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The neurogenic genes *egghead* and *brainiac* define a novel signaling pathway essential for epithelial morphogenesis during *Drosophila* oogenesis

Scott Goode[1,§], Michael Melnick[1,*], Tze-Bin Chou[1,†] and Norbert Perrimon[2]

[1]Department of Genetics, and [2]Howard Hughes Medical Institute, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, USA

*Present address: Laboratory of Cell Signaling, New England BioLabs, 32 Tozer Road, Beverly, MA 01915, USA
†Present address: Department of Zoology, National Taiwan University, Taipei, Taiwan, Republic of China
§Address correspondence to Scott Goode by e-mail: goode@rascal.med.harvard.edu

SUMMARY

***Notch* (*N*) and other neurogenic genes have been implicated in two fundamental processes, lateral specification of cell fates, and epithelial development. Previous studies have suggested that the neurogenic gene *brainiac* (*brn*) is specifically required for epithelial development (Goode, S., Morgan, M., Liang, Y-P. and Mahowald, A. P. (1996). *Dev. Biol.* 178, 35-50). In this report we show that *egghead* (*egh*), a gene with phenotypes identical to *brn*, encodes for a novel, putative secreted or transmembrane protein. We describe the role of *egh* and *brn* germline function in the morphogenesis of the follicular epithelium from the time it is born through the time that it migrates towards the oocyte late in oogenesis.**

By comparing the function of germline *egh* and *brn* to *N* during oogenesis, we have obtained direct evidence for the involvement of Notch in maintenance of the follicle cell epithelium, and the specificity of *brn* and *egh* in epithelial development during oogenesis. The most striking phenotype observed for all three genes is a loss of apical-basal polarity and accumulation of follicular epithelial cells in multiple layers around the oocyte. The spatiotemporal onset of this adenoma-like phenotype correlates with the differential accumulation of *egh* transcripts in the oocyte at stage 4 of oogenesis. In contrast to *N*, we find that *brn* and *egh* are essential for the organization, but not specification, of stalk and polar cells.

The expression patterns and functional requirements of *brn*, *egh*, and *N* lead us to propose that these genes mediate follicular morphogenesis by regulating germline-follicle cell adhesion. This proposal offers explanations for (1) the involvement of *egh* and *brn* in *N*-mediated epithelial development, but not lateral specification, (2) why *brn* and *egh* embryonic neurogenic phenotypes are not as severe as *N* phenotypes, and (3) how *egh* and *brn* influence Egfr-mediated processes. The correlation between the differential expression of *egh* in the oocyte and the differential requirement for *brn*, *egh*, and *N* in maintaining the follicular epithelium around the oocyte, suggests that Egghead is a critical component of a differential oocyte-follicle cell adhesive system.

Key words: Notch, epithelium, cell interaction, morphogenesis, neurogenic genes, oogenesis, signaling pathway, *Drosophila*

INTRODUCTION

Epithelia are one of the universal architectural units used in the development and maintenance of animal form. Epithelial cells are arranged in sheets that have distinct apical-basal polarity. The different shapes of organs develop from the regulated molding of epithelial structure via tissue-specific morphogenetic programs. These programs govern the complex integration of epithelial cell behaviors, including division, rearrangement, shape, shape changes and the recruitment or loss of cells from the epithelium.

As a model to understand epithelial morphogenesis, we are analyzing the simple monolayer follicular epithelium that develops in concert with the differentiating germline during *Drosophila* oogenesis. The follicular epithelium has developmental, morphological, and molecular properties of vertebrate epithelia (Fig. 1). Further, *Drosophila* oogenesis is amenable to genetic analysis, making it an attractive model for identifying molecules involved in epithelium formation. In this report we present the molecular and functional characterization of the gene *egghead* (*egh*), which encodes a novel molecule that is likely to be crucial for the adhesion of the follicular epithelium to germ cells throughout oogenesis.

