posterior end of a stage 1 follicle (region III of the germarium), *fng* expression appears to be restricted to the polar cells and stalk cells (Fig. 3B). Between stage 2 and 3 follicles, *fng* expression is detected in the polar and stalk cells (Fig. 3C,E,G). From stages 3 to 6 of oogenesis, *fng* expression is restricted to the polar cells (Fig. 3C,D). Overall, *fng-lacZ*^{RF58}expression appears similar to that of *fng* mRNA. However, the enhancer trap also exhibits staining in somatic cells at the anterior of the germarium (Fig. 3E) that is more difficult to detect convincingly above background by in situ hybridization. In addition, some enhancer trap staining is detected in main body follicle cells up to stage 3; we attribute this to the broad transcription of *fng* in the germarium, and the perdurance of β-galactosidase in these cells.

After stage 6, *fng* expression expands at the posterior end and by stage 8 is expressed in all of the follicle cells that contact the oocyte (Fig. 3F,J). At the anterior end, *fng* expression remains restricted to the polar cells (Fig. 3I,J). However, at stage 9 and 10a of oogenesis, *fng* expression is also detected in all of the follicle cells that will migrate towards the oocyte (Fig. 3J). Among the border cells (Fig. 1), *fng* is expressed only in the polar cells (Fig. 3I,J). In stage 10b follicles, *fng* expression becomes repressed in dorsal anterior follicle cells above the oocyte nucleus, but is maintained in all other follicle cells around the oocyte (Fig. 3K). As *fng* expression expands, *fng-lacZ*^{RF58}expression again lags *fng* expression detected by in situ hybridization, as during stages 7 and 8, not all cells that contact the oocyte express *lacZ* (Fig. 3I), and staining in most follicle cells is not detected until stage 10a (data not show).

Although occasionally some faint staining was detected in germline cells by in situ hybridization, the weak and variable nature of this staining leads us to infer that it reflects a background of nonspecific hybridization. This inference is also supported by the absence of any detectable expression in germline cells of *fng* enhancer trap lines (Fig. 3E,I).

Prior stage 5, Notch and Delta are expressed in all somatic cells (Bender et al., 1993; Xu et al., 1992). During stages 6 to 8, Notch is expressed most strongly in posterior cells, where *fringe* is, while Delta is repressed in these cells. Using antibodies directed against Serrate (Papayannopoulos et al., 1998), we determined that it is expressed similarly to Delta: in the germarium and stage 2 follicle it is expressed in all somatic cells (Fig. 3H), and later it is repressed in posterior cells (not shown).

Analysis of *fng* mosaic follicles

To elucidate the relationship between the requirements for *fng* in different populations of follicle cells and *fng* mutant phenotypes, and to analyze the consequences of complete loss of *fng*, we constructed genetic mosaics. These were generated by FLP-FRT-mediated mitotic recombination in animals heterozygous for *fng*^{13} (Irvine and Wieschaus, 1994). The resulting *fng* mutant clones were marked by the absence of expression of a Myc-tagged gene (π-*Myc*) or *lacZ*. Follicles exit the ovariole after they complete their development, hence clones that are induced in developing follicles are lost in about 7 days (transient clones) (Margolis and Spradling, 1995). However, recombination in stem cells generates a continuous supply of mutant tissue (permanent clones). To ensure that all mutant cells examined derived from clones that had been lacking in *fng* function throughout oogenesis, we restricted our analysis to permanent clones by waiting 10 days after the induction of recombination.

fng is not required in the germline

To rule out the possibility that *fng* functions in the germline, we identified mosaic follicles in which the somatic cells carried a wild-type copy of *fng*, while the germline cells were homozygous mutant. These follicles develop normally (Fig. 4A). By contrast, mosaics in which the germline cells carry a wild-type copy of *fng* but some somatic cells are homozygous mutant for *fng* exhibit defects (see below). Thus, *fng* is required only in the soma for oogenesis.

fng is required in the soma for follicle formation

Since each germarium contains two somatic stem cells, permanent clones result in ovarioles in which approximately half of the cells are homozygous and half are heterozygous. In wild-type controls, these clones are distributed such that individual follicles can be composed of a mixture π-*Myc*$^{+}$ and π-*Myc*$^{-}$ cells (mosaic follicles), or they can be composed entirely of π-*Myc*$^{+}$ cells or π-*Myc*$^{-}$ cells (Fig. 4B). Indeed, up

to three consecutive π-*Myc*$^+$ or π-*Myc*$^-$ follicles in a row can be observed (not shown). In contrast, among several hundred ovarioles analyzed, follicles composed entirely of *fng* mutant somatic clones were never observed. Thus, *fng* is absolutely required in the soma for follicle formation. Follicles that are mosaic for *fng* yet appear morphologically normal can sometimes be recovered. However, in all cases (>50 follicles scored) the *fng*- cells are excluded from the termini of the follicle. Conversely, follicles that include *fng*$^-$ cells at their termini, or in regions that we infer would normally have contributed to terminal structures, always exhibit defects in follicle formation or the development of polar and stalk cells. We have focused on requirements for *fng* during early oogenesis, and analysis of the relationship between the locations of wild-type and mutant cells and the defects observed define distinct requirements for *fng* in different subpopulations of cells.

