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hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in *Drosophila*

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SUMMARY

The *hedgehog (hh)* gene plays a role in regulating cell proliferation and specifying cell identity in diverse systems. We show that *hh* is expressed at the extreme apical end of *Drosophila* ovarioles in terminal filament cells and a newly identified group of associated somatic cells. Reducing or ectopically expressing *hh* affects somatic cells in region 2 of the germarium, 2-5 cells away from the cells in which Hh protein is detected. *hh* activity stimulates the proliferation of pre-follicle somatic cells, and promotes the specification of polar follicle cells. *hh* signalling during egg chamber assembly appears to be closely related to, or part of pathways involving the neurogenic genes.

Key words: *hedgehog*, oogenesis, cell proliferation, cell fate, *Drosophila*, polarity

INTRODUCTION

Oogenesis in *Drosophila* involves the association of a 16-cell germ line cyst with somatic cells to form an egg chamber, followed by the coordinated differentiation of the contributing cells into a mature egg (reviewed by King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993a; see Fig. 1). Egg chamber assembly takes place in the germarium, a structure at the tip of each ovariole, containing three recognized regions. The apical portion of the germarium (region 1) contains 2-3 germ line stem cells located below a stack of 6-10 somatic cells called the terminal filament, as well as 1-2 growing cysts (Lin and Spradling, 1993). Newly formed 16-cell cysts move posteriorly through region 2a while maintaining contact with thin, non-dividing 'inner germarium sheath' cells that line the germarium wall (Margolis and Spradling, 1995).

The events leading to egg chamber formation begin after cysts pass two somatic stem cells located at the border of region 2b (Margolis and Spradling, 1995) and become surrounded by inwardly migrating somatic cells. A few of the approximately 16 initial somatic cells that associate with the cyst become polar cell precursors and soon cease division (Margolis and Spradling, 1995). These pairs of specialized follicle cells eventually come to lie at the anterior and posterior poles of each egg chamber and help pattern the follicle and its oocyte (Ruohola et al., 1991; Gonzales-Reyes et al., 1995; Roth et al., 1995). The remaining pre-follicle cells continue to divide as the follicle moves further to the posterior, and may be joined by additional migrating cells as it prepares to bud from the germarium in region 3. Although our knowledge remains imperfect, it appears that most of the interleaving cells that separate a region 3 cyst from region 2 eventually end up as part of its follicle cell monolayer, while 5-8 cells differentiate as special 'stalk cells' that still connect the newly budded egg chamber to the germarium (Fig. 1). The rest of the ovariole consists of 6-7 progressively more mature egg chambers separated by their interfollicular stalks.

Intercellular signalling is required during multiple steps to pattern the developing egg chamber and oocyte (reviewed by Schupbach and Roth, 1994). In particular, soma-soma signalling dependent on the neurogenic genes *Notch (N)* and *Delta (Dl)* is needed to form egg chambers. Reduction in the activity of either gene results in the loss of interfollicular stalk cells and hyperplasia of the polar cells (Ruohola et al., 1991; Xu et al., 1992; Bender et al., 1993). The *daughterless (da)* gene is also required for egg chamber formation and da^- alleles exhibit dominant synergistic interactions with *N* and *Dl*, suggesting that these genes may be involved in the same genetic pathway (Cummings and Cronmiller, 1994). Reductions in any of these gene products produces compound egg chambers containing two or more germ line cysts, while stronger alleles or allele combinations block cyst encapsulation and egg chamber budding entirely. These neurogenic genes have been postulated to act as a conserved 'cassette' to specify follicle cell fates in a manner similar to their role in the peripheral nervous system (Ruohola et al., 1991). However, at least one neurogenic locus,

brainiac (brn) may act in a different manner (Goode et al., 1992). *brn* is required in the germ line for germ line cyst encapsulation, and appears to interact with *torpedo*, the *Drosophila* EGF receptor (DER) in the somatic cells (Goode et al., 1992). Unlike the reported effects of *N*, *Dl* and *da*, signalling mediated by *brn* and DER is also needed to pattern the dorsal-ventral axis of the egg chamber (Goode et al., 1992).

We have found that the segment polarity gene *hedgehog* (*hh*) is also required for egg chamber formation during oogenesis. *hh* activity is involved in the regulation of growth and patterning in a number of developing systems (reviewed by Ingham, 1995). *Drosophila hh* was first identified because of its embryonic phenotype (Nüsslein-Volhard and Wieschaus, 1980). It is required for correct intrasegmental patterning during *Drosophila* embryogenesis (reviewed by Perrimon, 1994). In leg and wing imaginal discs *hh* activity directs patterning and proliferation throughout the disc (Tabata and Kornberg, 1994; Basler and Struhl, 1994, Ingham and Feitz, 1995). In the developing eye imaginal disc *hh* is required for the progression of the morphogenetic furrow, a process involving the coordination of cell cycle synchronization, changes in cell shape, specification of cell identity and differentiation (reviewed by Heberlein and Moses, 1995). A number of vertebrate homologs of *hh* have been cloned, and one of these, *Sonic hedgehog* (*Shh*) is expressed in notochord and floorplate in the developing neural tube, Hensen's node, and the zone of polarizing activity (ZPA) in developing limb buds (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Reolenk et al., 1994). Expression of *Shh* in the notochord induces the differentiation of floorplate and motor neurons in the overlying neural tube, thereby polarizing the nervous system (reviewed by Ingham, 1995; Placzek, 1995). In the developing limb bud *Shh*-expressing cells mimic the polarizing activity of the ZPA, and through interactions with FGF-4, *Shh* is involved in the regulation of the cell proliferation required for limb outgrowth (reviewed by Perrimon, 1995; Johnson and Tabin, 1995). *hh*-family genes have thus been shown to regulate cell division, differentiation and cell identity in developing systems in both vertebrates and invertebrates.

MATERIALS AND METHODS

Fly stocks and culture

Drosophila genes and genetic symbols are described in FlyBase (1992). The following fly strains were used: the temperature sensitive allele $hh^{9K}/TM3$ (Jurgens et al., 1984), the strong allele $hh^{GS1}/TM3$ (Mohler, 1988), an enhancer trap insert in *hh* (*hhlacZ*) (Mohler and Vani, 1992; Wilson et al., 1989), *hs-hhM11* flies containing a construct with the entire *hh* coding region under the regulation of the *hsp70* promoter (Ingham, 1993), *UAS-Nhh* flies in which the 5′ end of the *hh* cDNA, encoding the N-terminal fragment of Hh, is cloned downstream of the yeast upstream activating sequences (UAS) (Feitz et al., 1995), *hs-GAL4* flies containing a construct expressing *GAL4* under the control of the *hsp70* promoter (Feitz et al., 1995), P(*lac,ry+*)A EIII/TM3 (h^{EIII}) flies with an enhancer trap insert in *hairy* (*h*) (a gift from A. Martinez Arias), and HSH33;HSH22 flies containing a construct with *h* regulated by the *hsp70* promoter on both second and third chromosomes (Ish-Horowicz and Pinchin, 1986). The enhancer trap lines *8360* and *PZ80* both of which mark polar cells, and *l(3)1344* which marks stalk cells carry the *PZ* transposon and were identified in a single *P* element mutagenesis screen (Karpen and Spradling, 1992; Spradling, 1993a).

Conditionally mutant *hh* flies were produced by crossing $hh^{9K}/TM3$ flies to $hh^{GS1}/TM3$ flies and growing the progeny at 17°C. The hh^{9K}/hh^{GS1} transheterozygotes (identified as non-*TM3*) were collected and 1-2 days after eclosion shifted to the restrictive temperature of 28°C or 29°C for 6 or 8 days prior to ovary dissection.

