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Fused-dependent Hedgehog signal transduction is required for somatic cell differentiation during *Drosophila* egg chamber formation

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SUMMARY

The *fused* gene encodes a serine/threonine kinase involved in Hedgehog signal transduction during *Drosophila* embryo and larval imaginal disc development. Additionally, *fused* mutant females exhibit reduced fecundity that we report here to be associated with defects in three aspects of egg chamber formation: encapsulation of germline cysts by prefollicular cells in the germarium, interfollicular stalk morphogenesis and oocyte posterior positioning. Using clonal analysis we show that *fused* is required cell autonomously in prefollicular and pre-stalk cells to control their participation in these aspects of egg chamber formation. In contrast to what has been found for Hedgehog and other known components of Hedgehog signal transduction, we show that *fused* does not play a role in the regulation of somatic stem cell proliferation. However, genetic interaction studies, as well as the analysis of the effects of a partial reduction in Hedgehog signaling in the ovary, indicate that *fused* acts in the classical genetic pathway for Hedgehog signal transduction which is necessary for somatic cell differentiation during egg chamber formation. Therefore, we propose a model in which Hedgehog signals at least twice in germarial somatic cells: first, through a *fused*-independent pathway to control somatic stem cell proliferation; and second, through a classical *fused*-dependent pathway to regulate prefollicular cell differentiation.

Key words: Oogenesis, Intercellular signaling, Prefollicular cells, Morphogenesis, Cell-cell adhesion, *Drosophila*

INTRODUCTION

Cellular interactions are crucial for cell fate determination, control of differentiation versus proliferation, and cell migration and adhesion during development of multicellular organisms. In particular, they are essential for coordinating and adjusting the timing between different cell developmental programs. *Drosophila* oogenesis presents an excellent system to study such interactions as proliferation and differentiation of somatic and germline cells have to be regulated in a coordinated fashion (reviewed by King, 1970; Spradling, 1993; Spradling et al., 1997).

In the *Drosophila* ovary, each oocyte develops inside an independent follicle also termed egg chamber. Egg chambers are formed in the anterior part of the ovariole, the germarium and mature progressively towards the posterior part of this autonomous unit of the ovary. The germarium has been divided into three distinct subregions according to morphological criteria. Anterior region 1 contains two to three germline stem cells (GSCs) identified by clonal analysis (Wieschaus and Szabad, 1979) and laser ablation studies (Lin and Spradling, 1993), and marked by the presence of a spherical cytoplasmic structure called the spectrosome (Lin et al., 1994). GSC division is asymmetric and generates both a daughter stem cell and a differentiated daughter cell called a cystoblast. Each cystoblast undergoes four rounds of mitosis with incomplete cytokinesis to produce a syncytium of 16 cystocytes known as a germline cyst. Complete cysts then mature through region 2a and become enveloped individually in region 2b by inwardly migrating somatic cells (or prefollicular cells) deriving from approx. two somatic stem cells (SSCs) lying at the border between regions 2a and 2b (Margolis and Spradling, 1995). The prefollicular cell population diverges soon after to give rise to interfollicular stalk cells (which individuate egg chambers), two pairs of polar cells (which mark anterior and posterior poles of the egg chamber) and follicular cells (which form a polarized epithelium around each egg chamber). Germline sister cells acquire different cell fates too, as in each cyst one single cell is determined to become the oocyte, while the 15 remaining cells will differentiate as nurse cells. Region 3 of the germarium corresponds to a stage 1 egg chamber (Spradling, 1993) that will bud off upon completion of stalk formation.

Egg chamber formation is thus a sequential process that requires coordination between somatic and germline differentiation programs, probably mediated by intercellular signaling between these two cell lineages. Although somatic cell differentiation in the germarium is a key step in this process, small cell size, intermingling between proliferation, migration and early differentiation events, and lack of specific early markers have impeded the precise elucidation of the prefollicular

cell differentiation program. Nonetheless, at least three crucial steps in prefollicular cell maturation are evident. Region 2a/2b prefollicular cells first specifically recognize mature 16-cell cysts and individuate them via morphogenetic events involving projections of cellular processes and probably also migration per se. In the absence of germline cells, prefollicular cells do not undergo any cell shape changes (Spradling et al., 1997). In addition, both secreted proteins (Brainiac, Egghead, Gurken/TGF-α) encoded by genes with germline specific function and transmembrane proteins expressed in prefollicular cells (Torpedo/EGFR) are thought to act as components of germen to soma signaling pathways required for correct encapsulation of mature 16-cell cysts by prefollicular cells (Goode et al., 1996a; Goode et al., 1996b; Goode et al., 1992; Rubsam et al., 1998). Region 2b intercyst cells then gain specific adherence properties: they accumulate DE-Cadherin and attract the oocyte posteriorly, thereby polarizing the egg chamber. This posterior positioning of the oocyte is mediated by homophilic interactions as DE-Cadherin function is required in both germline and somatic cells for this sorting-out process (Godt and Tepass, 1998; Gonzalez and St Johnston, 1998). Last, cell lineage divergence among prefollicular cells takes place in region 2b/3, allowing specification of polar cells, stalk cells and follicular cells. Recently, germen to soma Delta/Notch signaling has been shown to be required for polar cell differentiation (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). Lack of Notch, Fringe or Suppressor of Hairless in somatic cells, or of Delta in germline cells leads to an absence of polar cells. This is coupled with defective egg chamber individuation and abnormal stalk assembly, revealing the key role played by polar cells in early egg chamber formation.

The *fused* (*fu*) gene has been identified as a positive effector of the Hedgehog (Hh) signal transduction pathway in *Drosophila* embryonic and imaginal disc development (Alves et al., 1998; Ingham, 1993; Limbourg-Bouchon et al., 1991; Sanchez-Herrero et al., 1996). Although, previous analysis has revealed that *fu* mutations are associated with reduced female fecundity (Busson et al., 1988) and loosely characterized ovarian tumors (King, 1970), *fu* function in oogenesis has not been clearly defined. The *fu* gene encodes a serine/threonine kinase that can be subdivided into two domains, an N-terminal catalytic domain and a C-terminal putative regulatory region (Therond et al., 1996). However, no substrates for Fu kinase activity have been identified in any system. Several Fu partners in Hh signal transduction have been characterized in the embryo and wing imaginal disc: the transmembrane proteins Patched (Ptc) and Smoothened (Smo); and cytoplasmic proteins belonging to a multiprotein complex, namely Costal2 (Cos2), Suppressor of fused (Sufu), Cubitus interruptus (Ci) and Fu itself (reviewed by Murone et al., 1999). Genetic and molecular studies have led to a model in which binding of Hh to Ptc releases inhibition of Smo activity, which generates modifications in the properties of the regulatory cytoplasmic complex and, finally, results in the activation of the transcription factor Ci and subsequent transcription of target genes. In the ovary, Hh is secreted by anterior terminal filament cells and is required for SSC maintenance and proliferation (Forbes et al., 1996a; Zhang and Kalderon, 2001). Known components of the Hh signaling pathway are present in the ovary in addition to Hh [Ptc (Forbes et al., 1996b), Smo (F. B. and A.-M. P., unpublished), Cos2 (Vied and Horabin, 2001) and Ci (Forbes et al., 1996b)] and their activity is required in SSCs to regulate Hh signal transduction and, therefore, SSC proliferation (Zhang and Kalderon, 2000; Zhang and Kalderon, 2001). To date, no early function in prefollicular cell patterning has been clearly demonstrated for cytoplasmic transducers of Hh signaling.

We have undertaken an analysis of the *fu* mutant ovarian phenotype and report on the characterization of defects associated with ovarioles producing multicyst (two to several) and apposed egg chambers exhibiting advanced nurse cell and oocyte development. Our characterization of the *fu* so-called tumorous egg chamber phenotype will be presented elsewhere (F. B. and A. M. P., unpublished). Our analysis reveals that *fu* is required for at least three aspects of prefollicular cell morphogenesis during egg chamber formation: (1) prefollicular cell migration around germline cysts; (2) prefollicular cell intercalation during interfollicular stalk formation; and (3) posterior oocyte positioning in the egg chamber. Although we show that *fu* is expressed in both germline and somatic cells from the germarium onwards, clonal analysis demonstrates cell autonomous action of *fu* in prefollicular cells for all three aspects of proper egg chamber formation mentioned above. In contrast to other classical components of Hh signal transduction, we show that *fu* does not play a role in SSC proliferation. However, genetic interaction studies and the analysis of the effects of reduced Hh signal transduction in the ovary indicate that *fu* acts in the classical genetic pathway for Hh signal transduction in the ovary and that *fu*-dependent Hh signaling plays a role in prefollicular cell differentiation. This study therefore reveals that soma to soma signaling, in addition to soma-to-soma signaling, is necessary for prefollicular cells to adopt their characteristic dynamic morphogenetic properties.

MATERIALS AND METHODS

Fly strains

Several *fu* alleles were analyzed: fu^{1}, fu^{mH63} and fu^{JB3} mutations affect the N-terminal catalytic domain of the kinase and thus represent class I alleles; fu^{A} and fu^{G3} mutations affect the C-terminal regulatory region of Fu and thus represent class II alleles (Therond et al., 1996). fu^{mH63} is the strongest hypomorphic allele of *fu*. *fu* mutant ovaries contain abnormal chambers representative of all the phenotypic categories described in this report, irrespective of the class of the allele examined. Significant variability in female fecundity exists between *fu* mutant alleles, but there is no correlation between the severity of the ovarian phenotype and the class of *fu* allele (Busson et al., 1988). However, as previously described (King, 1970), we noticed an increase in the proportion of abnormal egg chambers with increasing age of the flies and increasing breeding temperature. Flies were raised at 25°C on standard media.

The *ptc-lacZ* (Alves et al., 1998), *ptc*-Gal4 (Bloomington Stock number 2017), hs-*hh* (Tabata and Kornberg, 1994), $Sufu^{LP}$ (Préat, 1992), $cos2^{WI}$/CyO (Sisson et al., 1997), hh^{ts2}/TM6 and hh^{AC}/TM3 (Ma et al., 1993) strains were used to test for a role of the Hh pathway. UAS-cos2/TM3 (K. Ho and M. Scott, unpublished) and UAS-Ci^{cell} (189.2) (Methot and Basler, 1999) were used for flip-out/Gal4 clonal analysis. Ci^{cell} encodes a truncated form of Ci shown to act as a constitutive transcriptional repressor (Methot and Basler, 1999).