Among mosaic follicles with defects in follicle formation, 75% (40/53) contained multiple 16-cell cysts (Fig. 4C). In all of these compound follicles (40/40), *fng*$^+$ cells are located at both extremities of the follicle (Fig. 4C,D), and *fng*$^-$ cells contribute only to non-terminal regions. These observations imply that *fng*$^-$ cells that would normally have contributed to terminal regions are impaired in their ability to migrate in between and separate germline cysts. In the remaining mosaic follicles with defects, germline cells are separated by follicle cells, but this separation is abnormal, leading to the formation of stalkless follicles. In 10% of cases (5/53), 16 cell germline cysts were separated into follicles by a bilayered epithelium of somatic cells (Fig. 4E). However, the epithelial cell layers were fused, and no stalk formed. In every case, the fused epithelial bilayer is composed entirely of *fng*$^-$ cells in the older follicle and a mixture of *fng*$^+$ and *fng*$^-$ cells in the younger follicle. In 15% of cases (8/53), cysts were separated by a monolayered epithelium that is composed entirely of *fng*$^-$ cells (Fig. 4F). Altogether, these observations indicate that *fng* is required for the normal separation of germline cysts into follicles.

fng is required autonomously for polar cell fate

In region IIb-III of the germarium, *fng* expression is maintained in the cells that separate adjacent follicles. The polar and stalk cells presumably belong to this population, as they end up between follicles in the vitellarium (Fig. 1). Polar and stalk cells derive from a shared population of precursor cells, and together they form a lineage distinct from that of other follicle cells (Tworoger et al., 1999). This allows for the formation of mosaic follicles in which only polar and stalk cell precursors are mutant for *fng*, and all other follicle cells are wild-type (or vice versa). We found 11 mosaic follicles in which we were able to infer that only polar-stalk precursors were mutant for *fng*, whereas all other follicle cells were wild type. These follicles were separated from adjacent follicles by at least some *fng*$^-$ stalk cells, although adjacent egg chambers appeared to have *fng*$^+$ polar cells (Fig. 5A). These follicles exhibit a consistent defect in which the epithelium is incomplete at the termini next to the *fng*$^-$ stalk cells, leaving some germline cells uncovered by follicle cells (Fig. 5A). Since this gap in the follicle epithelium occurs where the polar cells are normally found, it suggests that polar-stalk precursor cells mutant for *fng* are unable to differentiate into polar cells.

To test this, we used expression of Fas III as a marker of polar cell fate. Although a *neuralized-lacZ* enhancer trap line (*A101*), appears to be a more specific marker of polar cell fate during oogenesis (Ruohola et al., 1991), we were unable to construct the genotypes that would allow us to examine it in *fng* mosaics. Nonetheless, Fas III is expressed specifically in