In both invertebrates and vertebrates, cell adhesion molecules have been shown to play an essential role in epithelial organization (reviewed by Gumbiner et al., 1992). For example, in vertebrates and *Drosophila*, E-cadherin cell adhesion molecules are required for the formation and maintenance of epithelial structure, particularly in epithelia undergoing complex morphogenetic rearrangements (reviewed by Takeichi, 1995; Tepass et al., 1996; Uemura et al., 1996). Key mediators of cadherin function are cytoplasmic α- and β-catenin, which bind to both the cytoplasmic domain of cadherins and to f-actin filaments, providing a crucial link between adhesion and regulation of cell shapes and movements.

A second class of cell surface factors essential for epithelial development are receptor tyrosine kinases and their ligands. For example, vertebrate hepatocyte growth factor and p190^{MET} receptor tyrosine kinase induce chemotactic migration, proliferation, and differentiation of epithelial cells, and elicit epithelial tubule morphogenesis (Naldini et al., 1991). Epidermal growth factor receptor (Egfr) mutant mice suffer from multi-organ failure due to widespread impairment of epithelial development (Miettinen et al., 1995), and mice with reduced levels of one Egfr ligand, transforming growth factor α (TGFα), share some of these developmental abnormalities (Luetteke et al., 1993; Mann et al., 1993). Egfr and *grk* TGFα mutant *Drosophila* show similar abnormalities. Disruption of Egfr in follicle cells or TGFα signals in germ cells results in loss of dorsal-ventral patterned array of follicle cell shapes and impaired development of the follicular epithelium (Schüpbach, 1987; Goode et al., 1996). The discontinuities of the monolayer follicular epithelium observed in TGFα or Egfr mutant *Drosophila* are reminiscent of the extreme thinning observed in stratified epithelia of newborn Egfr mutant mice (Goode et al., 1996; Miettinen et al., 1995).

A third class of molecules essential for epithelial development are encoded by the neurogenic genes. Although intensively studied for their function in lateral specification (reviewed by Artavanis-Tsakonas et al., 1995), the *Drosophila* neurogenic genes *Notch* (*N*), *Delta*, *neuralized*, and *Enhancer of split* are also essential for the development of epithelial characteristics in embryonic tissues in which no obvious cell fate choices are in question. For example, loss of neurogenic function causes invaginating midgut rudiments to prematurely loose their epithelial characteristics during gastrulation; following gastrulation, the transition of midgut rudiments to form the midgut epithelium does not occur (Hartenstein et al., 1992). In *Xenopus*, expression of an activated form of the *N* homolog *Xotch* expands epithelial neural tube tissue and abolishes mesenchymal neural crest cells (Coffman et al., 1993). During *Drosophila* oogenesis, *N* and *Dl* are crucial for regulating bipotential cell fate decisions between follicle cells (Ruohola et al., 1991; Xu et al., 1992), but their role in the morphogenesis of the follicular epithelium remains to be investigated.

Previous studies have suggested that the neurogenic gene *brainiac* (*brn*) plays a specific role in epithelial development. Although the early embryonic *brn* phenotype is similar to that of mutants of the neurogenic genes, *brn* does not appear to be required for several bipotential cell fate decisions that utilize the *N* signaling cassette (Goode et al., 1996). *brn* encodes a novel, putative secreted protein that is required in the germline for establishing the follicular epithelium and for determining its dorsal-ventral polarity (Goode et al., 1992; Goode et al., 1996). *brn* mutations show synergistic genetic interactions with TGFα and Egfr mutations at each step in which these genes are essential in the morphogenesis of the follicular epithelium (see above), consistent with an essential requirement for *brn* in epithelial development. In this study, we directly characterize the function of *brn*, and an additional gene with identical phenotypes, *egh*, in epithelial development during oogenesis, and compare the requirement of *brn* and *egh* with the function of *N* in this process.