The 93F and A101 enhancer-trap lines (Bier et al., 1989; Ruohola et al., 1991) were used for tissue-specific β-galactosidase staining in interfollicular stalks and polar cells, respectively.

In our experiments, wild-type reference females correspond to *fu* heterozygous females that originate from the same line or cross as *fu* homozygous sisters, except in the case of *fu* in situ hybridization and

immunodetection experiments for which the Oregon R strain was used.

Egg chamber staining procedures

Immunocytochemistry was performed as described (McKearin and Ohlstein, 1995). The following antibodies were used in this study: mouse monoclonal anti-Orb 6H4 (1:30; Developmental Studies Hybridoma Bank [DSHB]), mouse monoclonal anti-Fas III 68BAC11 (1:30; Y. N. Jan, unpublished), rabbit anti-α-Spectrin (1:1000) (Byers et al., 1987), rat monoclonal anti-DE-Cadherin (1:20) (Oda et al., 1994), mouse monoclonal anti-Hts 1B1 (1:5; DSHB), rabbit polyclonal anti-Fu (1:200) (Robbins et al., 1997) and rabbit polyclonal anti-β-galactosidase (1:200; Boehringer). All the fluorescence-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories and used at a 1:200 dilution. Actin was labeled with rhodamine-conjugated phalloidin (Molecular Probes) at 0.1 μg/ml for 20 minutes in PBS. All samples were mounted in cytifluor (Kent).

For DAPI staining, tissues were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 30 minutes and rinsed twice, first in PBS with 0.1% Tween-20, then in PBS alone. Ovaries were placed for one night in PBS: glycerol (1:3), with 1 μg/ml of DAPI. β-galactosidase activity detection was performed as described (Grammont et al., 1997). Whole-mount in situ hybridization was performed as described previously (Doerflinger et al., 1999). Digoxigenin-labeled sense and antisense RNA probes for *fu* were synthesized from the D6 vector (Therond et al., 1999), using the RNA genius kit (Boehringer Mannheim).

Samples were examined either with a Leica DMR microscope or by confocal microscopy using a Leica DMR-BE microscope.

Clonal analysis

For analysis of fu^{G3} and fu^{JB3} alleles, FRT-mediated recombination events were induced in SSCs with an *e22c*-Gal4, UAS-*flp* line (Duffy et al., 1998) and revealed by loss of constitutively expressed *lacZ* from a *tub*-nls*lacZ* reporter construct (Goode and Perrimon, 1997). FRT19A nls-tub*lacZ*/Y; *e22c*-Gal4 UAS-*flp*/+ males were mated to FRT19A fu^{X}/FM6 females to produce FRT19A fu^{X}/ FRT19A *tub*-nls*lacZ*; *e22c*-Gal4 UAS-*flp*/+ females (fu^{X} denotes either fu^{JB3} or fu^{G3}). From the moment of the cross onwards, flies were kept at 25°C and dissected about 8 days after eclosion. For the fu^{mH63} allele, *y w* hsp-*flp* fu^{mH63}; FRT40A P[fu^{+}] *arm-lacZ*/FRT40A P[y^{+}] females were generated as described (Zhang and Kalderon, 2000). Flipase expression was induced by heat-shocking third instar larvae at 38°C for 1 hour. Dissection of 8- to 10-day-old females ensured that observed clones corresponded to stem cell clones.

Flip-out/Gal4 clones were produced by mating hsp-*flp*; UAS-Ci^{cell}/SM6-TM6 or hsp-*flp*; UAS-Cos2/SM6-TM6 males with Act>CD2>Gal4 UAS-GFP females (Neufeld et al., 1998; Pignoni and Zipursky, 1997) and heat-shocking late pupae at 37°C for 1 hour. Flies were thereafter kept at 25°C and dissected about 8 days after eclosion. Clones were marked by GFP staining.

Hh overexpression

To express Hh ectopically, we used a hs-*hh* transgene in which the *hh*-coding sequence has been placed under the control of the hsp70 promoter (Tabata and Kornberg, 1994). One-day-old flies were heat-shocked for 2 hours at 38°C and dissected and treated for X-gal staining 3 days later.

RESULTS

fu mutant ovarioles contain multicyst and apposed egg chambers

Analysis of *fu* mutant ovarioles using DAPI nuclear staining revealed the presence of egg chambers with more or less than 16 germ cells that exhibit a certain degree of nurse cell differentiation (Fig. 1B-F, arrowheads, compared with wild-type ovarioles, Fig. 1A). When an egg chamber contained less than 15 nurse cells, it was possible to find the complementary nurse cells in an adjacent chamber (Fig. 1D, arrowheads) suggesting that individual germline cysts were split in two during egg chamber formation in the germarium. In some of the egg chambers containing more than 16 germline cells, the varying degree of nurse cell polyploidy within a given chamber (Fig. 1E, arrowhead) suggested that cysts of different ages were developing together. In addition, the presence of two oocytes undergoing vitellogenesis in one chamber clearly demonstrated the multicyst nature of these chambers (Fig. 1F, arrows). This was confirmed by double detection of Orb protein, which accumulates specifically in the oocyte (Fig. 1G′, arrowhead), and actin, which is present in ring canals (Fig. 1G″), as some chambers contained two Orb-expressing oocytes each with four ring canals (Fig. 1H′, arrowheads, H″). Therefore, encapsulation of individual germline cysts is deficient in *fu* mutant ovaries. In addition, although multicyst egg chambers were enveloped by a regular follicular epithelium, in some cases the follicular epithelia of two adjacent chambers were apposed with no apparent intervening interfollicular stalk (Fig. 1C, arrow). Finally, these abnormal chambers and wild-type egg chambers were often present in the same ovariole (Fig. 1B). Although some of these ovarioles contained mature oocytes posteriorly, the posterior-most chamber in a significant proportion of these ovarioles contained advanced stage egg chambers with pycnotic nurse cell nuclei indicative of cell death (Fig. 1B, arrow).

fu mutant germaria exhibit impaired prefollicular cell encapsulation of germline cysts

We next looked for possible defects at the level of germline cyst encapsulation by prefollicular cells in the germarium of *fu* mutant ovarioles generating multicyst egg chambers. In region 2b of wild-type germaria, flattening of 16-cell germline cysts, such that they span the width of the germarium and arrange themselves in a linear fashion, is concomitant to separation of these cysts by the long, thin cytoplasmic processes extended by prefollicular cells (Fig. 2A-A″). These cell extensions accumulate several cytoskeletal and membrane proteins including Fasciclin III (Fas III; Fas3 – FlyBase), α-Spectrin and Hu-li tai shao (Hts) (Fig. 2A-A″, arrowheads, and data not shown). At the transition between regions 2b and 3, expression of these proteins is upregulated and concentrated apically and laterally in prefollicular cells that meet between two cysts and finally carry out the process of budding off of the egg chamber (Fig. 2A-A″, arrow).

In some *fu* mutant germaria, cells expressing high levels of Fas III characteristic of migrating prefollicular cells were present, but these cells all remained at the periphery of the germarium seemingly unable to migrate centripetally (Fig. 2B). In these germaria, cell processes normally extended by prefollicular cells towards the center of the germaria were not observed upon staining with antibodies against Fas III, α-Spectrin and Hts (Fig. 2B-B″ and data not shown). Consequently, germline cysts accumulate in region 3, as visualized by anti-α-Spectrin staining of the fusomes (Fig. 2B′,B″) and this severely delayed encapsulation presumably leads to inclusion of several cysts in one egg chamber.

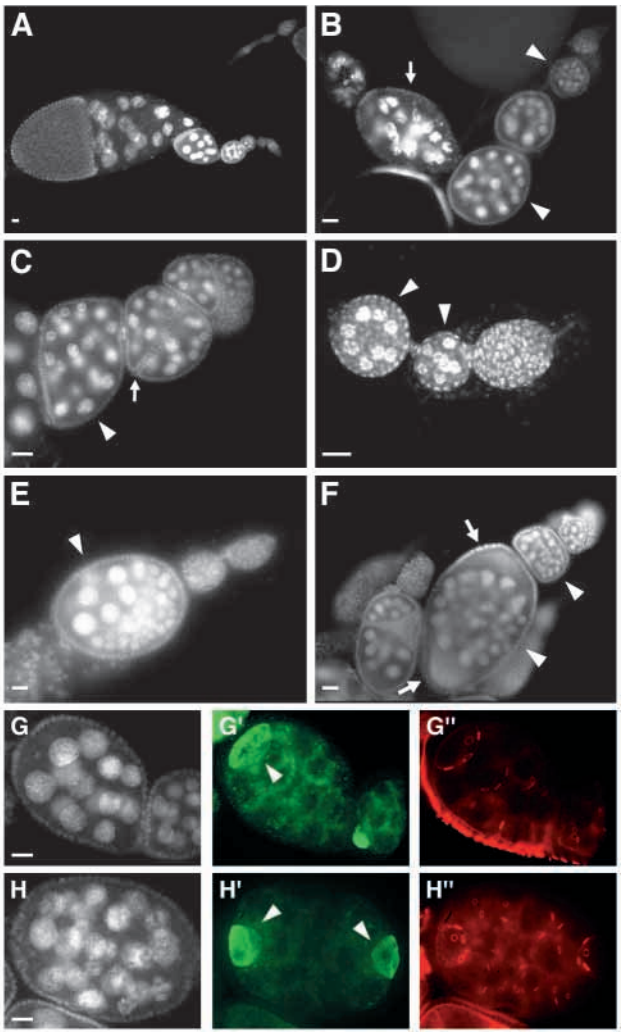

Fig. 1. *fu* mutations generate ovarioles with egg chambers containing abnormal numbers of germ cells. All ovarioles are oriented with anterior towards the top or right. (A-F) DAPI staining of wild-type (A), fu^{JB3} (B-D,F) and fu^{1} (E) ovarioles. Arrowheads indicate compound egg chambers (B,C,E,F) or chambers with less than 16 germline cells (D). (B) Arrow indicates a degenerating egg chamber. (C) Arrow indicates apposed egg chambers. (F) Arrows indicate two vitellogenic oocytes in the same egg chamber. (G-H) Wild-type (G) and fu^{JB3} (H) egg chambers stained with DAPI (G,H), anti-Orb antibodies (G′,H′) and phalloidin (G″,H″). Arrowheads indicate Orb-expressing oocytes. Scale bars: 10 μm.