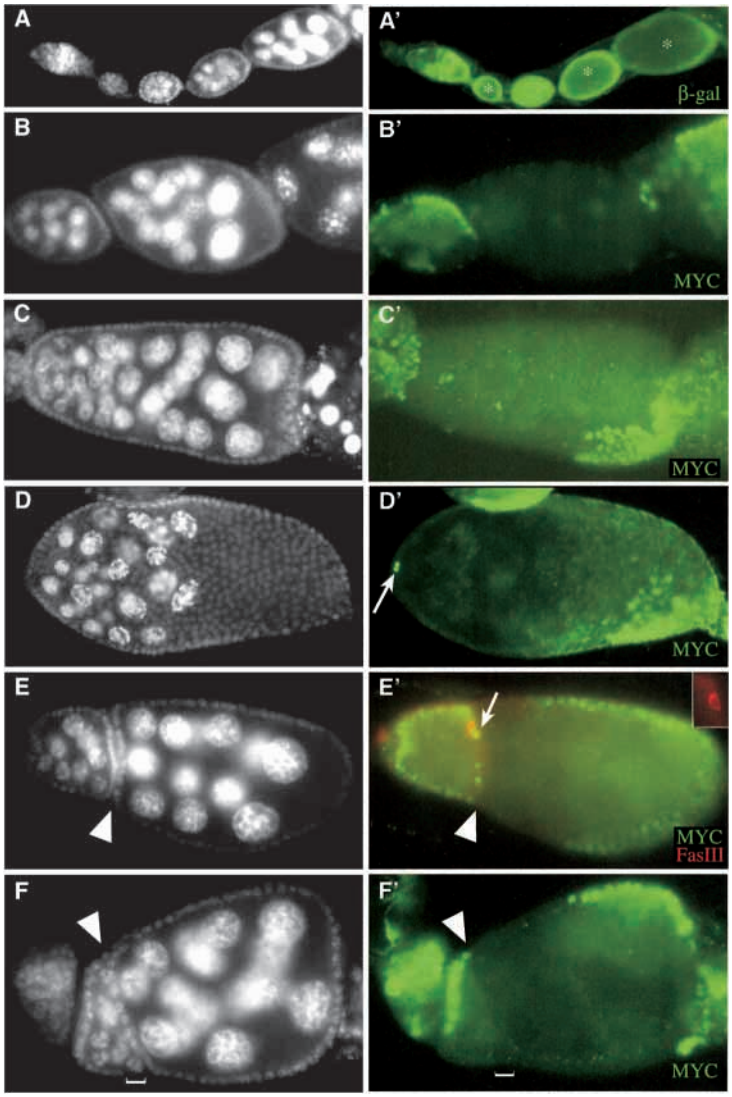

Fig. 4. Compound and stalkless follicles in *fng*^{13} clones. *fng*^{13} (A,C-F) and wild-type control (B) clones marked with *arm-lacZ* (A) or π-*Myc* (B-F). Left panels show nuclei (white), while right panels show clone markers (green). In the right-hand column, figures show separate images of the same follicle. Arrows indicate polar cells, arrowheads point to the absence of interfollicular stalks. In E', Fas III staining is also shown (red). (A) *fng*^{13} clones in the germline, asterisks mark mutant cysts. (B) In wild type, a follicle with an entirely π-*Myc*$^-$ epithelium can form. (C-F) *fng*^{13} clones in the soma. (C,D) Compound follicles with *fng*$^+$ cells at both extremities. In D, only the polar cells are *fng*$^+$ at the anterior. (E) Stalkless follicles separated by a fused bilayered epithelium. Two posterior polar cells (inset) are present in the epithelium of the younger follicle. (F) Stalkless follicles separated by a monolayer epithelium. The epithelium of the younger follicle is discontinuous (bracket).

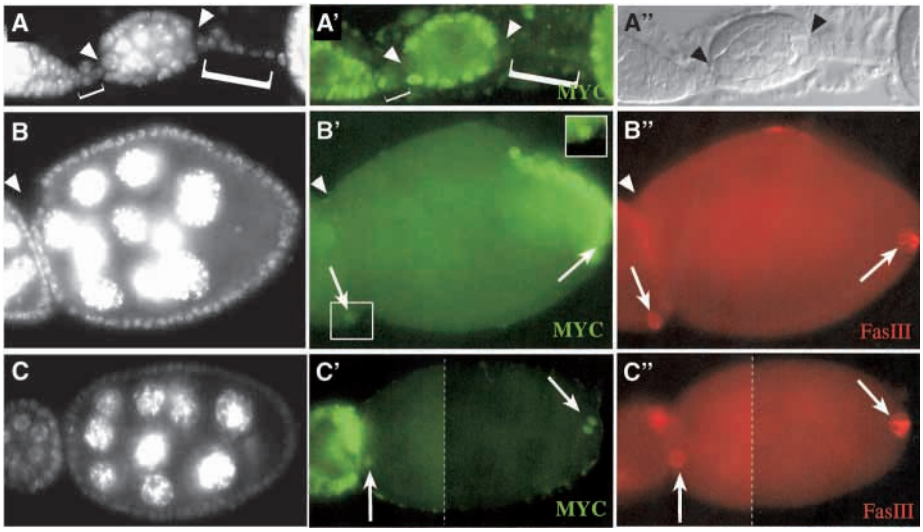

Fig. 5. *fng* is required to specify polar cells. *fng*^{13} clones marked with π-Myc (green) and stained for nuclei (white) and Fas III (red). Arrows indicate polar cells; arrowheads point to the absence of polar cells. (A) Stage 2 follicle with *fng*$^{+}$ main-body follicular cells and *fng*$^{-}$ stalk cells, A" is a Nomarski image. Brackets indicate abnormal stalks. (B) Stage 6 follicle with *fng*$^{-}$ cells at the anterior and no anterior Fas III-positive cells. The more anterior follicle (partially visible) has only two *fng*$^{+}$ cells at its posterior (staining intensity enhanced in inset); they are polar cells and the follicle appears normal. (C) Stage 7 follicle with *fng*$^{-}$ main-body follicle cells and *fng*$^{+}$ polar cells. The image is a composite from two different focal planes of the same follicle, fused at the broken line.

the polar cells from stages 3 to stage 10A of oogenesis in wild-type egg chambers (Ruohola et al., 1991), and even in *Notch* mutants appears to be a reliable marker of polar cell fate from stages 3 to 7 (see below). Fas III staining was absent from *fng*$^{-}$ follicle cells, and importantly was absent from mosaic follicles with gaps in the anterior or posterior follicular epithelium (Fig. 5B). This observation implies that *fng* is required for normal polar cell fate.

We also identified six mosaic follicles in which all of the main-body follicle cells were *fng*$^{-}$, and only the polar and stalk cells at each extremity were *fng*$^{+}$ (Fig. 5C). Strikingly, all of these follicles appear normal and include Fas III-expressing polar cells (Fig. 5C). Thus, expression of *fng* exclusively in the polar-stalk lineage is sufficient for follicle formation. Moreover, we found 15 examples in which *fng*$^{+}$ polar cells formed at both ends of a stalk, but most or all the stalk cells were *fng*$^{-}$ (Fig. 6A). Thus, *fng* is not required within the stalk for polar cell formation either. The strict correlation between Fas III staining and the presence of two terminal *fng*$^{+}$ cells indicates that *fng* is required cell-autonomously within the polar cells for their differentiation. This conclusion is also supported by the observation that only two *fng*$^{+}$ cells are required to begin or end a compound follicle (Fig. 4D).

fng functions non-autonomously to define the number and organization of stalk cells

fng is also transiently expressed in stalk cells, and *fng* hypomorphs can have abnormally long and disorganized stalks. When all of the main-body follicle cells are *fng*$^{-}$, but all of the polar and stalk cells are *fng*$^{+}$, the stalk develops normally (6/6). In contrast, mosaics in which all of the main-body follicle cells are *fng*$^{+}$ but some of the stalk and polar cells are *fng*$^{-}$ usually have elongated and/or disorganized stalks (Fig. 5A). Thus, the requirement for *fng* for stalk development maps to the polar-stalk lineage.