Heat shock regime

Flies with a single copy of the *hs-hh* construct, and in some experiments a copy of a cell-specific *lacZ* marker, were put in a vial of food with dry yeast and tissue paper. The tubes were immersed in a 37°C water bath for 1 hour at twelve-hourly intervals over a period of 3 days. Ovaries were dissected and stained at different time points after heat shock.

Germarium transplants

Germarium transplants were carried out as described by Lin and Spradling (1993). Donor females containing two copies of the *hs-hh* construct were heat shocked in a 37°C water bath for 1 hour immediately prior to dissection and transplantation of germaria into the abdomens of 5- to 6-day old ovo^{D1} females. The injected flies were kept at 24°C for 6 days and heat shocked for 1 hour at 37°C once each day. The ovarioles produced by the transplanted germaria were dissected out and stained with DAPI.

Histochemical and immunological staining

Antibody staining

Antibody staining was carried out as described by Lin et al. (1994) using a protocol based on the embryonic protocol described by Patel et al. (1989). Two different anti-Hh antisera were used, each directed against the N-terminal fragment of the Hh protein; Ab1, generated against residues 83-160 (Lee et al., 1994) was used at a 1:100 dilution; Ab2 raised against residues 1-224 (Taylor et al. 1993) was used at 1:500. Anti-Vasa polyclonal antibody (Hay et al., 1988) was used at a 1:2000 dilution. TRITC-conjugated AffilPure™ donkey anti-rabbit secondary antibodies from Jackson ImmunoResearch Laboratories were used at a 1:200 dilution.

There was a discrepancy in the staining pattern of the two anti-Hh polyclonal antibodies. In addition to the pattern of staining described here, Ab1 also weakly stained the nuclei of somatic and germ line cells in the germarium, and the germinal vesicle of stage 2 to stage 7 egg chambers. The fragment of Hh protein used to generate Ab1 was within the fragment used to generate Ab2, suggesting that this nuclear staining may not be specific to Hh but a cross reaction of Ab1 with a nuclear protein.

DAPI staining and β-galactosidase staining were carried out as described by Lin and Spradling (1993). All stained samples were mounted in 50:50 PBS:glycerol and examined under a Zeiss Axiophot microscope equipped with Nomarski and epiflourescent optics. X-gal-stained ovarioles were photographed using 160T Ektachrome slide film, DAPI and antibody-stained ovarioles were photographed with 400T Ektachrome slide film.

RESULTS

hh is expressed in specific somatic cells at the anterior tip of the ovariole

Our studies of *hh* expression revealed that the cellular structure and behavior of the terminal filament was more complex than previously realized. In young females, the base of the terminal filament consists of a pair of flatter cells we term basal cells (Fig. 1C; H. L. and A. C. S., unpublished observation). The terminal filament is associated with another group of 2-6

somatic cells which are usually positioned asymmetrically on one side of the germarium tip, capping the underlying germ line stem cells. These cells, which we refer to as cap cells (Fig. 1C), appear tightly associated with one another as well as with the basal cells of the terminal filament. As flies age, there is a change in the organization of these non-mitotic cells. The cap cells, basal cells and 1-2 proximal terminal filament cells condense into a tight cluster within the tip of the germarium, while the remaining terminal filament cells form another more apical cluster partially separated from the germarium tip (Fig. 1D-G).

To analyze the expression of the *hh* gene in ovaries, we first studied the expression of an enhancer trap insert in *hh* (*hhlacZ*) which faithfully reproduces the pattern of the endogenous *hh* gene in both embryos and imaginal discs (Mohler and Vani, 1992). Strong expression is seen in terminal filament and cap cells at the tip of every germarium (Fig. 1B). Much lower levels of expression occur in inner sheath cells and in a few anterior follicle cells later in egg chamber development (Fig. 1B). Two different polyclonal antibodies both directed against the amino half of the Hh protein (Taylor et al., 1993; Lee et al., 1994) were used to stain ovaries from flies between 1 and 9-days old. After staining with either antibody, fluorescence and confocal microscopy revealed that the Hh protein was localized at the surface of terminal filament and cap cells (Fig. 1C-G). Hh protein was not detected in the inner germarium sheath or anterior follicle cells with either antibody. Thus, the *lacZ* expression observed in these cells appeared to be an artifact of the *hhlacZ* enhancer trap line. However, we could not rule out that *hh* is expressed in inner sheath and anterior follicle cells at levels below the detection limit of the antibodies used.

The level of Hh expression varies within the terminal filament and cap cells and is modulated over time. In 1-day old flies, Hh protein can be detected in all terminal filament and cap cells. Staining is extremely weak in the distal half of the terminal filament, but much stronger in more proximal cells, including basal and cap cells (Fig. 1C,D). Staining is unevenly distributed over their cell surfaces, and includes bright aggregates that may be analogous to the plaques of Hh protein described in embryonic ectoderm (Taylor et al., 1993) and imaginal discs (Tabata and Kornberg, 1994). As flies age, the level of *hh* expression in the distal cells of the terminal filament decreases further. In 9-day old flies, when cap cells, basal cells and some of the proximal terminal filament cells condense into a cluster within the germarium tip, *hh* is expressed strongly in these cells, and expression may be completely lost from the more distal terminal filament cells (Fig. 1F).

To investigate the origin of *hh* expression in germaria, *hhlacZ* expression was analyzed in larval gonads. In mid-third instar larval ovaries, when terminal filament and ovary differentiation begin, *hhlacZ* is expressed in a band of cells above the presumptive germ line stem cells about one third of the way from the apical end. These cells continue to express *hhlacZ* as they interdigitate to form terminal filaments during the late third instar (Fig. 1H). Much lower levels of expression are also