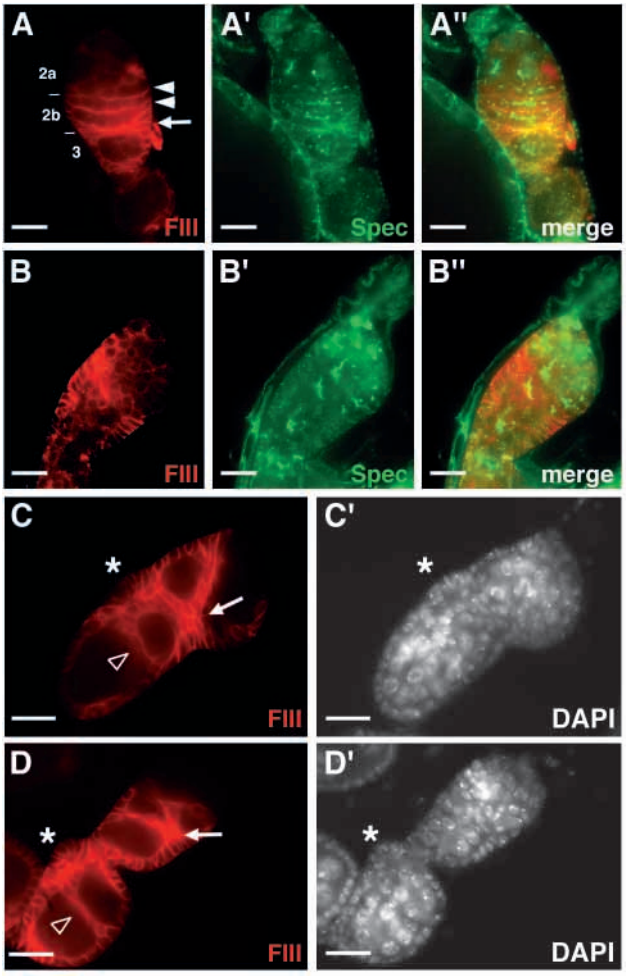

Fig. 2. *fu* is required for correct encapsulation of germline cysts by prefollicular cells in the germarium. All germaria are oriented with the anterior towards the top or top right-hand corner. (A-B″) Double labeling of wild-type (A-A″) and fu^{JB3} (B-B″) germaria with anti-Fas III (A,B) and anti-α-Spectrin (A′,B′) antibodies, both of which stain prefollicular cells and their descendants (anti-α-Spectrin also stains fusomes, the branched cytoplasmic structures that link all the cystocytes of a developing cyst). FIII denotes Fas III. Spec denotes α-Spectrin. Basal membranes are defined by contacts with the peripheral basal lamina. Arrowheads in A indicate prefollicular cells extending cell processes centripetally and the arrow indicates prefollicular cells that have begun the intercalation process (germarial region 2b/3). (C,D) fu^{JB3} germaria stained with anti-Fas III antibodies. (C′,D′) Corresponding DAPI nuclear staining. Arrows in C,D indicate irregular intercalation between prefollicular cells. Arrowheads in C,D indicate somatic cells, within egg chambers, weakly staining for Fas III. Asterisks in C,C′,D,D′ highlight peripheral aggregates of somatic cells. (D′) In the focal plane shown here, ~15 nurse cell nuclei are visible in the budding egg chamber, whereas additional nurse cell nuclei are visible in other focal planes of this chamber (data not shown). Scale bars: 10 μm.

In many *fu* mutant germaria, some prefollicular cell migration between germline cysts was evident, but the dynamics of Fas III expression in these cells, their morphology and subsequent encapsulation were all abnormal. Once again, the long, thin prefollicular cell processes containing Fas III were largely absent (Fig. 2C,D). Prefollicular cells exhibiting strong Fas III staining were observed that had migrated between germline cysts, but these cells were often somewhat cuboidal, showing more lateral than apical Fas III staining, irregular encapsulation of cysts and limited intercalation for stalk formation (Fig. 2C,D, arrows). In addition, cells staining weakly for Fas III were found grouped together at the exterior of germaria or of newly formed chambers (Fig. 2C,D, asterisks), which may represent abortive stalk formation.

Even under these unfavorable circumstances, some egg chamber budding does occur (Fig. 2D). Interestingly, when newly formed multicyst chambers were observed (>16 germ cells, Fig. 2D′), they showed internal compartmentalization by

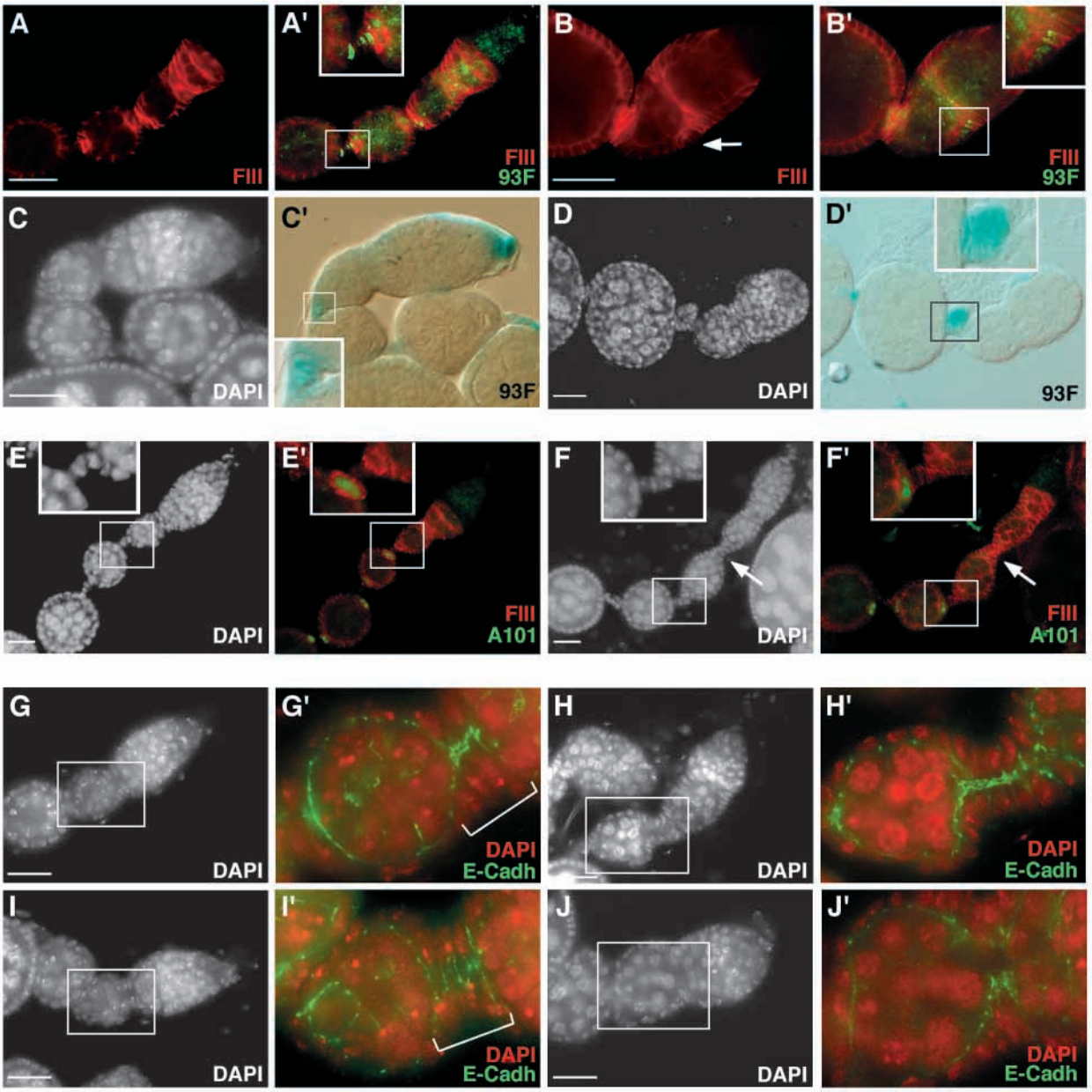

Fig. 3. *fu* mutations affect stalk morphogenesis. All ovarioles are oriented with the germaria towards the right. (A,B) 93-F/+ (A) and fu^{JB3}; 93F/+ (B) ovarioles double-stained with anti-Fas III antibodies (A,A′,B,B′, red) and anti-β-galactosidase antibodies (A′,B′, green). FIII denotes Fas III. Arrow in B and insert in B′ indicate Fas $III^{low}/93F^{+}$ peripheral somatic cells. (C′,D′) Expression of the 93F enhancer-trap line after X-gal detection of β-galactosidase activity in wild-type (C′) and fu^{JB3} (D′) ovarioles. (C,D) Corresponding DAPI nuclear staining. (E-F′) A101/+ (E) and fu^{JB3}; A101/+ (F) ovarioles triple-stained with DAPI (E,F), anti-Fas III antibodies (E′,F′, red) and anti-β-galactosidase antibodies (E′-F′, green). Arrows in F,F′ indicate interfollicular cells arranged in a double row. (G-J) Wild-type (G,I) and fu^{JB3} (H,J) ovarioles stained with DAPI. (G′-J′) Magnification of the boxed area of G-J germaria, double-stained with DAPI (red) and anti DE-Cadherin antibodies (green). Ovarioles were dissected either from 7- to 8-day-old females (A-D) or from younger 3-4 day old females (E-J). Scale bars: 10 μm.