To determine where *fng* is required, we compared the structure of stalks in the presence or absence of polar cells. Stalks in which the polar cells at both ends appeared to be *fng*$^{+}$, but most or all the stalk cells were *fng*$^{-}$, were normal or displayed weak defects. Among 15 abnormal examples (Fig. 6A), the length ranged from eight to ten cells, except for one stalk of 17 cells. Among 19 mosaics in which polar cells appeared to be absent from one end of the stalk, the length varied from nine to 22 cells. Moreover, organization of the stalk was more severely disrupted, as the cells can form clusters and their nuclei are not properly oriented (Figs 5A, 6B). These morphological defects are observed in both *fng*$^{+}$ and *fng*$^{-}$ cells, indicating that the influence of *fng* on stalk development is not strictly autonomous. We also examined the fate of these cells by looking at an enhancer trap line, *93F*, that directs expression specifically in stalk cells from stage 2 through the end of oogenesis (Ruohola et al., 1991). Cells within mosaic stalks can express *93F* (Fig. 6B) Analysis of *93F* expression also identifies 'pseudo-stalk' cells, which express 93F but do not form a morphologically visible stalk, within the epithelium of stalkless follicles (Fig. 6C).

Overexpression of *fng* can generate an extra polar cell

To determine whether expression of *fng* could be sufficient to specify polar cells, we used the Flip-out Gal4 technique (Ito et al., 1997) to generate clones of cells ectopically expressing *fng*. These clones were marked by co-expression of GFP, and polar cell fate was assessed by examining the expression of Fas III or *A101*; both gave similar results. These markers can be expressed in more than two cells at the end of a follicle prior to stage four, but in stage four and older follicles are only expressed in the two polar cells. In over 100 follicles examined, control (GFP-expressing) FLP-out clones we never associated with extra polar cells (not shown). Strikingly, 12% (19/157) of stage 4 and later follicles with Flip-out *fng*$^{+}$ clones contained three polar cells (Fig. 7A) and 0.6% (1/157) contained four. These polar cells are always adjacent to each other, and are at the termini of the follicle. This suggests that the ability to respond to the presence of *fng* overexpression is restricted to the polar-stalk cell lineage. Consistent with this, in the remaining 138 follicles, main-body follicle cells over-expressed *fng*, but they did not adopt a polar cell fate regardless of whether *fng* overexpression was (Fig. 7B) or was not (Fig. 7C) present in polar cells. These results also indicate that overexpression of *fng* in polar cells does not exert a non-autonomous induction of polar fate in main-body follicle cells.

Moreover, the ability of *fng* to induce polar cells is restricted even within the polar-stalk lineage; as apart from one exception, only a single additional cell was transformed.

Intriguingly, extra polar cells only form when the polar-stalk lineage is mosaic for ectopic *fng* expression. If all of the polar and stalk cells overexpress *fng*, then only the normal complement of two polar cells form at each end of the stalk (>100 follicles scored). Moreover, in all cases where an extra polar cell forms, one of the polar cells does not overexpress *fng*, and the remaining two or three do.

Surprisingly, aside from the induction of an extra polar cell, follicles and stalks in which *fng* is mis-expressed are usually completely normal. We have also ectopically expressed *fng* under the control of a variety of different Gal4 drivers that are expressed in specific patterns (see Materials and Methods) without observing any consistent defects in follicle development. Thus, despite the apparently complex regulation of *fng*, it appears that the spatial and temporal patterning of *fng* expression is not essential for normal oogenesis.

Notch is required to specify polar cell fate

Earlier studies of *Notch* function during oogenesis have ascribed to it an influence on polar cell fate that is exactly opposite to what we have observed for *fng*. They have suggested that Notch functions to limit the number of cells that can adopt the polar cell fate, analogous to its role in limiting the number of proneural cells during neurogenesis (Ruohola et al., 1991). This inference was based on the analysis of ovaries mutant for a hypomorphic allele of *Notch*, N^{ts1}. To define the requirements for *Notch* more clearly, we generated somatic clones for a null allele of *Notch*, N^{55e11}, and analyzed their influence on the expression of three different polar cell markers: Fas III, *A101*, and E Cadherin (Niewiadomska et al., 1999; Ruohola et al., 1991). We were unable to recover follicles containing *Notch* mutant cells among permanent clones, presumably because *Notch* has essential functions in the germarium. Consequently, we induced transient clones, and analyzed the follicles two days later.

In stage 8 or older follicles, all $Notch^-$ cells, regardless of their location, autonomously express Fas III (Fig. 8A). Based on the normal rate of development (King, 1970; Spradling, 1993), the progenitors of these cells were in stage 1 or older follicles at the time of clone induction. As polar cells stop dividing around region IIb of the germarium (Tworoger et al., 1999), we infer that the progenitors were main-body follicle cells and not polar cells. The expression of Fas III in $Notch^-$ cells does not correspond to a transformation to polar cell fate, as Fas III expression is not detected until stage 8, the cells continue to proliferate (Fig. 8A), and they do not express *A101* (Fig. 8B).