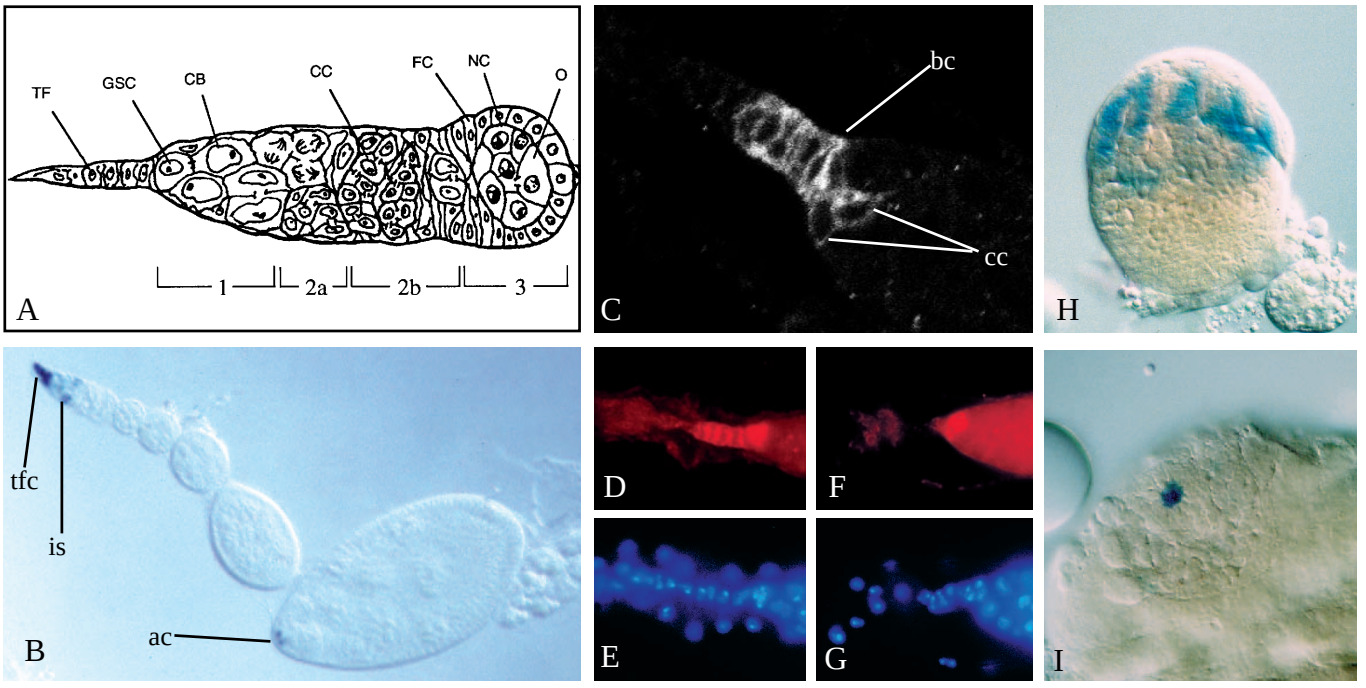

Fig. 1. *hh* expression in *Drosophila* ovaries and testes. (A) Germarium structure including regions 1-3. CB, cystoblast; CC, cystocyte; FC, follicle cell; GSC, germ line stem cell; NC, nurse cell; O, oocyte; TF, terminal filament. (B) An ovariole expressing *hhlacZ* stained with X-gal. The terminal filament and cap cells are strongly labeled (tfc) while inner sheath cells (is) and some cells at the anterior of stage 9 and older egg chambers (ac) stain weakly. (C) Hh protein visualized in the germarium of a 5-day old adult using confocal immunofluorescence microscopy. Only the terminal filament, region 1 and region 2a of a single germarium are shown. 'Cap' cells (cc) are labeled below the basal cells (bc) of the terminal filament. Hh protein is localized at cell surfaces and is less abundant in distal terminal filament cells. (D-G) Reorganization of the terminal filament with age. The terminal filament and region 1 are compared from a 1-day old fly (D, Hh protein; E, DNA) and 9-day old fly (E, Hh protein; G, DNA). At 9 days *hh* expression is confined to the more proximal cell cluster. (H) *hhlacZ* expression in l3 larval ovary. Developing terminal filaments are labeled. (I) *hhlacZ* expression in the l3 testis, showing labelling of the somatic hub cells.

seen in the region posterior of the terminal filaments, which contains the germ line cells intermingled with some somatic cells. The *Drosophila* testis also contains a group of non-mitotic somatic cells closely associated with the germ line stem cells. These 'hub' cells may have some analogy with terminal filament cells (reviewed by Fuller, 1993). *hhlacZ* is expressed in hub cells of testes from third instar larvae (Fig. 1I), and Hh protein is detectable in the hub cells of adult testes although at much lower levels than in the terminal filaments (data not shown).

hh is required for somatic cells to encapsulate germ line cysts

To analyze the function of *hh* in the adult ovary, flies *trans*-heterozygous for the temperature sensitive allele hh^{9K} and the amorphic allele hh^{GS1} were used to modulate the level of *hh* activity. When hh^{9K}/hh^{GS1} flies (hereafter referred to as hh^{ts} flies) grown at 18°C were shifted to the restrictive temperature for 6-8 days, compound egg chambers were produced which appear to have resulted from a failure in the encapsulation of germ line cysts by somatic cells. Wild-type flies grown under the same conditions and hh^{ts} flies grown at 18°C were fertile and had ovaries with normal morphology.

After 6 days at 28°C most of the ovarioles from hh^{ts} females had abnormal germaria (Fig. 2). Although the terminal filament, cap cells and growing cysts in region 1 appear normal, region 2 is filled with round germ line cells amongst which individual cysts are difficult to distinguish (Fig. 2C-F). Staining with an anti-Vasa antibody, which specifically marks the germ line, showed that each germarium contains a cluster of closely associated germ line cells (Fig. 2D) and in some cases revealed that these clusters consist of several cysts wrapped around one another. Separated disc shaped cysts typical of the fully enveloped cysts normally present in region 2b were rarely present. Follicle cells were not seen invaginating in region 2, and interdigitating cells resembling those that normally separate stage 1 egg chambers from the germarium were only detected rarely. No increase in somatic cell death was observed in these germaria (data not shown). Therefore, lowering *hh* activity seems to reduce the number of somatic cells in the germarium which invaginate between adjacent cysts, resulting in the failure of germ line cysts to acquire the cell envelopes characteristic of region 2b follicles.

Weakly affected germaria continue to produce large, abnormal egg chambers that contain more than 15 germ line cells (Fig. 2E-F). Most of the large follicles appear to be formed by enveloping multiple 16-cell cysts because: (1) they contain 2 or more groups of 15 nurse cells which are temporally coordinated in their development; (2) the nurse cell to oocyte ratio remains 15:1; (3) similar follicles produced by females bearing certain *brn* and *top* alleles retain normal cystocyte interconnections, based on phalloidin staining (Goode et al., 1992). In the multiple cyst follicles, one oocyte is usually located at the posterior of the compound chamber while the other(s) are located medially or anteriorly. A few older chambers lacked sufficient supernumerary oocytes to match the number of nurse cells; in others the germ line cells had proliferated irregularly, resembling those produced by tumorous mutants (King, 1970). The significance of these chambers is unknown, but their proportion was lower under conditions more restrictive to *hh* expression.

Severely affected ovarioles were also produced that lack any budded egg chambers. In some ovarioles, a small germarium is attached to a giant chamber which contains all the cysts that would normally be separated into egg chambers along the length of the ovariole (Fig. 2G). In other ovarioles, the terminal filament and cap cells are attached directly to a giant sac containing many germ line cells (Fig. 2H). The giant chambers usually contain a single posterior oocyte, several more anterior oocytes, but not enough in total to match the large number of