somatic cells weakly staining for Fas III (Fig. 2D, open arrowhead). These cells have not intercalated to form a stalk, nor have they formed a follicular epithelium. Multicyst chambers of this type were present ‘in-the-making’ in region 3 of the germarium (Fig. 2C, open arrowhead, C′), which may be eventually budded off by more anterior prefollicular cells that stain strongly for Fas III (Fig. 2C, arrow). More posteriorly in the ovariole, this partitioning of multicyst egg chambers by somatic cells was not observed, suggesting that these cells may be eventually degraded (data not shown). Therefore, defective prefollicular cell behavior in region 2b of *fu* mutant germaria (Fig. 2B-D) is probably what leads to the inclusion of several cysts in one chamber.

fu mutant ovarioles exhibit abnormal interfollicular stalk formation

In order to look at interfollicular stalk cell specification, we used the 93F enhancer trap line, which expresses the *lacZ* gene

specifically in these cells once stalk morphogenesis is complete (Fig. 3A′, insert, for β-galactosidase immunodetection and C′, insert, for X-Gal staining). Concomitant to 93F upregulation in interfollicular stalks, Fas III expression diminishes significantly (Fig. 3A,A′, insert). In *fu* mutant germaria that exhibit egg chamber budding defects, we found small, peripheral groups of cells expressing both low levels of Fas III compared with neighboring prefollicular cells (Fig. 3B, arrow, see also Fig. 2C,D, asterisks), and the 93F interfollicular stalk cell marker (Fig. 3B′, insert). Therefore, these stalk cells appear to be specified as such, but they are set aside, unable to fulfill their role in budding off of individual egg chambers. In addition, we found that stalk-like structures between egg chambers in *fu* mutant ovarioles express the 93F stalk cell marker, although these stalks exhibit an abnormal morphology. Instead of presenting the wild-type linear arrangement of five to seven oval-shaped cells (Fig. 3C′, insert), *fu* mutant stalks were comprised of aggregates of round cells arranged in a ball shape (Fig. 3D′, insert). Therefore, *fu* mutations do not appear to affect stalk cell specification, at least with respect to the 93F marker, but rather their morphogenetic properties during egg chamber budding.

In order to examine the possible basis of the stalk morphogenesis defect in *fu* mutants, we looked at the expression of cytoskeletal and cell membrane proteins that have been shown to exhibit polarized localization in pre-stalk cells during stalk formation. We performed this analysis on younger *fu* mutant females (3-4 days old instead of 7-8 days), because we found that in doing so it was possible to look at stalk formation defects in the absence of the more severe encapsulation anomalies. In wild-type germaria, only one egg chamber is observed being budded off by pre-stalk cells which express Fas III laterally and completion of egg chamber budding is associated with the formation of a fully mature anterior stalk (Fig. 3A,E,E′, inserts). In *fu* mutant ovarioles from young females, Fas III staining reveals more than one egg chamber in the process of budding at a time (Fig. 3F′). The budding process seems significantly delayed with respect to wild type, as evidenced by the age of the germline cysts in the budding egg chambers (compare Fig. 3E,F). However, Fas III in the pre-stalk cells exhibits normal lateral localization (Fig. 3F′, arrow). Finally, the newly formed stalks are arranged in two rows of cells instead of one (compare Fig. 3E, insert with Fig. 3F, arrow and insert) and Fas III expression perdures abnormally (compare Fig. 3E′,F′, inserts). Interfollicular stalks in the more mature regions of these *fu* mutant ovarioles, however, exhibited downregulation of Fas III and a relatively normal morphology, suggesting that the initial anomalies eventually resolve themselves (data not shown).

We also examined the expression of the cell-cell adhesion protein, DE-Cadherin, which has been shown previously to be polarized apically in prefollicular cells during egg chamber budding (Fig. 3G,G′). By examining expression of this protein in many wild-type germaria, with the intention of visualizing all stages of egg chamber budding, including those that may occur rapidly, we identified a transition in the expression pattern of DE-Cadherin in pre-stalk cells that seems to correspond to the initiation of intercalation between these cells. First, prefollicular cells that have displaced themselves centripetally between germline cysts make apical cell-cell membrane contacts that accumulate DE-Cadherin specifically (Fig. 3G′, bracket). The staining is observed as two apical bands separated by a space suggesting that these cells first make contacts with adjacent cells rather than opposing cells. Although the nuclei of these cells have moved centripetally they are not fully apical as yet. In what appears to be a subsequent step, as pinching off of the stage 1 egg chamber by pre-stalk cells has progressed further (Fig. 3I), DE-Cadherin expression changes dramatically (Fig. 3I′, bracket). It appears as an apical zig-zag expression pattern that probably corresponds to new lateral surface contacts established upon intercalation between these cells. The nuclei of these cells are now also positioned more apically and their appearance by DAPI staining is more diffuse than before, perhaps in preparation for the intercalation process. In contrast to wild-type animals, in *fu* mutants, a high proportion of germaria displayed the pre-intercalation arrangement of pre-stalk cells, these cells expressing strong apical DE-Cadherin (Fig. 3H,H′), while the initiation of intercalation, characterized by a zig-zag DE-Cadherin expression pattern, was largely absent. These results suggest that pre-stalk cells in *fu* mutants are compromised in their ability to initiate the intercalation process, which would lead to delayed egg chamber budding. However, apical polarization of DE-cadherin is not affected in *fu* mutant pre-stalk cells (Fig. 3H′). In fact, even in *fu* mutant germaria in which prefollicular cells have not completed their centripetal migration between germline cysts, DE-Cadherin is nonetheless expressed and localized apically (Fig. 3J,J′). It is possible that DE-Cadherin accumulation is somewhat excessive in *fu* mutant pre-stalk cells compared with wild type, in particular in those cells that are in contact with the germline (compare Fig. 3G′ with 3H′ and data not shown). However, these experiments do not allow us to determine whether this excess apical accumulation is the cause of delayed intercalation or rather the result.

Given that stalk cells and polar cells are closely linked by lineage (Tworoger et al., 1999), we also looked at polar cell specification in *fu* mutants using anti-Fas III antibodies and the A101 enhancer trap line. In wild-type ovaries, these two markers are expressed in polar cell pairs at each pole of the egg chamber, as of the stage 2 egg chamber, which is fully budded from the germarium (Fig. 3E', insert). In relatively young *fu* mutant females, the first egg chamber that fully buds off the germarium also expresses these polar cell markers appropriately (Fig. 3F′, insert); however, this egg chamber is considerably older than the stage 2 egg chamber expressing these markers in wild-type ovarioles. Therefore the delay in stalk formation in *fu* mutants is also accompanied by a delay in polar cell specification. In older *fu* mutant females, which contained multicyst and apposed types of egg chambers, several pairs of cells expressing the Fas III and A101 polar cell markers were observed, with a good correlation between the number of germline cysts in these chambers and the number of pairs of polar cells (data not shown). Finally, polar cell specification occurs in *fu* mutant clones induced in somatic stem cells and their descendents using several *fu* alleles, including the pupal lethal strong hypomorphic allele, fu^{mH63} (see below and Fig. 5A′, insert). Therefore, *fu* is not required for either stalk or polar cell specification.

fu is expressed from the germarium onwards, both in germline cells and in somatic cells

Previous analysis of *fu* transcript distribution in the ovary using

low-sensitivity DNA probes (Therond et al., 1993) showed late germline expression of *fu* (beginning at stage 8), consistent with its maternal requirement for early embryogenesis. Given that we show here that *fu* mutations also affect early oogenesis, in particular egg chamber formation, we used more sensitive assays to determine whether *fu* is expressed earlier than stage 8 and whether it is expressed in germ cells, somatic cells, or both. In situ hybridization experiments using a *fu* antisense RNA probe revealed strong expression of *fu* starting in the mid-germarium, corresponding to both mature 16-cell germline cysts and prefollicular and follicular cells (Fig. 4A). In addition, this analysis indicated expression of *fu* in young egg chambers in both the germline (nurse cells and oocyte) and surrounding follicle cells (Fig. 4A). In situ hybridization experiments using the appropriate *fu* sense RNA probe gave no detectable signal (data not shown). In parallel, immunocytochemical analysis of the ovary was carried out using an anti-Fu polyclonal antibody (Fig. 4B,B′, different confocal sections of the same ovariole). Fu protein distribution completely overlapped that of *fu* transcripts in the ovary, and was detected in the cytoplasm of both germline and somatic cells. In addition, the higher sensitivity of the immunostaining allowed detection of Fu in the anterior portion of the germarium, including somatic terminal filament and cap cells (Fig. 4B′, bracket) and underlying germline stem cells (Fig. 4B′, region 1). Specific recognition of Fu protein by this antibody has been demonstrated previously in both embryonic extracts and embryos in situ (Robbins et al., 1997; Therond et al., 1996; Therond et al., 1999). Although no specific *fu* mutant allele is available that abolishes *fu* expression completely, several *fu* mutant alleles have been shown to accumulate reduced levels of *fu* transcripts and/or protein compared with wild type (Robbins et al., 1997) (P. Thérond, PhD thesis, University of Paris VII, 1991). Consistent with this, immunodetection of Fu protein in ovaries from fu^1 mutant females revealed a strong overall reduction in signal compared with wild type (Fig. 4C,D). Therefore, as is the case in the embryo and imaginal discs (Alves et al., 1998; Therond et al., 1993; Therond et al., 1999), *fu* expression in the ovary is ubiquitous, not being restricted to either cell lineage, somatic or germline.

fu mutant prefollicular cell clones display defects in migration over germline cysts and stalk formation

In order to remove *fu* function specifically in somatic cells of the ovary, *fu* mutant mitotic cell clones were induced in SSCs using the FLP/FRT system (see Materials and Methods). Loss of either *arm-lacZ* or *tub-lacZ* reporter expression was used to mark the mutant clones. The induction of relatively large clones that included the anterior region of the follicular epithelium of an egg chamber (Fig. 5A′,B′) was associated with the production of egg chambers with abnormal numbers of germline cells (Fig. 5A,B). The multicyst nature of these egg chambers (n=19) was evidenced by the presence of two oocytes as visualized with DAPI (Fig. 5B, arrows) or anti-Orb staining (data not shown).