In younger follicles (stages 4-5), *Notch* mutant clones at either end of the follicle result in loss of polar cells (Fig. 8C and data not shown). The progenitors of these cells were in regions IIb-III of the germarium at the time of clone induction. Thus, *Notch* is required to define polar cell fate during follicle formation. We were unable to identify any mosaic follicles in which only polar cells or only main-body follicle cells were $Notch^-$, presumably because these types of mosaics are much rarer when transient clones are generated. However, we found two mosaics with very few $Notch^+$ cells at each extremity. In both cases, the polar cells were $Notch^+$ and the follicles looked normal (Fig. 8D). This suggests that, as for *fringe*, *Notch* activity is required autonomously in the polar cells, and that its function in these cells is sufficient for follicle formation. Consistent with the hypothesis that Notch is activated specifically within the polar cells, a transcriptional target of Notch signaling in many different tissues, *E(spl)* mβ-*lacZ* (Cooper et al., 2000), is expressed specifically within the polar cells from stages 2 to 5 of oogenesis (Fig. 7D).

To determine whether activation of Notch could be sufficient

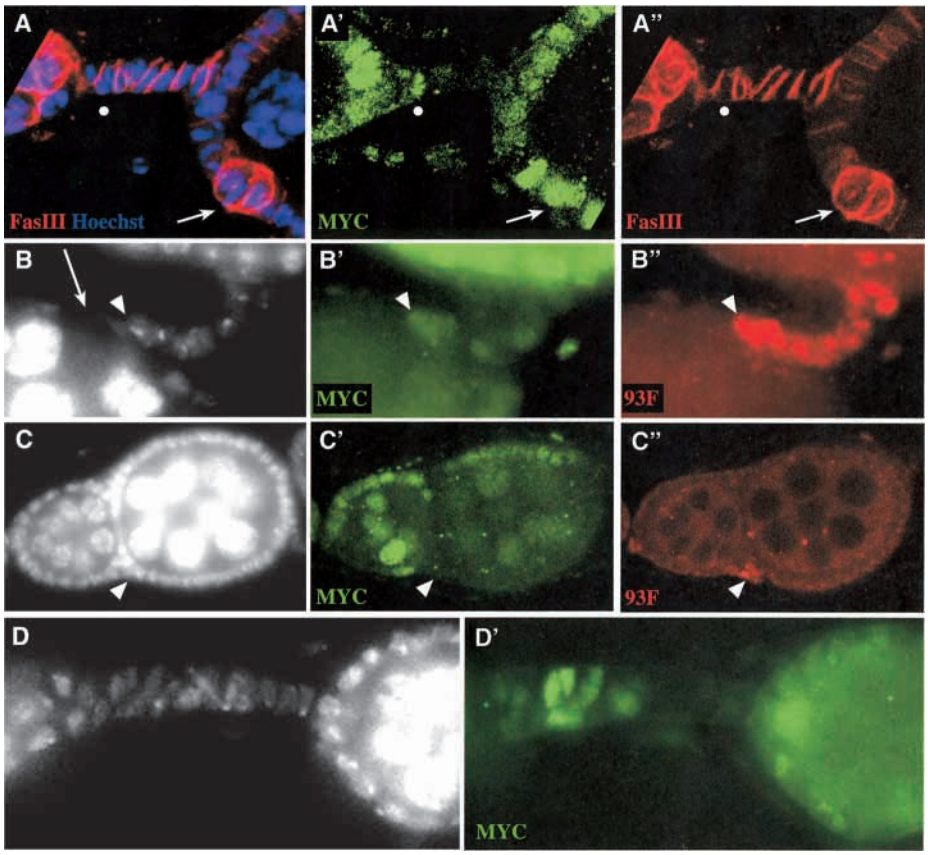

Fig. 6. Requirements for *fng* and *Notch* in stalk cells. (A-C) fng^{13} or (D) N^{55e11} clones marked with π-Myc (green) and stained for nuclei (white or blue) and P3F or FasIII (B,C, red). (A) Stalk with eight fng^- cells and 1 fng^+ cell (white dot). Although FasIII staining is never detected in fng^- cells in the follicular epithelium, it persists in fng^- cells in the stalk. Arrow points to polar cells. (B) Stalk with 11 cells, four are fng^+. All express 93F (arrowhead). Arrow indicates a gap into the follicle epithelium, presumably due to the absence of polar cells. The adjacent stalk cell is fng^+ (arrowhead). (C) Stalkless follicles with four 93F-expressing cells (arrowhead). (D) Stalk with >15 cells that is mosaic for *Notch*.