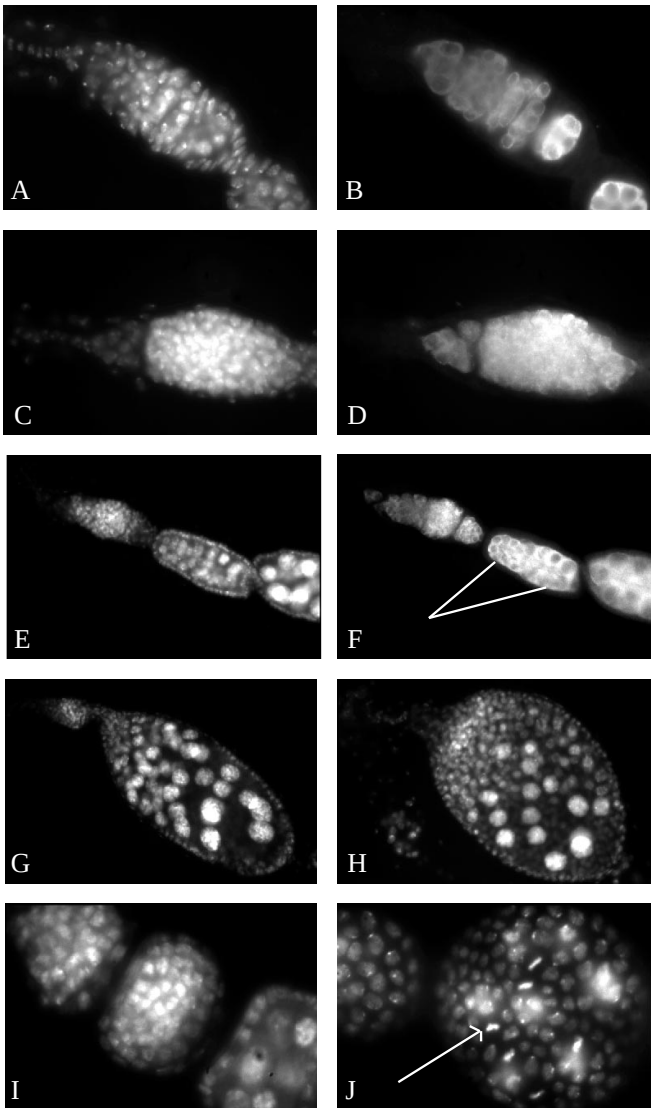

Fig. 2. Reduced *hh* activity blocks cyst encapsulation. Ovarioles from wild-type (A,B) and hh^{9K}/hh^{GS1} flies grown at the restrictive temperature (C-J) stained with DAPI or anti-Vasa antiserum (B,D,F). All the hh^{ts} ovarioles are from females kept at 28°C for 6 days, except H where treatment was at 29°C for 8 days. Somatic cell invagination is reduced in hh^{ts} germaria and germ line cysts fail to separate (C-H). More than one cyst is often incorporated into egg chamber that do form (E,F). The arrows indicate two cysts within a single chamber. Strongly affected ovarioles may retain (G) or lack (H) a clearly demarcated germarium, and contain multiple developing 16-cell cysts (E-H) or small germ line cells similar to ovarian tumors (C,D,I). Mitotic follicle cells are more frequent in stage 2-5 hh^{ts} egg chambers (J). The arrow indicates a somatic cell mitotic figure.

nurse cells present. Clusters of small undifferentiated germ line cells are sometimes seen. The proportion of severely affected ovarioles increased under more severe restrictive conditions. Following 6 days at 28°C, 49% of ovarioles had compound egg chambers containing two to four germ line cysts (Fig. 2E-F) while chamber formation failed completely in only 5% (Fig. 2G). In contrast, after 8 days at 29°C, only 21% of ovarioles contained compound egg chambers while 72% of the ovarioles analyzed were more severely affected.

Similar effects on egg chamber budding have been previously observed following disruption of several neurogenic genes (Ruohola et al., 1991; Xu et al., 1992; Goode et al., 1992; Cummins and Cronmiller, 1994). However, follicle cells from hh^{ts} flies at the restrictive temperature exhibit a further abnormality in post germarial chambers that was not reported by these authors. In young egg chambers with either normal or multiple complements of germ line cells, an increased number of mitotic figures is sometimes present in the follicle cell layer. A maximum of 10 mitotic figures were seen in a single stage 4-5 chamber (Fig. 2J); yet, in wild-type chambers follicle cell mitotic figures are less frequent, and no more than two per chamber are usually observed.

Ectopic *hh* expression in the ovary generates an excess of somatic cells

To further analyze the role played by *hh* in egg chamber formation, flies containing a construct in which the *hh* gene is under the control of the heat shock promoter hsp70 (Ingham, 1993) were used to look at the effects of ectopic *hh* expression. Flies carrying a single copy of the *hs-hh* construct were heat shocked for 1 hour twice a day for 3 days and their ovaries were dissected immediately and 3 days after the end of the final heat shock (flies in which *hh* was ectopically expressed using this construct will be referred to subsequently as *hs-hh* flies). Ectopic expression of *hh* in this way results in a dramatic increase in the number of somatic cells contributing to each ovariole with excess somatic cell accumulating between the egg chambers (Fig. 3). A much smaller increase (2-3 fold) in the average number of stalk cells was also seen following heat treatment of control animals.

Germaria containing *hs-hh* constructs were transplanted into non-transgenic hosts to determine if somatic cell proliferation required *hh* expression in ovarian cells. Flies containing two copies of the *hs-hh* construct were used as donors to maximize the amount of ectopic *hh* produced. Host flies containing transplanted germaria were grown at room temperature for 6 days and heat shocked daily for one hour before the transplants were dissected for analysis. The same effects on ovariole morphology were seen in ovarioles from transplanted germaria as those

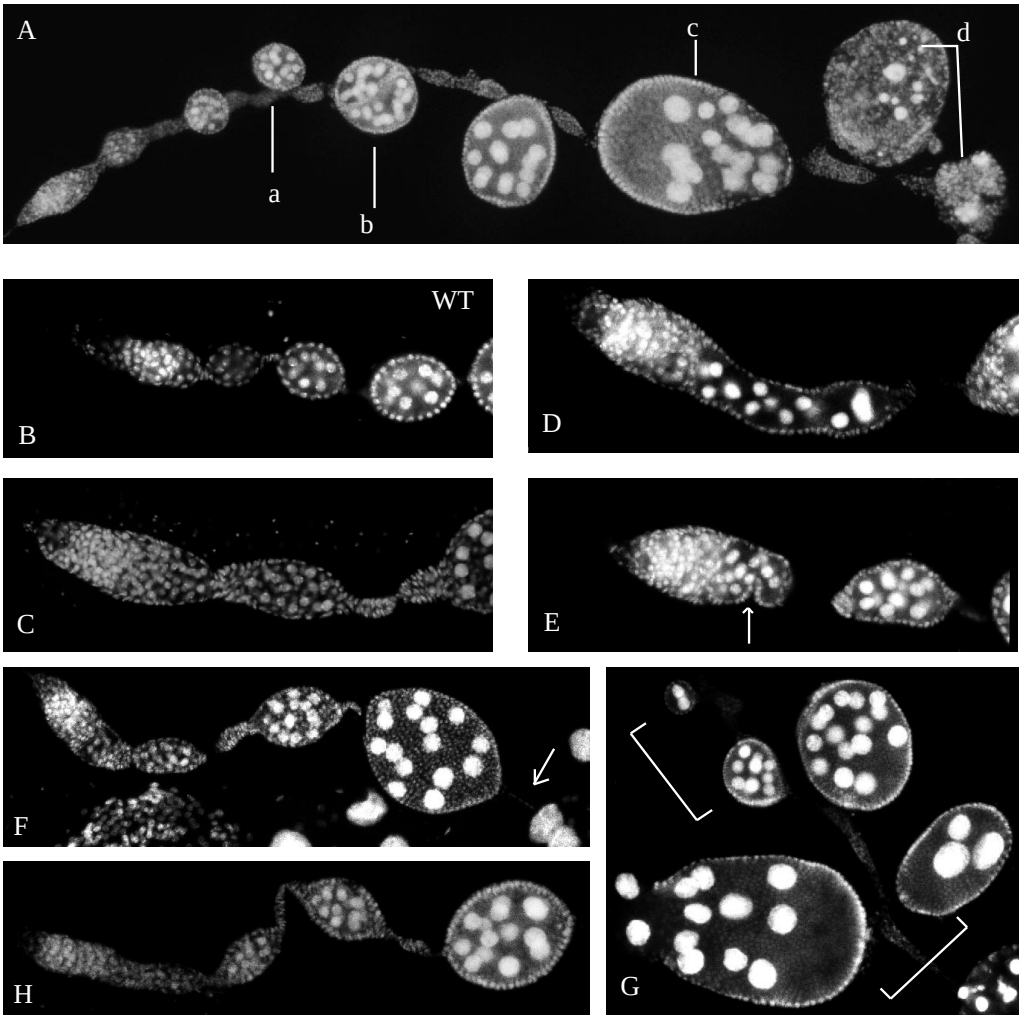

Fig. 3. Ectopic *hh* expression increases somatic cell proliferation in the germarium. Ovarioles and egg chambers stained with DAPI are shown from wild-type (B), *hs-hh* germaria transplanted into hosts and analyzed after 6 days of development and heat treatment (A,G), and *hs-hh* ovarioles analyzed after 3 days of heat treatment (C,D,E,F). Chambers in hs-hh ovarioles are separated by giant stalks (A) and display additional abnormalities. Egg chambers may bud from a cable of somatic cells (a), and their polarity is sometimes disrupted (a-b) or reversed (c). Distal chambers eventually degenerate (d). Frequently, cysts are split between two consecutive egg chambers (G, brackets). B-E are at the same magnification. Germaria are frequently swollen (compare C and B), and budding is delayed as revealed by the size of the developing nurse cells (D,E). Chambers may become elongated (D) and split by invaginating cells (E, arrow). (F) The location of expanded and normal stalks (arrow) indicates that excess proliferation occurs in the germarium (see text). (H) An ovariole from a female in which the N-terminal portion of Hh was expressed for 3 days.

heat shocked in situ, indicating that ectopic expression of *hh* within the ovariole is sufficient to cause these defects (Fig. 3A).