In addition, long, disorganized stalks were observed (Fig. 5C,C′, arrows) from which egg chambers showed an off center attachment. Interestingly, in the great majority of cases observed, these stalks were composed of fu^+ cells, while *fu* mutant cells were found as part of the follicular epithelium of the adjacent chambers (Fig. 5C′). We next examined germarial regions 2b/3 more closely and observed that segregation of fu^+ and *fu* cells already occurs at this point. fu^+ prefollicular cells were observed that had migrated centripetally over a germline cyst (Fig. 5D,D′, asterisks), while adjacent *fu* prefollicular cells remained at the periphery (Fig. 5D,D′, arrows). *fu* cells thus appear compromised in their capacity to migrate over germline cysts, and the asymmetric budding by fu^+ cells is probably what leads to the generation of abnormally shaped and mispositioned fu^+ stalks. The differential capacities of fu^+ and *fu* cells were also observed in rare mosaic stalks where *fu* and fu^+ cells were present together (Fig. 5E,E′). Both *fu* and fu^+ stalk cells take on a flattened shape but do not intercalate, remaining as two independent stacks of cells instead. These results indicate that *fu* function is necessary in prefollicular cells in order for them to acquire adhesive and/or migratory properties, allowing them to encapsulate germline cysts and intercalate.

fu mosaic egg chambers contain mislocalized oocytes

Competition between *fu* and fu^+ cells generated in mosaic ovarioles revealed an additional property of *fu* prefollicular cells, as mosaic ovarioles were shown to contain chambers with perturbed anteroposterior asymmetry. Indeed, although the oocyte is invariantly found at the posterior pole of wild-type egg chambers, mosaic epithelia were often associated with a mislocalized oocyte, as revealed by anti-Orb immunostaining (compare Fig. 6A-A″ with 6B,B′ arrows). Strikingly, we noticed that the position of the misplaced oocyte is not random, since mislocalized oocytes orient themselves with high fidelity towards fu^+ cells (82.5%, n=40), independently of where these cells are located. In particular, laterally (Fig. 6A-A″), as well as anteriorly localized oocytes (data not shown) could be found. Therefore, this observation indicates a failure of posterior *fu* follicle cells either to drive early oocyte sorting out or to maintain the posterior position of the oocyte. As posterior localization of the oocyte has been shown to occur at the transition from germarial region 2b to region 3 in wild-type ovaries (Godt and Tepass, 1998; Gonzalez and St Johnston, 1998), we next examined germaria of mosaic ovarioles and found that oocytes were already mispositioned as of this stage (Fig. 6C,C′, arrows). In addition, similar defects in early posterior positioning of the oocyte are observed in *fu* mutant females (data not shown), though less frequently than in mosaic ovarioles where *fu* and fu^+ cells are in competition. These results therefore suggest that removing *fu* function in prefollicular cells prevents oocytes from correctly reaching the posterior of germline cysts.

Somatic stem cell proliferation is not affected in *fu* mutant ovarioles

As it has previously been shown that Hh signaling is required in the ovary for SSC maintenance and proliferation, we tested whether mutations in the *fu* gene affect SSC self-renewing divisions.

Somatic stem cell clones were generated using three different *fu* alleles including the strong hypomorphic fu^{mH63} allele. No significant difference in the frequency or in the size of clones was observed between *fu* and control stem cell clones generated in parallel according to a chi-square test (Table 1,

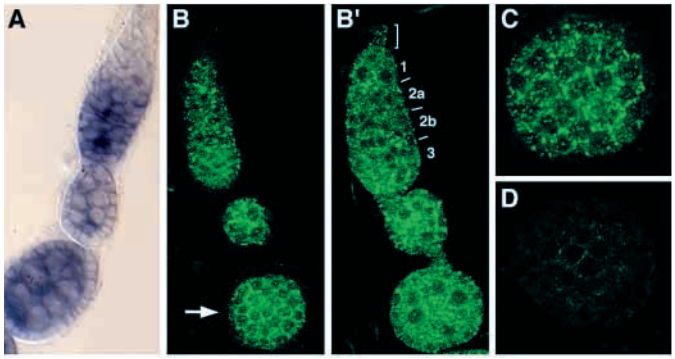

Fig. 4. *fu* is expressed in both the germline and somatic cells of the ovary. All ovarioles are oriented with the germaria towards the top or top right-hand corner. (A) Distribution of *fu* transcripts in a wild-type germarium and stage 2 and 3 egg chambers. (B,B′) Two different confocal sections of a wild-type germarium and stage 2-3 egg chambers stained with anti-Fu antibodies. Confocal section in B corresponds to follicular somatic cells (revealed by their 'honey comb-like' staining, arrow) and confocal section in B′ to germline cells and anterior-most somatic cells. (C,D) Confocal sections of wild-type (C) and fu^1 (D) stage 4 egg chambers stained with anti-Fu antibodies, showing reduced levels of Fu protein accumulation in *fu* mutant follicular cells compared with wild type. Identical confocal settings and image processing with Adobe Photoshop were used for C,D.

α=0.15). Even in 8- to 10-day-old females, *fu* mutant cells were typically found spread throughout mosaic ovarioles (Figs 5, 6, and data not shown), suggesting that neither SSC nor follicular cell division rates are affected in *fu* mutant ovarioles.

The proliferating activity of *fu* SSCs was next more precisely quantified by scoring the number of dividing somatic cells in germarial regions 2 and 3 of *fu* mutant and wild-type females. Mitotically dividing cells were stained specifically with anti-phosphoHistone3 (PH3) antibodies and germarial somatic cells were identified using anti-Fas III antibodies. As previously described (Zhang and Kalderon, 2001), $PH3^+$/$FasIII^-$ cells lying in region 2a/2b, immediately anterior to the limit of Fas III staining, were considered as SSCs. Thus, a reduction in the SSC proliferating activity should result in a decrease in the number of region 2a/2b $PH3^+$/$FasIII^-$ cells and, in turn, in a reduction in the number of germarial region 2b/3 $PH3^+$/$FasIII^+$ cells. Comparative analysis of fu^{JB3} and wild-type ovarioles revealed that no such reduction was observed either in region 2a/2b, or in regions 2b and 3 of *fu* germaria (Table 2). In fact, *fu* females contained rather more dividing cells in germarial regions 2 and 3 than did wild-type females, which we interpret as a consequence of egg chamber maturation and budding defects accompanied by the enlargement of the corresponding regions in mutant females (for example, see Fig. 2C). Taken together, these results suggest that, unlike Hh, Fu kinase activity is not required in the ovary for SSC proliferation.

fu functions as a Hh signal transducer in the ovary

As described above, Hh signal transduction involved in SSC proliferation does not seem to require Fu kinase activity. Therefore, we tested whether the requirement for *fu* in prefollicular cell differentiation involves the classical Hh signaling pathway.

Table 1. Relative proportions of $lacZ^+$, mosaic and $lacZ^-$ ovarioles generated after flipase induction

	$lacZ^+$	Mosaic	$lacZ^-$	*n*
fu^+	85	23	6	114
fu^{mH63}	91	24	1	116

The frequency of fu^+ somatic stem cell clones was compared with the frequency of fu^{mH63} clones generated in parallel. $lacZ^+$ represents ovarioles with no induced clones; mosaic represents partially clonal ovarioles; and $lacZ^-$ represents ovarioles entierely composed of stem cell clones. Eight-day-old females dissected 16 days after heat-shock were analyzed.

Table 2. Proliferating activity of *fu* germarial somatic cells

	2a/2b	2b	3	*n*
fu^+	0.01	0.26	0.53	146
fu^{JB3}	0.00	0.57*	0.72	108

*Statistically different from wild-type value (P<0.01).

The mean number of $PH3^+$ cells per ovariole of 3- to 4-day-old females is given for each region of the germarium. Region 2a/2b corresponds to $PH3^+$/$FasIII^-$ somatic stem cells.

With this aim in view, we tested whether *fu* mutations could affect the transcription of *ptc*, a classical Hh target gene. For this, a *ptc-lacZ* enhancer trap expressed in the same cells as endogenous *ptc* in embryos and in the stripe of strongest *ptc* expression in wing discs (Alves et al., 1998) was used. In the ovary, *ptc-lacZ* is expressed in a subset of *ptc*-expressing cells (anterior somatic cells) (Fig. 7A). This construct is responsive to variations in Hh levels since a strong increase in *ptc-lacZ* expression is observed after ectopic induction of *hh* using a hs-*hh* transgene (Fig. 7B). Strikingly, this ectopic transcription does not occur in a *fu* mutant context (Fig. 7C). In fact, even basal ptc-*lacZ* expression was abolished in *fu* females, which was confirmed using another P-element reporter (*ptc*-Gal4) also inserted in the *ptc* locus (data not shown). Therefore, *fu* seems to be necessary downstream of *hh* for the activation of this ovarian somatic *ptc* enhancer.