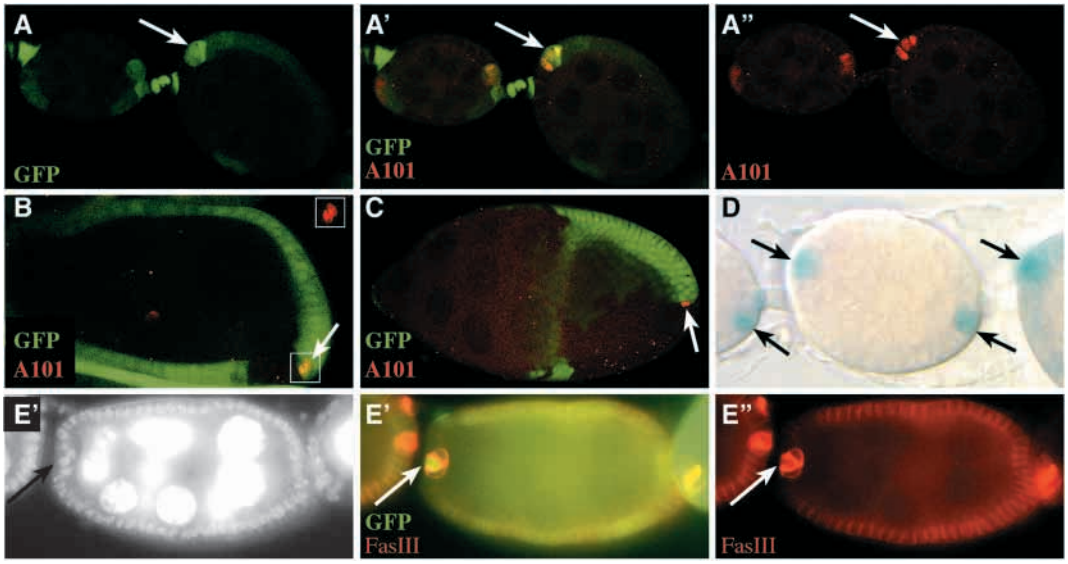

Fig. 7. Ectopic activation of Notch can induce extra polar cells. Arrows indicate polar cells. (A-C) Flip-out clones expressing *fng* marked with GFP (green) and stained for expression of *A101* (β-galactosidase, red). (A) Stage 4 follicle with three polar cells. (B) Stage 8 follicle with ectopic *fng* expression including the posterior polar cells, only two polar cells form. Inset shows *A101* expression for the boxed region. (C) Stage 10A follicle with ectopic *fng* expression in follicle cells adjacent to the posterior polar cells, only two polar cells form. (D) X-gal stain of stage 3 wild-type follicle with *E(spl)mβ-lacZ* expressed specifically in the polar cells. (E) Stage 5 follicle with Flip-out clones expressing activated Notch, marked with GFP and stained for Fas III (red). Three polar cells form (arrow). Lateral follicular cells also express activated-Notch, but are not transformed into polar cells.

to induce polar cell fate, we induced Flip-out clones that expressed a constituitively activated form of Notch. Eight out of 29 (27%) of follicles expressing activated-Notch had one or two extra-polar cells (Fig. 7E). As for *fng*, these cells are found at the termini of the follicle, adjacent to the normal polar cells, and are observed only when polar cells are mosaic for Notch activation. Main-body follicle cells that overexpress activated Notch do not adopt a polar cell fate (Fig. 7E). Thus, ectopic activation of Notch can lead to extra polar cells, but only within a small group of competent cells.

Earlier studies have suggested that stalkless follicles result from reduction of *Notch* function, whereas long, abnormal stalks result from ectopic activation of *Notch* (Bender et al., 1993; Larkin et al., 1996). However, we have observed that long, abnormal stalks can form in *fng* mutants. As our analysis implies that this *fng* phenotype is influenced by polar cell specification, and *Notch* is required to specify polar cells, we analyzed stalk formation in *Notch* mutant clones. Indeed, long (on average 15 cells), morphologically abnormal stalks occur when one of the polar-stalk precursors is mutant for *Notch* (Fig. 6D).