The morphology of the ovarioles containing an excess of somatic cells suggested that somatic cells in region 2-3 of the germarium were likely targets of ectopic *hh* (Fig. 3C-E). The terminal filament and cap cells of germaria in *hs-hh* ovarioles remain neatly stacked, and region 1 looks fairly normal. However, region 2 is swollen and densely packed with cells, and individual disc-shaped cysts typical of region 2b in wild type germaria are difficult to distinguish. Region 3 often contains more than one cyst as well as large numbers of somatic cells. Cysts in region 3 appear to be delayed in budding from the germarium since they often have polyploid nurse cells and oocyte nuclei characteristic of later stages (Fig. 3C-E). These region 3 egg chambers are frequently misshapen; they are often very elongated (Fig. 3D) and occasionally appear to be constricted by the inward migration of somatic cells between cystocytes rather than around the entire cyst (Fig. 3E, arrow). Constrictions like this probably give rise to the split cysts seen in *hs-hh* ovarioles where 2 adjacent chambers together contain 15 nurse cells of apparently identical age (Fig 3G, brackets).

The chambers that have separated from the germarium in *hs-hh* ovarioles are connected by giant stalks which contain from 25 to over 130 cells, in contrast to the 5-6 cells between egg chambers in wild type ovarioles (Fig. 3A,C,F,G). Tubes of follicle cells appear to pinch off both ends of the young egg chambers just posterior of the germarium (Fig. 3C,F). These tubes of cells then constitute the giant stalks which separate the egg chambers in *hs-hh* ovarioles. In more severely affected ovarioles a continuous cable of somatic cells lies along one side of the egg chambers (Fig. 3A). In some cases egg chambers appear to bud from a mass of excess somatic cells. Stalk size and the incidence of split chambers rises with increasing copy number of the *hs-hh* construct and longer exposure to heat shock. This suggests that the somatic cells in the germarium respond in a dosage dependent fashion to increased *hh* activity.

The time course of giant stalk formation and recovery was analyzed to determine which somatic cells were stimulated to proliferate by *hh* mis-expression. After 3 days of the heat shock regime previtellogenic chambers are separated by very large stalks, but stage 10 and older chambers remain connected by stalks of normal length (Fig. 3F). In wild-type flies grown at 24ºC it takes about three days for a germ line cyst to move from region 1 to region 3 of the germarium, and a further three days for a stage 1 egg chamber to reach stage 10 (King, 1970; Lin and Spradling, 1993). As ectopic *hh* expression does not significantly affect the rate of chamber production (see below) the time course of giant stalk formation suggests that giant stalks arise between chambers that are in the germarium at the time of ectopic *hh* expression. When ovaries were allowed to recover at 24ºC for three days following heat shock treatments, the largest stalks were usually seen between stage 5 and older egg chambers, as predicted. However, even after an additional seven days of recovery, stalks were still somewhat larger than normal, suggesting that full recovery requires an extensive period. These experiments imply that *hh* has an effect on somatic cell proliferation during chamber formation and budding, but not on follicle cells surrounding post-germarium egg chambers.

hh activity stimulates the proliferation of pre-follicle cells rather than stalk cells

In order to determine whether the giant interfollicular stalks observed in *hs-hh* ovarioles result from the hyperplasia of true stalk cells, an enhancer trap line (‘stalk line’) that specifically labels stalk cells was used (Fig. 4). In wild-type ovaries, expression of the stalk line switches on in the somatic cells interdigitating between region 2 and the budding region 3 follicle (Fig. 4A, arrow). In *hs-hh* ovarioles, stalk line expression is absent from region 3 (Fig. 4B, arrow) and from the giant stalks formed between young chambers (Fig. 4D).

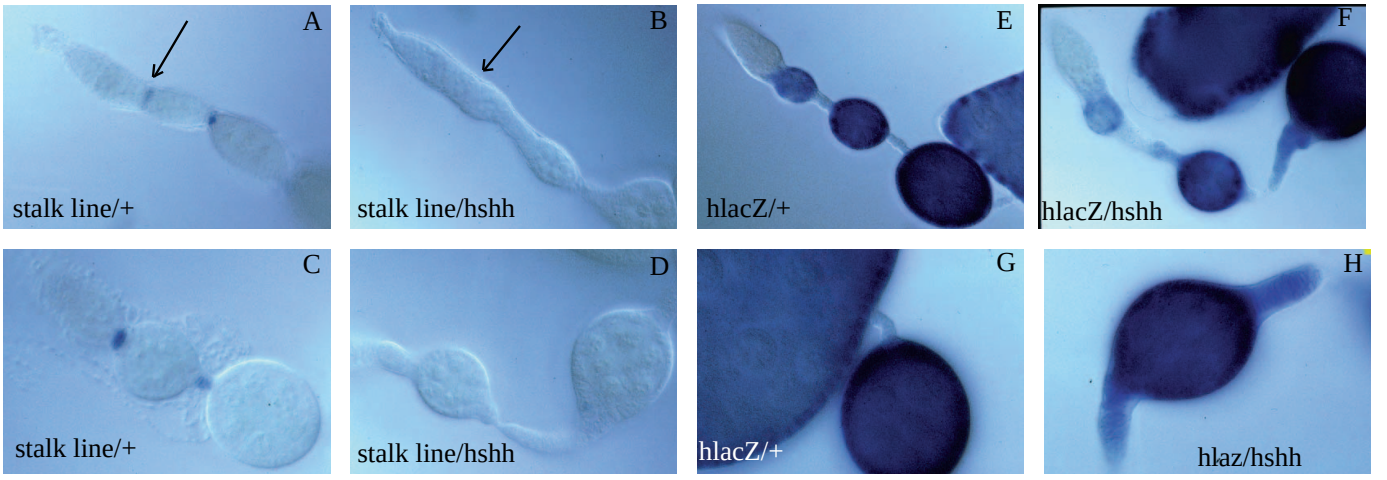

Fig. 4. Cells in giant interfollicular stalks express follicle cell but not stalk cell markers. The expression of a stalk-specific enhancer trap line *l(3)1344* and *hairyacZ* (*hlacZ*) line in ovarioles from wild-type flies and flies containing one copy of the *hs-hh* construct subjected to the standard heat shock regime were analyzed by X-gal staining. In the wild-type germarium (A,C), the stalk cell marker is confined to interfollicular stalk cells and their precursors in region 3. In *hs-hh* ovarioles (B,D), the stalk specific marker is not expressed. The arrows in A and B indicate the region 2b region 3 border where stalk line expression normally switches on. In wild-type ovarioles (E,G), the follicle cell marker *hlacZ* is initially expressed in region 3 of the germarium. Expression is then up regulated in follicle cells and down regulated in stalk cells. In *hs-hh* ovarioles (F,H) *hlacZ* is expressed in most giant stalk cells. Expression is weak or absent in cells near the middle of the stalks.

The absence of stalk line expression argues that the giant stalks do not result from hyperplasia of true stalk cells.