In embryo and imaginal discs, at least two other intracellular components of the Hh pathway, *cos2* (*cos* – FlyBase) and *Su(fu)*, have been shown to interact genetically with *fu* in a dose-dependent manner. Previous work showed that removing one copy of *cos2* leads to a partial suppression of both embryonic and wing *fu* phenotypes (Préat et al., 1993). We therefore investigated whether this was the case for *fu* ovarian phenotypes and found that fu^1/fu^1; $cos2^{WI}$/+ and fu^{JB3}/fu^{JB3}; $cos2^{WI}$/+ females exhibit a significantly lower proportion of abnormal ovarioles compared with their fu^1/fu^1 and fu^{JB3}/fu^{JB3} sisters (Fig. 7D). *Su(fu)* was identified as an extragenic semi-dominant suppressor of the adult wing *fu* phenotype but has no phenotype by itself (Préat, 1992). In addition, *Su(fu)* amorphic mutations fully suppress the *fu* embryonic segment polarity phenotype and pupal lethality. Although the $Su(fu)^{LP}$ amorphic mutation has also been described to fully suppress the *fu* ovarian phenotype, we reinvestigated this point under strictly controlled growth conditions with two different class I *fu* alleles (fu^1 and fu^{JB3}). The $Su(fu)^{LP}$ mutation rescued the fu^1 and fu^{JB3} ovarian phenotypes, but only partially because homozygous fu^1; $Su(fu)^{LP}$ females still exhibited a significant proportion of abnormal ovarioles (Fig. 7E). These experiments

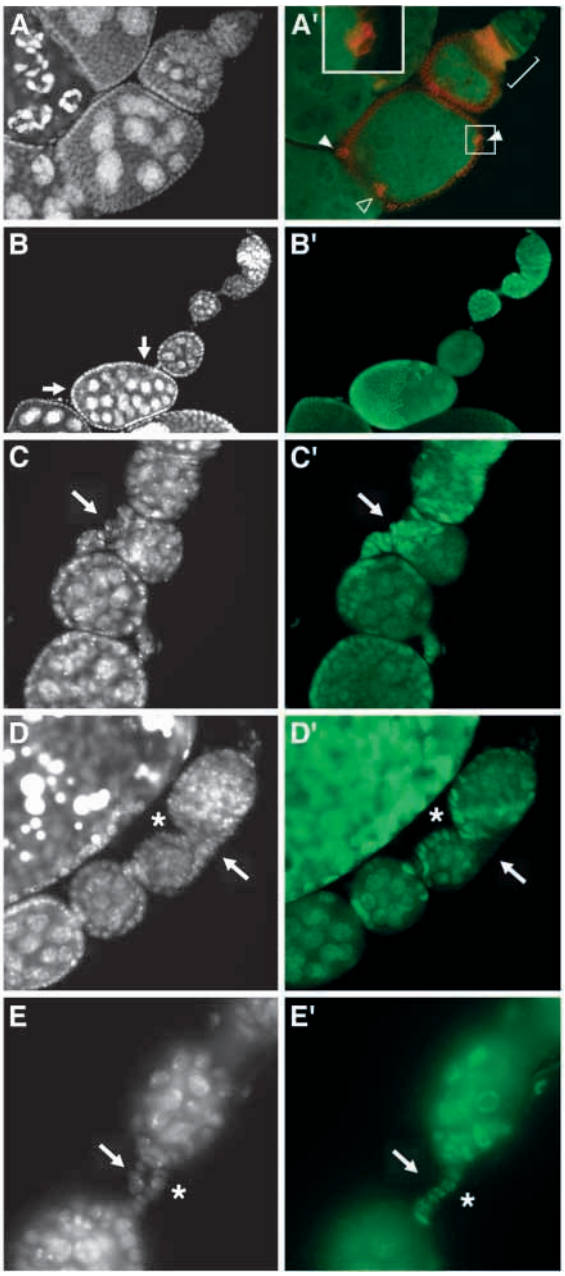

Fig. 5. *fu* function is required in prefollicular cells for centripetal migration and stalk formation. All ovarioles are oriented with the anterior-most region towards the top or top right-hand corner. (A,A′) Mosaic ovariole in which fu^{mH63} mutant follicle cell clones lacking an *arm-lacZ* reporter construct have been induced. Ovariole is triple-stained with DAPI (A), anti-β-galactosidase (A′, green) and anti-Fas III (A′, red) antibodies. fu^{+} somatic cells are found only in the most anterior part of the ovariole (prefollicular cells, bracket). Follicular cells exhibit weak Fas III, but no β-galactosidase staining. White arrowheads point to pairs of polar cells belonging to the second egg chamber, whereas the open arrowhead indicates anterior polar cells belonging to the more posterior egg chamber (as determined after superposition of DAPI and β-galactosidase images, data not shown). Polar cells presented in insert are not in the same focal plane as follicular and germline cells shown in A and A′. (B-E′) Mosaic ovarioles in which *fu* mutant follicle cell clones lacking a *tub-lacZ* reporter construct have been induced. Ovarioles are double-stained with DAPI (B-E) and anti-β-galactosidase antibodies (B′-E′). (B,B′,E,E′) fu^{G3} mosaic ovarioles. (C-D′) fu^{JB3} mosaic ovarioles. (B) Arrows indicate the two small oocyte nuclei present in the multicyst egg chamber. (C,C′) Arrows indicate lateral accumulation of fu^{+} cells in stalk-like structures. (D-E′) Arrows indicate *fu* mutant somatic cells and asterisks indicate fu^{+} cells.

therefore suggest that the *fu-Su(fu)* antagonism exists during oogenesis, even if it seems to be more complex than in other systems.

We then asked whether overexpression of wild-type Ci could at least partially restore *fu* ovarian phenotypes, as is the case for the *fu* wing phenotype (Alves et al., 1998). Using the flip-out/Gal4 system, we generated ovarioles exclusively composed of Ci-overexpressing somatic cells (see Materials and Methods). These ovarioles did not exhibit any obvious defects (Table 3). However, somatic Ci overexpression significantly rescued *fu* ovarian phenotypes, as fu^{JB3} hsp-*flp* /fu^{JB3}; UAS-*Ci*/+; Act-Gal4 UAS-GFP/+ females exhibited a higher proportion of normal ovarioles compared with their fu^{JB3} hsp-*flp*/fu^{JB3}; SM6/+; Act-Gal4 UAS-GFP/TM6 sisters (Table 3).

Taken together, these results suggest that *fu* acts in the ovary as a positive effector of a Hh signal transduction pathway involving Su(fu), Cos2, Ci and *ptc* as a transcriptional target to control early somatic cell development in the ovary.

Reducing Hh signaling phenocopies *fu* ovarian phenotypes

This is the first time that a loss-of-function study unambiguously reveals a role for components of the Hh signal transduction pathway in somatic cell differentiation and egg chamber formation. Indeed, removing the function of Smo and Ci, two positive Hh signal transducers, results in a strong and early block in SSC proliferation, therefore hindering clonal analysis and interpretation of induced phenotypes. In order to circumvent this problem and to confirm this new function of Hh signaling, we tested several other genetic contexts allowing a partial reduction in Hh signaling. We first generated females with hypomorphic combinations of *hh* alleles (hh^{ts2}/hh^{ts2} and hh^{AC}/hh^{ts2}) and examined their ovarioles 4-5 days after shifting them to the restrictive temperature (29°C). As previously reported (Forbes et al., 1996a), we observed several ovarian defects including germaria exhibiting disorganized encapsulation (Fig. 8A) and multicyst egg chambers (Fig. 8B). However, such phenotypes cannot be solely interpreted as resulting from a deficit in somatic cell number as we found large groups of disorganized somatic cells, resembling those found in *fu* mutant ovarioles, at the periphery of *hh* mutant germaria and multicyst egg chambers (Fig. 8A′,B′, arrows). This suggests that *hh* mutant prefollicular cells are also defective in their capacity to migrate, individuate germline cysts and intercalate to form normal stalks.

Next, we reduced Hh signal transduction in prefollicular cells by overexpressing either a negative regulator of the transduction pathway (Cos2), or a constitutive inhibitor form of Ci (Ci^{cell}). In both cases, using the flip-out/Gal4 system to generate cell clones, we obtained ovarioles which contained large clones, or even all somatic cells, overexpressing Cos2 or Ci^{cell}, as visualized with the UAS-GFP reporter (Fig. 8C′-F′, green). The proliferative capacity of cells within such clones

Table 3. Restoration of *fu* ovarian phenotypes after somatic overexpression of wild-type Ci

	Normal	Abnormal	*n*
fu^{JB3} hsp-*flp*/fu^{JB3}; Sp/+; Act-Gal4 UAS-GFP/+	5.21%	94.79%	120
fu^{JB3} hsp-*flp*/fu^{JB3}; UAS-Ci/+; Act-Gal4 UAS-GFP/+	50.78%	49.22%	106
fu^{JB3} hsp-*flp*/FM3; UAS-Ci/+; Act-Gal4 UAS-GFP/+	100.00%	0.00%	94

Scored ovarioles were divided into two categories: ovarioles containing exclusively wild-type egg chambers (normal) and ovarioles containing at least one abnormal egg chamber (abnormal). Under our experimental conditions, all or almost all somatic cells overexpress Ci in a given ovariole, which is the reason we use the shorthand Act-Gal4 in the genotypes. Note that the sole somatic overexpression of Ci did not induce any detectable ovarian phenotype.

thus does not seem to be affected. However, these somatic overexpression clones were associated with various defects including multicyst (Fig. 8C,E, arrowheads) and apposed (Fig. 8C,E, arrows) egg chambers, disorganized encapsulation in the germarium and abnormal stalk formation (Fig. 8D′,F′).

Altogether, our results are consistent with the existence of an ovarian *fu*-dependent Hh signaling pathway directly involved in prefollicular cell morphogenesis during egg chamber formation.

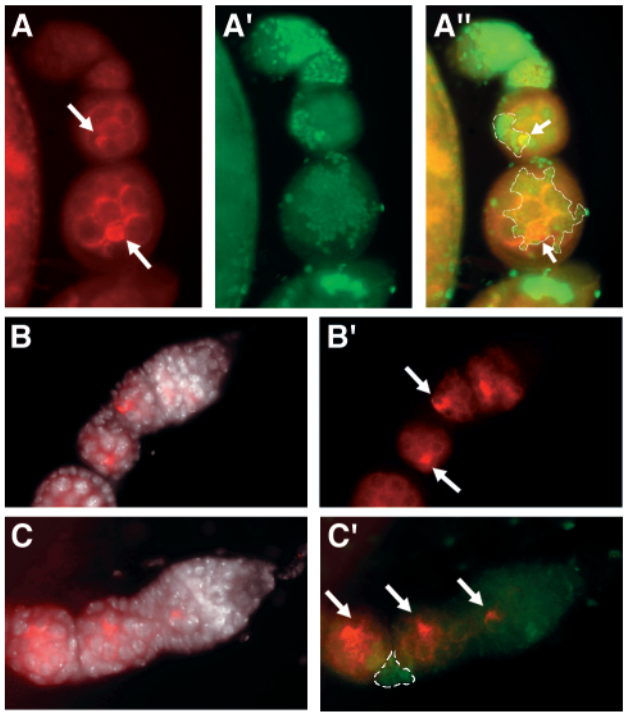

Fig. 6. *fu* function is required in prefollicular cells for posterior oocyte positioning. All ovarioles are oriented with the anterior-most region towards the top or top right-hand corner. (A-A″) fu^{G3} mosaic ovariole double-stained with anti-Orb (A, red) and anti-β-galactosidase (A′, green) antibodies. (A″) Merge. fu^{+}-expressing follicle cells are delimited by broken lines. (B,C) Wild-type (B) and fu^{G3} mosaic germaria (C) triple-stained with DAPI (B,C), anti-Orb (B,B′,C,C′, red) and anti-β-galactosidase (C′, green) antibodies. fu^{+}-expressing follicle cells in C′ are delimited by broken lines and correspond primarily to stalk cells. Arrows in B′ indicate posteriorly localized Orb-expressing oocytes and arrows in A, A″,C′ to mislocalized Orb-expressing oocytes. Note that, in addition, the normal posterior subcellular localization of the Orb crescent within the oocyte (arrows in B′) is perturbed in *fu* mosaic ovarioles (arrows in C′).