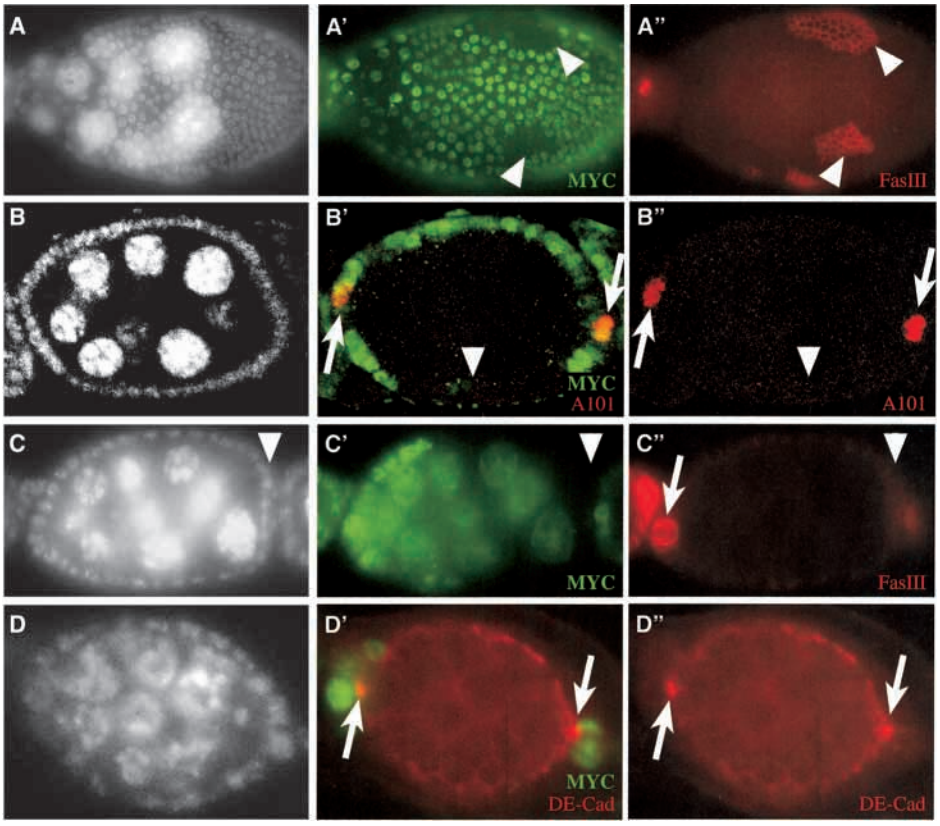

Fig. 8. *Notch* is required to specify polar cells. (A-D) N^{55e11} clones marked with π-Myc (green), and stained for nuclei (white) and Fas III (A,C), *A101* (B) or E-Cadherin (D) (red). In all panels, arrows indicate polar cells and arrowheads point to $Notch^{-}$ clones. (A) Stage 8 follicle. $Notch^{-}$ cells express Fas III. (B) Stage 5 follicle. No *A101* expression is detected in $Notch^{-}$ cells within the follicular epithelium. (C) Stage 5 follicle. No Fas III expression is detected in the $Notch^{-}$ cells within the follicular epithelium. The arrowhead points to the absence of Fas III-expressing polar cells. (D) Stage 3 follicle with only ten $Notch^{+}$ cells at the anterior and three $Notch^{+}$ cells at the posterior. The follicle appears normal and includes polar cells.

DISCUSSION

fringe is expressed in a complex and dynamic pattern throughout oogenesis, and we have investigated its functions by analyzing hypomorphic alleles, and by creating clones mutant for a null allele. Although a suggestion that *fng* functions during early oogenesis has been reported recently in a study that focussed on expression of an antisense construct (Zhao et al., 2000), our studies provide the first clear determination of the requirements for *fng* during early oogenesis. Our results further provide new insights into the earliest steps in follicle cell patterning, the requirements for different follicle cell types in separating cysts into follicles, and the role and regulation of the *Notch* signaling pathway during early oogenesis.

Specification of polar cells

Cell-lineage studies have revealed that somatic cells in the germarium become subdivided into two groups: the precursors of the polar and stalk cells, and the precursors of the main-body follicle cells (Tworoger et al., 1999). The molecular basis for this subdivision is unknown, and it has not been possible to distinguish the two groups until after follicles exit the germarium. However, our studies reveal that *fng* is expressed in a group of cells along the border between regions IIb and III of the germarium. As this corresponds to a group of cells that separate cysts, and *fng* is expressed exclusively in the polar and stalk cells from the earliest time at which they are morphologically recognizable (between stage 1 and 2 follicles), we suggest that this stripe of *fng* expression in the germarium includes the progeny of polar-stalk precursors. Although this would make *fng* the earliest known gene whose expression is largely restricted to polar-stalk cells, the observation that *fng*$^{-}$ cells can be recovered in stalks at later stages of development indicates that *fng* is not actually required to specify this lineage.

However, *fng* is required autonomously to specify polar cell fate. The observations that *fng* expression becomes restricted to the polar cells, and that overexpression of *fng* can generate an extra polar cell, further imply that *fng* is a key player in polar cell specification. Nonetheless, other factors must also contribute to polar cell specification, as *fng* is expressed in a larger group of precursors within the germarium, and as overexpression of *fng* in most cases can generate only a single extra polar cell.

Polar cells play a key role in follicle formation

In genetic mosaics, the requirement for *fng* in follicle formation maps to the polar cells. Our analysis of *fng* mutant clones thus identifies key requirements for the polar cells in enclosing and separating germline cysts. However, the phenotypes observed depend not just on the presence of polar cells in a single follicle, but also on the presence of polar cells in the adjacent follicle.

The main-body follicle cells of each egg chamber are separated from the next by a polar-stalk unit consisting of a pair of polar cells, a stalk, and another pair of polar cells (Fig. 1). As more than one polar-stalk precursor participates in the formation of each unit (Tworoger et al., 1999), a polar-stalk unit could be mosaic for *fng* function in our experiments. When only one pair of polar cells forms, adjacent follicles are still separated. However, one of two morphological defects occurs. In some cases, the egg chamber without polar cells appears open, with a gap in the epithelium. In other cases, stalkless follicles form, separated by a bilayered epithelium. Notably, the observation that normal egg chamber formation actually depends on the specification of two pairs of polar cells per polar-stalk unit implies that polar and stalk cells acquire distinct fates prior to follicle formation.

More severe phenotypes are observed when all of the cells in a polar-stalk unit are *fng*$^{-}$, and no polar cells can form. In most cases, this results in the formation of a compound follicle. The compound follicle ends whenever polar cells form. Moreover, normal follicles appear to form as long as *fng*$^{+}$ polar cells form, even if all other cells are *fng*$^{-}$. These observations imply that the polar cells have the capacity to recognize distinct cysts, to migrate in between them, and to separate them. Although in some cases adjacent cysts can be separated even without any polar cells, this separation is composed of only a monolayer of epithelial cells. Thus, the occasional ability of the main-body follicular cells to migrate between cysts is not sufficient to generate independent follicles.