To determine if the excess cells have the characteristics of follicle cells we examined the effect of ectopic *hh* expression on ovaries marked with the follicle cell specific marker *hairylacZ* (*hlacZ*). In wild-type ovarioles, *hlacZ* is activated initially in the somatic cells in region 3 of the germarium. Expression is then up-regulated in the follicle cells of stage 2 egg chambers and down regulated in the stalk cells (Fig. 4E,G). In *hs-hh* ovarioles *hlacZ* is activated in region 3 of the germarium and is highly expressed in follicle cells as in wild-type controls. Strong staining is frequently seen throughout the giant stalks, although a small number of non-staining cells are often present in the middle of these stalks (Fig. 4F,H). These results support the notion that excess follicle cells accumulate between egg chambers to form the giant stalks. Despite its presence in these cells, *hairy* does not itself appear to be playing a direct role since ectopic expression of *h* using a *hs-h* construct (Ish-Horowicz and Pinchin, 1987) did not affect somatic cell number (data not shown).

Ectopic expression of *hh* affects the positioning of the oocyte within egg chambers

In addition to causing the overproduction of follicle cells, ectopic expression of *hh* also affects the position of the oocyte within egg chambers formed during the period of ectopic expression. Following 3 days recovery from the standard heat shock regime a significant proportion of *hs-hh* ovarioles contain egg chambers with oocytes at the side or in the middle of the egg chamber. These defects are most clearly seen in stage 5 and older egg chambers by the position of the GV and later of yolk droplets (Figs 3A, 5A). Nurse cells are normally distributed in a gradient of increasing ploidy from anterior to posterior within the egg chamber. In *hs-hh* chambers with abnormally positioned oocytes, the distribution of nurse cell ploidy corresponds to the new oocyte position such that the largest nurse cell nuclei are those closest to the oocyte.

Ectopic expression of *hh* affects polar cell differentiation

The polarity of the egg chamber and oocyte is thought to depend on polar cell pairs and other specialized follicle cells at the anterior and posterior ends of the egg chamber (Ruohola et al., 1991). Polar cells are specified shortly after cyst encapsulation, prior to egg chamber budding (Margolis and Spradling, 1995). It is possible that the polarity defects seen in *hs-hh* ovarioles result from the early mis-specification of follicle cell identities that are important for the establishment

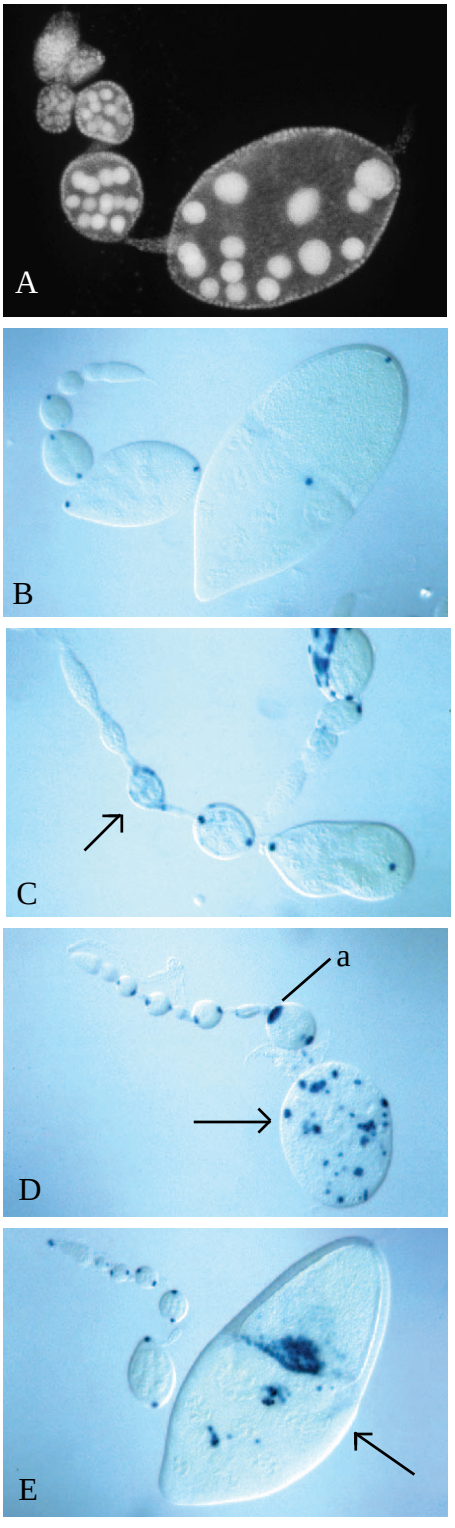

Fig. 5. Ectopic *hh* expression causes defects in egg chamber polarity and produces extra polar cells. (A) Polarity defects in a *hs-hh* ovariole after 3 days of recovery from a 3-day heat shock regime. In the oldest egg chamber, the oocyte is mis-positioned, the largest nurse cells are not located at the posterior, and the normal pear shape of the chamber is altered (DAPI staining). (B-E) Extra polar cell production in *hs-hh*. Ovarioles containing polar cell-specific enhancer traps are stained with X-Gal to reveal the location of polar cells in ovarioles from wild type (B) or following 3 days ectopic *hh* expression followed by recovery for 0 days (C), 3 days (D), or 6 days (E). The presence of many extra cells expressing the polar cell marker is evident in chambers that were in region 2 of the germarium during the time of ectopic *hh* expression (arrows C,D,E). During recovery, the youngest affected egg chambers exhibit polar cell hyperplasia that is restricted to the poles (D), with the anterior pole being most severely affected (arrow a). Low levels of expression of the polar cell marker are also seen in the giant stalks separating the affected egg chambers (C,D). In the stage 10B egg chamber shown after 6 days of recovery (E), the extra marked cells have migrated to the anterior of the oocyte.

of egg chamber polarity. To look for abnormalities in follicle cell identity that result from *hh* mis-expression, two polar cell-specific enhancer trap insertions, *PZ80* and *8360*, were analyzed in *hs-hh* ovarioles and in wild-type controls that had been exposed to the standard heat shock regime and dissected immediately, 3 days and 6 days after the end of heat shock.

In wild-type ovarioles both enhancer traps specifically mark pairs of polar cells at the egg chamber termini, starting at stage 2 (Fig. 5B). Occasionally, in these young chambers, three anterior or posterior follicle cells were marked. In *hs-hh* ovarioles dissected immediately after a 3-day heat shock, the polar cell markers were ectopically activated in many cells throughout the follicle cell layer of the youngest post-germarium egg chambers, usually in only one or two chambers following the germarium (Fig. 5C). Staining was observed in a large proportion of the follicle cells surrounding these chambers, and some staining was also observed in the somatic cells accumulating between the chambers. Older chambers, stage 7 onwards, generally had normal polar cells. This suggests that the egg chambers in which polar cell specification was affected were probably in region 2 at the start of ectopic *hh* expression.

This conclusion was further supported by examining identically treated flies that were allowed to recover at 24°C for 3 days before dissection. In their ovarioles, stage 5-10 egg chambers that would still have been in region 2 at the end of the heat shock regime were covered with multiple patches of cells expressing the polar cell marker (Fig. 5D). In young chambers, the expression of the marker had returned to normal. One or occasionally two chambers lying between the normal and strongly affected chambers displayed polar cell hyperplasia that was limited to the poles of the egg chamber, with the anterior pole being more severely affected than the posterior (Fig. 5D**,** arrow). These chambers were presumably exposed to declining levels of *hh* activity while forming in the germarium. After a further 3 days of recovery at 24°C, hyperplasia of polar cells was absent even though stalks between egg chambers were still slightly larger than in wild type (Fig. 5E). A few stage 10 chambers were present that appeared to have developed from chambers with anterior hyperplasia. In these chambers, the excess polar cells migrate through the nurse cells like normal anterior polar cells (Fig. 5E). These kinetics of polar cell hypertrophy reinforce the conclusion that ectopic expression of *hh* effects polar cell specification in region 2 of the germarium.