DISCUSSION

fused mutations affect the morphogenetic properties of prefollicular cells but not the specification of stalk, polar and follicular epithelial cells

Egg chamber formation in the *Drosophila* ovary requires a somatic cell developmental program that involves: (1) somatic stem cell self-renewing divisions; (2) prefollicular cell morphogenesis for germline cyst encapsulation, anchoring/positioning of the oocyte posteriorly, and interfollicular stalk formation; and (3) prefollicular cell differentiation into three cell types, stalk, polar and follicular epithelial cells. However, it is not clear as yet how prefollicular cell morphogenesis and differentiation are integrated. Our study of *fu* mutant ovaries, which produce ovarioles containing multicyst and apposed egg chambers, revealed that, in contrast to what has been shown for other components of Hh signal transduction, *fu* function is not required for the first step of this program, the proliferation of somatic stem cells (see below). In addition, unlike other genes that, when mutated, lead to defective egg chamber formation (e.g. mutations that affect components of the Notch/Delta signaling pathway), *fu* is not required for the third step in this program, stalk and polar cell specification and formation of the follicular epithelium. Rather, our analysis revealed several specific defects in prefollicular cell behavior during egg chamber formation, all involving cell-cell recognition and adhesion, cell shape changes, and cell motility.

Germline cyst encapsulation requires extension of cellular processes by prefollicular cells in regions 2a/2b of the germarium, such that they can recognize and adhere to mature 16-cell germline cysts, and subsequently migrate centripetally between individual cysts. Interfollicular stalk formation requires that pre-stalk cells in regions 2b/3 lose heterotypic adhesion to germline cells and gain homotypic adherence and the capacity to intercalate (Tworoger et al., 1999). The effector molecules implicated in these processes have not been characterized to a great extent, though several surface membrane and cytoskeletal proteins that have been shown to exhibit dynamic expression patterns in prefollicular cells and their descendants are likely to be involved. For example, several proteins (actin, Fas III, Hts, α-Spectrin, Filamin and others) are localized specifically to the cellular processes that prefollicular cells extend over germline cysts, and most of these are subsequently concentrated apically in pre-stalk cells just before their intercalation. Once the interfollicular stalk is formed, the expression of some of these proteins is downregulated in stalk cells (Fas III), while other proteins are expressed laterally in these cells (actin, Hts, α-Spectrin and PS1-β integrin) (Gonzalez and St Johnston, 1998; Lin et al.,

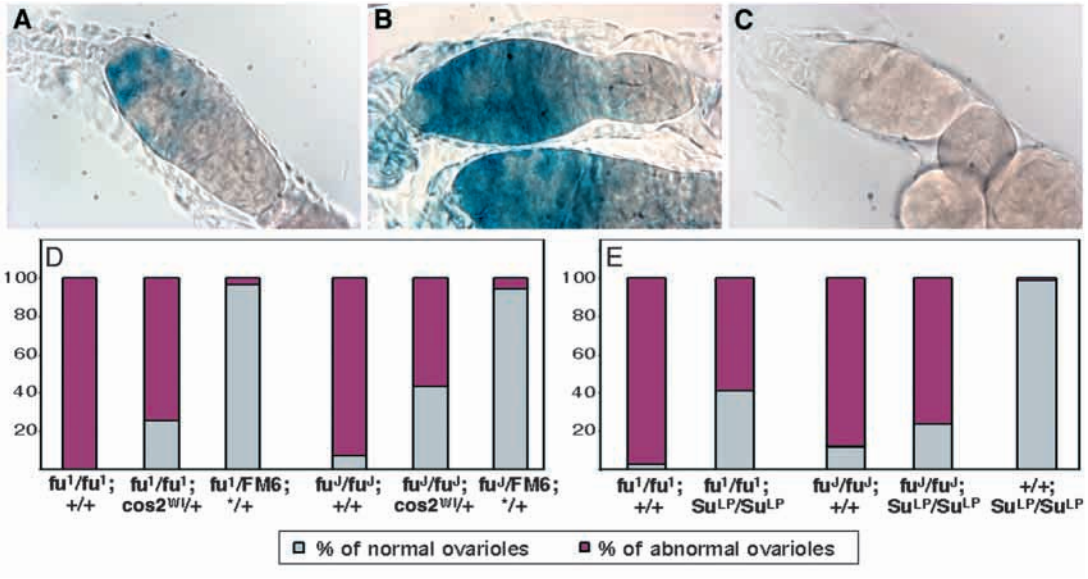

Fig. 7. *fu* interacts genetically with components of the Hedgehog pathway in the ovary. (A-C) Expression of a *ptc-lacZ* enhancer-trap in wild-type (A), hs-*hh* (B) or fu^{1}/fu^{JB3}; hs-*hh*/+ germaria. Ovarioles are oriented with anterior towards the left. (D,E) Removing one copy of *cos2* (D) or two copies of *Sufu* (E) restores the *fu* ovarian phenotype. Class II *fu* alleles were not tested in these experiments because fu^{II}; $cos2^{WI}$/+ and fu^{II}; $Su(fu)^{LP}$ flies die as late pupae, displaying a strong *cos2* phenotype. fu^{J} denotes the fu^{JB3} allele. (D) Females were grown at 25°C and dissected 5-8 days after eclosion. *indicates either $cos2^{WI}$ or + (these two different genotypes were not distinguishable in our test). Flies came from the same cross for a given allele. (E) Females were grown at 21°C until late pupal stage, then shifted to 25°C and dissected 8 days after eclosion. Females originated from separate crosses performed in parallel. The total number of ovarioles examined for a given genotype was at least 150, except for fu^{1} homozygous flies in E (n=39).

1994; Niewiadomska et al., 1999; Ruohola et al., 1991; Tanentzapf et al., 2000; Zarnescu and Thomas, 1999). Finally, proper expression of the DE-Cadherin, Armadillo/β-Catenin and α-Catenin cell-cell adhesion complex at the membrane of both the posterior follicle cells and the oocyte probably mediates contact between these two cell types and posterior positioning of the oocyte (Godt and Tepass, 1998; Gonzalez and St Johnston, 1998).

We show here that, in *fu* mutant ovarioles, encapsulation of multiple cysts in a single egg chamber is associated with absence of prefollicular cell extensions around germline cysts and impaired centripetal migration of these cells. In addition, stalk formation in *fu* mutants is, in less affected individuals (young females with normal egg chambers), slow/delayed and, in more severely affected individuals (older females with multicyst egg chambers), very irregular (leading to abnormal stalk morphology). By following the expression of DE-Cadherin, which marks the apical membrane of pre-stalk cells, we show that, in *fu* mutants, pre-stalk cells that have migrated centripetally between germline cysts are blocked before the intercalation process. Induction of *fu* mutant clones in prefollicular cells led to the same types of encapsulation and stalk morphogenesis defects, indicating cell autonomous function in these cells for these processes. This study highlighted the impaired ability of *fu* mutant prefollicular cells to migrate between germline cysts and to participate to interfollicular stalk formation. This mosaic analysis also showed that *fu* mutant and wild-type cell populations have a tendency to remain segregated, implying that surface differences between these cells prevent their intermixing. Finally, *fu* function in prefollicular cells is implicated in another process involving specific cell-cell interactions, posterior positioning of the oocyte in the egg chamber. Taken together, these results suggest a function for *fu* in prefollicular cells for appropriate expression of one or several surface membrane or cytoskeletal proteins necessary for several aspects of prefollicular cell morphogenesis during egg chamber formation. Although we examined the expression of a number of cytoskeletal and membrane proteins in prefollicular cells in *fu* mutants (for example, DE-Cadherin, Fas III and others; data not shown), so far it has not been possible to relate the anomalies observed to a loss in expression or in polarized localization of any of these proteins. Interestingly, *fu* and other components of Hh signal transduction have been implicated in other developmental processes that involve establishing dynamic and differential cell-surface properties. For example, proper migration of germ cells during embryogenesis and their coalescence with somatic gonadal precursor cells to form the primitive gonad involves Hh expression in these somatic cells and function of components of classical Hh signal transduction in the germ cells (Deshpande et al., 2001). Dahmann and Basler (Dahmann and Basler, 2000) also showed that opposing outputs of Hh signaling play a role in establishing differential cell affinities and thereby defining the anteroposterior compartment boundary in wing imaginal discs. This study also demonstrated that a difference in the level of DE-Cadherin expression alone was sufficient to maintain two wing disc cell populations segregated. However, the actual cell adhesion effectors that may be regulated by differential Hh signal transduction in wing development, as is the case for germ cell migration and egg chamber formation, remain to be determined.