Several other genes, including *brainiac*, *egghead*, *toucan*, *daughterless*, *hedgehog* and *cut*, have been identified as playing important roles in the packaging of cysts into separate follicles (Bender et al., 1993; Cummings and Cronmiller, 1994; Forbes et al., 1996; Goode et al., 1996; Goode et al., 1992; Grammont et al., 1997; Jackson and Blochlinger, 1997; Xu et al., 1992). *hedgehog* is required for the normal proliferation of somatic cells in the germarium (Forbes et al., 1996; Zhang and Kalderon, 2000), so it is possible that *hedgehog* mutation leads to the formation of compound follicles because of a decrease in the number of polar-stalk precursors. Three of these genes, *brainiac*, *egghead* and *toucan*, are required in the germline (Goode et al., 1996; Goode et al., 1992; Grammont et al., 1997). Determination of their roles may provide new insights into the interactions between germline and somatic cells required to organize a follicle.

Specification and organization of the stalk

fng is also required for stalk formation, and this requirement maps in genetic mosaics to the polar-stalk lineage. However, the requirement is complex in two respects. First, the influence of *fng* on stalk formation is non-autonomous, and appears to map largely to the polar cells. In the complete absence of polar cells, no stalk forms, while stalks that are composed almost entirely of *fng* mutant cells can form as long as wild-type polar cells exist at each end. Moreover, stalks exhibit more severe defects whenever polar cells at one end are missing. Even in cases where abnormally long stalks have *fng*$^{+}$ polar cells at each end, it is conceivable that the long stalk phenotype relates to a requirement for *fng* in the polar cells. That is, we hypothesize that *fng*$^{-}$ cells that would ordinarily have been fated to be polar cells may instead be incorporated into the stalk, and that polar-stalk precursors may continue to join the stalk until *fng*$^{+}$ polar cells arrive.

Second, in some cases *fng* mutant clones result in stalkless follicles, but in other cases they result in long stalks. This observation, together with our analysis of *Notch* mutant clones, indicates that there is no direct correlation between stalk length and Notch activity. Instead, we suggest that the number, timing

and/or location of polar cells within the polar-stalk lineage at the time of stalk formation is crucial to stalk organization.

fng expression and the regulation and function *Notch* during early oogenesis

The phenotypes observed in *fng* mutant clones are also observed in *Notch* mutant clones. Moreover, as for *fng*, the requirements for *Notch* appear to map to the polar cells. While our results confirm that *Notch* plays important roles in separating follicles and organizing stalks, they overturn previous conclusions about the nature of this requirement. Most notably, it had been suggested that loss of *Notch* function leads to extra polar cells (Ruohola et al., 1991). Although after stage 8 Fas III becomes expressed in *Notch* mutant cells, this occurs regardless of their location and in the absence of other aspects of polar cell fate. Instead, our results imply that *Notch* is required for polar cell specification, and that *Notch* is required only in these cells for the separation of germline cysts into distinct follicles. Furthermore, the specific expression of *E(spl)mβ-lacZ* in the polar cells confirms that Notch is activated within these cells, and the induction of extra polar cells by activated Notch demonstrates that the activation of Notch can be sufficient to specify polar cells within a competent subpopulation of somatic cells.

In the *Drosophila* wing and eye, as well as in cultured mammalian cells, *fng* has been shown to potentiate the activation of Notch by Delta and to inhibit the activation of Notch by Serrate (Fleming et al., 1997; Hicks et al., 2000; Panin et al., 1997; Papayannopoulos et al., 1998). Notch, Serrate and Delta all appear to be expressed ubiquitously in follicle cells throughout the germarium. In many circumstances, expression of Notch ligands can exert an autonomous inhibition of Notch activation (e.g. de Celis and Bray, 1997; Micchelli et al., 1997). It has been hypothesized that *fng* may potentiate Delta signaling by allowing Notch activation within ligand-expressing cells (Irvine and Vogt, 1997; Panin and Irvine, 1998). Similarly, we propose that in the germarium, *fng* expression overrides autonomous inhibition to allow Notch activation by Delta within ligand-expressing cells, thereby positioning Notch activation to a subset of cells (the polar cells) within a broad domain of Notch and ligand expression. This is consistent with the observation that *Notch* and *fng* are both positively required within polar cells for them to adopt their fate, and that *fng* expression soon becomes restricted to these cells.

Although *fng* is expressed in a discrete pattern that correlates with its genetic requirements, ectopic expression of *fng* during oogenesis has surprisingly little effect. This contrasts with the very dramatic effects on Notch activation associated with *fng* mis-expression during imaginal development (reviewed in Irvine, 1999). The limited consequences of *fng* mis-expression are also surprising in light of the observation that *mirror* (*mirr*) mutant ovaries have compound follicles (Jordan et al., 2000). It has been hypothesized that the *mirr* phenotype derives from de-repression of *fng* transcription. *mirr* expression is complementary to *fng*, and ectopic expression of *mirr* represses *fng* expression and generates phenotypes similar to those observed in *fng* mutants (Jordan et al., 2000). We suggest that the simplest resolution to this discrepancy would be the existence of co-factors that are required for *fng* function and are co-regulated by *mirr* during oogenesis. As FNG is involved in the synthesis of an O-linked tetrasaccharide (Moloney et al., 2000), the enzymes that are responsible for catalyzing other steps in its synthesis are candidate FNG co-factors, and it will be interesting to determine whether they too are expressed in discrete patterns.

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