Ectopic expression of *hh* does not affect the rate of germ line stem cell division

Ablation experiments suggest that a signal from terminal filament cells negatively controls the division rate of the adjacent germ line stem cells (Lin and Spradling, 1993). As *hh* is both expressed in the terminal filament cells and encodes a known signalling molecule it seemed plausible that the rate regulating signal might be encoded by *hh*. This model predicts that the relative excess of somatic cells following increased *hh* activity would result from reduced germ cell production rather than from increased somatic cell proliferation. The rate of germ line cyst production from individual germaria can be quantitated following transplantation into sterile host females. To test this hypothesis, *hs-hh* germaria were transplanted, subjected to daily heat shocks, and the number of egg chambers produced over a 6-day period compared to transplanted ry^{506} control germaria treated in the same manner. No significant differences were observed. ry^{506} germaria produced 11.2±0.8 chambers, while hs-*hh* germaria produced 11.8±3.3 chambers. Thus, ectopic expression of *hh* under these conditions does not have a significant effect on the rate of germ line stem cell division.

The effects on somatic cell proliferation are mediated by the N-terminal portion of the Hh protein

The *Drosophila hh* gene encodes a 46×10^3 M_r protein with a signal peptide sequence. Signal sequence cleavage and autoproteolytic cleavage releases a 19×10^3 M_r N-terminal fragment and a 25×10^3 M_r carboxy fragment (Lee et al., 1994). In the systems where the N-terminal fragment has been tested it has been shown to contain all the activity of the full length protein except the autoproteolytic activity (Fan et al., 1995; Feitz et al., 1995; Marti et al., 1995; Porter et al., 1995; Roelink et al., 1995; reviewed by Concordet and Ingham, 1995).

To test whether this is also the case in the *Drosophila* ovary, flies containing a construct in which the N-terminal fragment of Hh (N-Hh) is linked to a UAS sequence were crossed to flies containing a heat inducible *Gal4* construct. Ubiquitous expression of N-Hh was achieved by exposing the progeny containing one copy of each construct to the standard heat shock regime. Ectopic expression of the N-Hh results in a very similar phenotype to the ectopic expression of the full length protein (Fig 3H). This indicates that as in the other systems the cleaved N-terminal fragment of the Hh protein appears to have the same activity as the full length protein.

DISCUSSION

hh expression in apical somatic cells may regulate egg chamber formation

Our experiments showed that in the adult *Drosophila* ovary *hh* is expressed in a small group of cells at the apical end of each germarium (Fig. 1). Hh protein could only be detected in the terminal filament cells, and 2-6 adjacent somatic cells that we call ‘cap cells’. The basal 1-2 terminal filament cells directly contact the underlying germ line stem cells near the location of specialized regions of germ cell cytoplasm known as ‘spectrosomes’ that are rich in the membrane skeleton proteins Hts and α-spectrin (Lin et al., 1994; Lin and Spradling, 1995). Cap cell expression was frequently asymmetric with respect to the anterior-posterior axis of the ovariole, and appeared to vary between germaria. These expression patterns suggested possible roles for *hh* in germ cell proliferation and indicated that departures from anterior/posterior axial symmetry arise much earlier than stage 9 when the dorsal-ventral axis of the egg chamber is established (Gonzalez-Reyes et al., 1995; Roth et al., 1995).

Studies of *hh* function revealed that apical *hh*-expressing cells act primarily as a signalling center controlling the proliferation and behavior of other somatic cells within the germarium. Reducing *hh* activity in apical cells affects somatic cells located in region 2 of the germarium, a distance of 2-5 cell diameters (see Fig. 1A). Furthermore, mis-expressing *hh* in more posterior cells, using heat-inducible copies of the gene, drastically disrupts egg chamber formation but has little effect

on pre-existing egg chambers. The rate of germ line stem cell division is not affected by altering *hh* expression. However, it remains possible that apical *hh* signalling requires ongoing germ cell division and serves to coordinate somatic cell behavior with germ line cyst production. Consistent with the idea of a coordinating function, terminal filament cells differentiate in late third instar larval ovaries (King, 1970; Godt and Laski, 1995) and begin to express *hh* as germaria first start to form (Fig. 1H).

hh activity in cells at the tip of the germarium is required for the continued envelopment, budding and polarization of egg chambers. *hh* diffusing from the germarium tip may directly affect the behavior of more posterior cells, or it may act indirectly by activating secondary signalling molecules such as *wg* and *dpp*. These alternatives are presently under investigation (Z. F. et al., unpublished). At least two processes that contribute to egg chamber formation might be primary targets of *hh* signalling. First, somatic cells must proliferate at a sufficient rate to provide enough cells to surround each new cyst and to separate nascent follicles from the germarium. Second, multiple somatic cell sub-types, including polar cells, main-body follicle cells and stalk cells and their precursors must be specified. Our experiments suggest that *hh* signalling influences both these processes.

hh regulates the proliferation of somatic cells in the germarium

hh activity clearly affects somatic cell proliferation following ectopic expression. Egg chambers derived from germaria expressing *hh* ectopically are separated by giant stalks containing up to 20 times more cells than wild-type stalks (Fig. 3). In principle, either a decreased rate of germ line cyst production or an increased rate of somatic cell proliferation could explain the observed increases in the soma:germ line ratio. However, no decrease in germ line cyst production was seen in transplanted *hs-hh* germaria. Consequently, the excess somatic cells must result from increased somatic cell proliferation in these ovarioles.

Normally, follicle cells divide approximately every 9.6 hours following their birth in region 2 of the germarium, and grow at a similar rate in chambers up to stage 6 (Margolis and Spradling, 1995). However, only somatic cells within the germarium appear to be susceptible to *hh*-induced over-proliferation. Under normal circumstances, budded egg chambers may not be exposed to *hh*-mediated signals, and they may lack the necessary machinery for this response. Within the germarium, we could not determine whether all or only some of the somatic cells responded to *hh* activity. However, the supernumerary cells behaved and expressed markers characteristic of follicle cells rather than stalk cells, excluding the possibility that the giant stalks result from hyperplasia of the normal stalk cell population.

While ectopic *hh* expression increased somatic cell proliferation in the germarium, this does not reveal whether *hh* fulfills a similar requirement in normal ovaries. When *hh* expression was reduced, somatic cells failed to invaginate and encapsulate germ line cysts and fewer somatic cells were present in the ovariole. As increased somatic cell death was not observed, and there was no evidence for the accumulation of cells that had proliferated but were unable to invaginate, it is reasonable to propose that the reduction in somatic cell number in hh^{ts} ovarioles results from reduced cell proliferation. The effects of *hh* on cell proliferation are dosage dependent. Both an increase in the copy number of the *hs-hh* construct and an extended period of heat shock cause a more extensive accumulation of excess somatic cells. Stronger reductions in *hh* activity caused fewer cysts to acquire follicle cell layers. This suggests that under normal conditions the level of *hh* controls the number of pre-follicle cells available to encapsulate germ line cysts. Dosage dependent effects of *hh* have also been observed in the developing imaginal discs (Ingham and Feitz, 1995).

The production of excess follicle cells can explain the structure of *hs-hh* ovarioles

The stimulation of cell proliferation by ectopic *hh* can explain the morphological defects we observed. It has been suggested that invaginating pre-follicle cells have a high affinity for the surface of newly formed 16-cell germ line cysts (Goode et al., 1992). Expressing *hh* throughout the germarium appears to result in increased numbers of these 'germ line sticky' invaginating cells relative to the number of available cysts. The pressure to maximize surface contacts between these excess pre-follicle cells and cysts might produce the elongated and split germ line cysts in *hs-hh* germaria (Fig. 3).