Fu-independent Hh signal transduction in SSCs controls their proliferation

In the ovary, Hh signals from the terminal filament and cap cells and is required for SSC proliferation and subsequently for egg-chamber budding (Forbes et al., 1996a; Forbes et al., 1996b). SSC self-renewing properties are not maintained in the absence of Hh signaling, whereas excessive Hh signaling produces supernumerary stem cells (Zhang and Kalderon, 2001). In addition to the membrane receptors Ptc and Smo, Ci

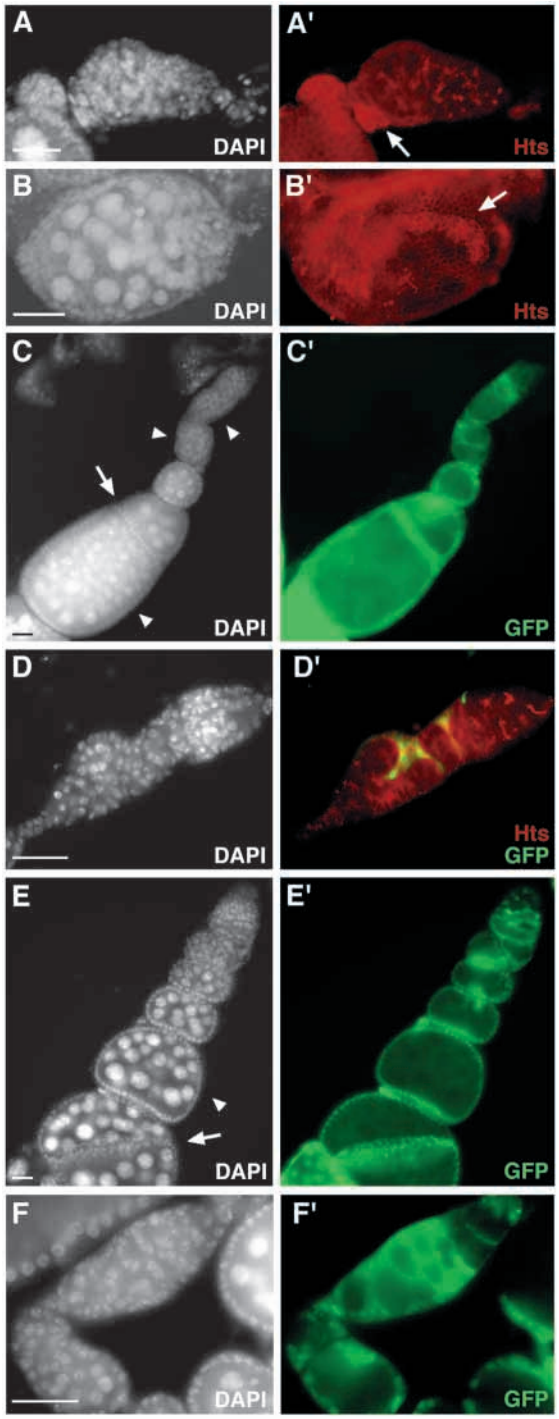

Fig. 8. Reduction in Hedgehog signaling levels leads to abnormal prefollicular cell behavior. All ovarioles are oriented with the anterior-most region towards the top or top right-hand corner. hh^{ts2}/hh^{ts2} (A,A′) and hh^{AC}/hh^{ts2} (B,B′) ovarioles dissected from 5-day-old females placed at 29°C immediately after eclosion and double-stained with DAPI (A,B) and anti-Hts antibodies (A′,B′). Hts is normally present in spectrosomes and fusomes of germline cells as well as in all somatic cells as of germarial region 2a/2b (Lin et al., 1994). In particular, Hts accumulates at high levels in interfollicular cells. Arrows in A′,B′ indicate somatic cells aggregated at the periphery of the ovarioles. (C-D′) DAPI (C,D) and GFP (C′,D′, green) staining of hsp-*flp*/+; Act>CD2>Gal4,UAS-GFP/UAS-*cos2* ovarioles. Germarium in D′ was additionally stained with anti-Hts antibodies (red). (E-F′) DAPI (E,F) and GFP (E′,F′, green) staining of hsp-*flp*/+; Act>CD2>Gal4,UAS-GFP/UAS-Ci^{cell} ovarioles. All prefollicular cells and their descendants are GFP^+ in C′ and E′, whereas both GFP^+ and GFP^- prefollicular and follicular cells are present in D′ and F′. (C,E) Arrows indicate apposed egg chambers, arrowheads indicate multicyst egg chambers. Scale bars: 10 μm.

has been implicated in this process as a component of Hh signal transduction. However, in a *hh* loss-of-function context, SSC proliferation is restored by induction of low levels of somatic Hh signaling in SSC (achieved by removing protein kinase A function, an inhibitor of Ci activity, in these cells) (Zhang and Kalderon, 2000). The authors therefore suggested that, as in wing imaginal disc development, where *fu* activity is required for transducing high but not low levels of Hh signaling, *fu* activity may not be endogenously required for regulation of SSC division (Alves et al., 1998; Sanchez-Herrero et al., 1996; Vervoort et al., 1999). Our results obtained upon induction of *fu* mutant clones, as well as the quantitation of the mitotic activity of somatic cells in *fu* mutant germaria, confirm that *fu* is not necessary for SSC proliferation, suggesting that Hh signals to SSC through a Fu-independent pathway.

Fu-independent Hh signal transduction has already been reported in other systems (Suzuki and Saigo, 2000; Therond et al., 1999). In the ventral ectoderm of the embryo, for example, Hh is secreted in a single row of cells at the parasegmental boundary and signals in both anterior and posterior directions, leading to the expression of the *ptc* gene in all neighboring cells. Interestingly, unlike *smo* and *ci*, *fu* function is required solely in anterior cells to transduce Hh signaling (Therond et al., 1999). In the embryo as well as in the ovary, the differential requirement of Fu kinase activity can be interpreted either as a differential sensitivity of cells to Hh signal intensity and/or of target genes to Ci activation levels, or as the existence of position-specific modulators or even effectors of Hh response. Use of unconventional transducers has already been suggested for Hh signal transduction in posterior compartment cells of the wing imaginal disc (Ramirez-Weber et al., 2000), Boldwig's organ cells (Suzuki and Saigo, 2000) and ovarian germline cells (Vied and Horabin, 2001).

Fu-dependent Hh signal transduction in prefollicular cells regulates egg chamber production

Our study reveals that *fu* endogenous function is required in prefollicular cells for acquisition of specific morphogenetic properties (see above). In addition, we provide several lines of evidence for a role for *fu* in a classical Hh signal transduction pathway within prefollicular cells for their participation to egg chamber formation. First, *fu* and *hh* mutant ovarian phenotypes overlap as both result in aberrant somatic cell behavior and formation of multicyst and apposed egg chambers (this study) (King, 1970). Second, *fu* is necessary, downstream of *hh*, for the expression of an ovarian somatic *ptc-lacZ* enhancer-trap. Third, *fu* ovarian phenotypes can be partially suppressed by removing either one or two copies, respectively, of two negative regulators of Hh signaling [*Cos2* and *Su(fu)*], or by overexpressing the transcription factor Ci. Last, the morphogenetic defects described for *fu* mutant prefollicular cells can be phenocopied by somatic overexpression of either

Cos2 or the inhibitory Ci^{cell} proteins. We therefore propose a model in which Hh signals at least twice in germarial somatic cells: first, through a *fu*-independent pathway to control SSC proliferation; and second, through a classical *fu*-dependent pathway to regulate early aspects of prefollicular cell differentiation. Therefore, *fu* loss-of-function mutations, which we show only affect prefollicular cell morphogenesis, allow the analysis of the role of Hh signal transduction in this process specifically.

Interestingly, previous studies focusing mostly on the effects of excessive Hh signal transduction in the ovary also indicated that two different stages of somatic ovarian cell development in the germarium are targeted by this signaling molecule (Zhang and Kalderon, 2000): early on (region 2a/2b), SSC proliferation and oocyte posterior positioning are affected; and later (region 2b/3) there is an apparent delay in the prefollicular cell development program, which, when combined with early effects on SSC proliferation, leads to the formation of giant stalks comprising poorly differentiated prefollicular cells between early egg chambers, delayed polar cell specification (stage 4 instead of 2) and an excess of these cells, and continued follicular epithelial cell division after stage 6. In *fu* mutants there is no effect on SSC or follicular cell proliferation, but some of the defects affecting prefollicular cells are similar, including non-posterior oocyte positioning, delayed prefollicular cell differentiation leading to delayed egg chamber budding and delayed polar cell specification. In addition, both somatic *fu* and *ptc* mutant clones show striking segregation from wild-type cells, *fu* mutant clones preferentially localized to the follicular epithelium, whereas *ptc* mutant clones localized to the stalks. These results indicate that cellular differences in Hh signal transduction levels, whether reduced (*fu*) or increased (*ptc*) compared with wild-type levels, affect the cell-cell recognition and adhesive properties of prefollicular cells. Taken together, these studies show that there is an overlap between the ovarian phenotypes associated with a reduction and an increase in Hh signaling, indicating that crucial levels of Hh signaling are required for prefollicular cell morphogenesis.

Nonetheless, *fu* mutations do not completely arrest egg chamber budding, rather causing a delay in several aspects of the prefollicular cell developmental program, including stalk and polar cell specification. Even *fu* mutant clones induced in prefollicular cells using the strong hypomorphic allele, fu^{mH63}, did not provoke more severe anomalies than the other alleles used in this study. These results suggest that prefollicular cell development does not depend solely on *fu*-dependent Hh signaling and that there is possibly some redundancy in the regulation of this process. Indeed, other studies have shown the importance of germline-emitted signals, in particular the secreted molecules Egghead, Brainiac and Gurken/TGFα, for the encapsulation of germline cysts by prefollicular cells (Goode et al., 1996a; Goode et al., 1996b; Goode et al., 1992; Rubsam et al., 1998). In addition, specification of polar and stalk cells via germline-to-soma signaling involving Delta/Notch, is also necessary for proper egg chamber formation (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). It is possible then that the correct timing of events in the mid-germarial region for proper encapsulation and egg chamber budding is achieved by two signaling sources, the terminal filament (Hh signaling) and mature 16-cell germline cysts (Egghead, Brainiac, Gurken/TGF-α, and Delta signaling). The integration of all of these signals by prefollicular cells would be necessary for these cells to go through their developmental program in the appropriate time frame, thus allowing synchronous germline cyst maturation and encapsulation by prefollicular cells.

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