The germ line-associated somatic cells form an epithelial monolayer of follicle cells as the budding takes place. This involves the formation of specialized cell-cell junctions (Piefer et al., 1993). The greater strength of these epithelial interactions relative to the strength of soma-germ line adhesion might be responsible for the loss of contact of excess follicle cells with germ line cysts in *hs-hh* ovarioles that results in the formation of giant stalks. Furthermore, in the most severely affected ovarioles, the strong tendency of maturing follicle cells to arrange themselves as an epithelium could explain how chambers surrounded by a monolayer are eventually able to bud out of a vast excess of somatic cells. This epithelium forming property of follicle cells does not appear to be affected by *hh* activity.

hh affects polar cell specification and stalk cell formation

hh is likely to influence the specification of at least two cell types, polar cells and stalk cells. Polar cells appear ectopically throughout egg chambers exposed to elevated levels of *hh* during their formation (Fig. 5C-D). The altered location and greatly increased numbers of polar cells suggests that *hh* expression promotes this cell fate. In contrast to polar cells, stalk cells were decreased in number or absent altogether when *hh* activity was reduced or expressed ectopically. Stalk cells are likely to differentiate under the influence of intercellular interactions during the normal budding process (Ruohola et al., 1991). The absence of stalk cells in *hs-hh* ovarioles may reflect abnormal cellular interactions within the context of so many extra somatic cells, rather than a direct affect of *hh* on stalk cell specification. Ovarioles with reduced *hh* levels may not contain a sufficient number of available cells for budding to take place, thereby precluding stalk cell formation.

The effects of *hh* activity on cell proliferation and specification are probably responsible for the observed alterations in cyst structure and polarization. In *hs-hh* ovarioles, abnormally positioned germ line cells are seen in egg chambers formed during the period of ectopic *hh* expression, but not in pre-

existing egg chambers (Fig. 5A). Abnormal cyst orientation is readily recognized by the absence of the oocyte from its normal position at the posterior end of the egg chamber. The effects of *hh* on cyst orientation might be a secondary consequence of the disruptions in follicle cell number and identity. If polar cells, which are known to be specified early, play a role in cyst orientation in region 2b, then the presence of excess and abnormally positioned polar cells in these chambers might disrupt normal polarization.

hh may interact with neurogenic genes during egg chamber formation

The ovariolar defects observed in ovaries with reduced levels of *hh* are very similar to those caused by reductions in *N, Delta*, and *da*, and in certain *brn, top* double mutants (Ruohola et al,. 1991; Goode et al., 1992; Xu et al., 1992; Bender et al., 1993; Cummings and Cronmiller, 1994). *hh* and the neurogenic genes may be required for a common pathway during egg chamber formation. However, their expression patterns differ greatly. *N, Dl* and *da* are expressed in the somatic cells in which they are required; N and Dl proteins accumulate at high levels in invaginating cells beginning in region 2b (Ruohola et al,. 1991; Xu et al., 1992; Bender et al., 1993), while Da protein is strongly detected in region 3 cells (Cummings and Cronmiller, 1994). *Brn* function is required in germ line cells for signals mediated by the torpedo EGF-receptor (Goode et al., 1992). In contrast, *hh* expression is confined to a small group of cells located at the tip of the ovariole. It therefore appears that *hh* acts upstream of the neurogenic genes, at least temporally.

It may be that region 2b somatic cells require inputs from several different signalling systems prior to initiating their inward migration. These would include a germ line signal mediated by the DER tyrosine kinase receptor, and a *hh*-dependent signal sent from the terminal region of the ovariole. The neurogenic genes might mediate further communication between the somatic cells that process these inputs, initiate the decision to invaginate, and coordinate the complex movements and cell fate decisions that are ultimately required to generate a newly budded egg chamber.

Egg chamber production involves the specification of several somatic cell types, including polar cells and stalk cells. Reduced activity of *N* and *Dl* causes the production of an increased number of polar cells at the ends of egg chambers, as well as the loss of the stalk cell fate (Ruohola et al., 1991). We propose that *hh* signalling specifies the proper anterior-posterior orientation of polar cell precursors in region 2b, while cell-cell interactions mediated by *N* and *Dl* ensure that only two cells maintain this fate, in an analogous manner to the action of these genes other systems (Ruohola et al., 1991). This model would explain why excess polar cell produced under conditions of reduced *N* activity remain clustered at the poles of the egg chamber, while the excess polar cells induced by generalized *hh* expression are spread in small groups throughout the follicular epithelium. The specification of a limited number of polar cells at the poles of the egg chambers may be a prerequisite for normal stalk cell specification and/or budding. If so, some of the common defects observed in these processes in *hh* depleted ovarioles and neurogenic mutants may result from the disruption of polar cell specification by this pathway.

As well as their requirement for cyst encapsulation, the *brn* gene (Goode et al., 1992), and possibly *N* (Xu et al., 1992) are also required to maintain a continuous follicle cell monolayer surrounding the egg chamber as it develops. In contrast, disrupting *hh* expression did not produce discontinuities in the follicle cell monolayer. Rather, an increased number of mitotic follicle cells was seen in postgermarial egg chambers in hht^{ts} ovarioles. This suggests that transient gaps resulting from incomplete encapsulation may be filled by extra divisions. The ability of epithelia lacking *hh* but not those deficient in *brn* or *N* to repair discontinuities probably reflects a distinct ongoing requirement for the neurogenic genes after follicle cells have formed an epithelial monolayer.

Regulation of cell proliferation and cell identity by *hh* may be a feature of diverse systems

We have shown that *hh* regulates the proliferation as well as the identity of somatic cells in the germarium. Ectopic expression of the N-terminal fragment and the full-length Hh protein produced a very similar phenotype (Fig. 6). This is consistent with the findings in other systems that *hh* activity is confined to the N-terminal portion of the protein (reviewed by Concordet and Ingham, 1995).

The role of *hh* in specifying cell identity has been well documented (for reviews, see Johnson and Tabin, 1995; Ingham, 1995). Recent studies of *hh* function in *Drosophila* imaginal discs and in vertebrates are also starting to reveal a role of *hh* in regulating cell proliferation. For example, in *Drosophila* wing imaginal discs, the reduction of *hh* activity results in a drastic reduction in the size of both anterior and posterior compartments, while the ectopic expression of *hh* causes expansion of the anterior compartment with disrupted patterning in this compartment (Basler and Struhl, 1994; Capdevila and Gurrero, 1994; Tabata and Kornberg, 1994; Ingham and Feitz, 1995). Similarly, a requirement of *hh* for cell proliferation has been proposed in the vertebrate limb bud where *hh* is involved both in determining the polarity of the limb and regulating its outgrowth (reviewed by Johnson and Tabin, 1995). In *Drosophila* eye discs, however, ectopic expression of *hh* in the mitotically active region anterior to the morphogenetic furrow causes the cessation of cell division and formation of an ectopic morphogenetic furrow (reviewed by Heberlein and Moses, 1995). This implies an opposite role of *hh* in controlling the proliferation state of these cells. A potentially similar role of *hh* may also occur during mammalian CNS development, where *hh* specifies motor neuron differentiation, which is also associated with the cessation of mitosis (reviewed by Ingham, 1995). Thus, *hh* may be involved in the coordination of cell proliferation and specification in diverse systems. Our results illustrate that, in *Drosophila* ovary *hh* is not only involved in the specification of early somatic cell fate, but is also clearly required for the proliferation of these cells. As markers of early somatic cell identity become available, the *Drosophila* ovary will become an even more effective model for further analyzing the role of *hh* in regulating both cell proliferation and differentiation.

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