We find that *egh* encodes a novel, putative secreted or transmembrane protein. *egh* expression in the germline correlates with morphogenesis of the follicular epithelium throughout oogenesis. In the absence of *egh* or *brn* function, formation of the follicular epithelium is less efficient, frequently resulting in a failure to individuate germline cysts. These genes are also essential for maintaining the epithelial characteristics of the follicular epithelium once it is formed. When *brn* or *egh* germline function is disrupted, follicle cells lose their apical-basal polarity and accumulate in multiple layers, particularly around the oocyte. Further, *egh* and *brn* are essential for the efficient migration and maintenance of border cells and main body epithelial cells when they move towards the oocyte during the late phases of oogenesis. These results suggest that *egh* and *brn* are germline components of a differential oocyte-follicle cell adhesive system.

Strikingly, we find that neither *brn* nor *egh* is essential for regulating the bipotential cell fate decisions that *N* has previously been implicated in during oogenesis. In contrast, we find that in addition to its role in cell fate specification, *N* is also essential for maintaining the epithelial characteristics of the follicular epithelium. Since *brn egh* double mutant animals display phenotypes no more severe than either mutant alone, we suggest that these genes function in a novel signaling pathway specific for epithelial morphogenesis. We propose that Brainiac and Egghead, originating from the germline, collaborate with Notch on the apical surface of follicle cells to mediate germline-follicle cell adhesion, and discuss our results in this context to explain how these genes may influence Egfr mediated processes.

MATERIALS AND METHODS

Genetics

All *egh* mutations used in this study are described in Table 1. *brn* mutations are described by Goode et al. (1992, 1996). Most *brn* and *egh* mutations were recombined with FRT^{101}, and germline clone (GLC) analysis was completed as described by Chou and Perrimon (1992). For *egh* mutations *egh^{62d18}* and *egh^{65h5}*, GLC analysis was completed using X-irradiation as described by Perrimon et al. (1989). Except for experiments involving conditional mutations, all experiments were completed at 25°C on standard *Drosophila* medium. *N^{ts1}* phenotypes were analyzed following a shift of females to 32°C for 36 hours. *ovo^{D1rs1}* is described by Oliver et al. (1987).

Histology and microscopy

For all stainings except with αPS1 integrin sera, ovaries were fixed for 20 minutes in 4% ultrapure, methanol-free, EM-grade formaldehyde (Polysciences, Inc.) in PBS, rinsed in PBS, then teased apart into ovarioles. F-actin stainings were completed as described by Goode et al. (1996). Antibody and propidium iodide staining were completed as described by Orsulic and Peifer (1994). α-PS1-integrin stainings were completed on unfixed, non-permeabilized tissue as described by Brower et al. (1984).

For double stainings with 4.1 and phosphotyrosine (PT) sera, tissue was first incubated with monoclonal 4.1 (Fehon et al., 1994) and a rhodamine-conjugated secondary, then incubated with anti-PT monoclonal antibody directly conjugated to a fluorescein fluorophore (US Biochemical) at a concentration of 1:100. All tissues were analyzed with a conventional epifluorescence, or a Biorad laser scanning confocal microscope, attached to a Zeiss Axiophot microscope.

Embryonic Hoyer's mounts, sections, and HRP-antibody stainings were completed as described by Goode et al. (1992).

Follicle cell division patterns

Mitotic follicle cells were scored using f-actin and DNA stained

Table 1. *egh* alleles

Allele	Mutagen	Lethal phase*	References
9PP4	EMS	pharate adult	Perrimon et al., 1989
DF944	EMS	pharate adult	Lefevre and Watkins, 1986
62d18	EMS	pharate adult	Robbins, 1983
63e4	EMS	pupal	Robbins, 1983
65h5	EMS	pupal	Robbins, 1983
GA107	X-ray	pupal	Lefevre, 1981

*Experiments were conducted at 25°C. All alleles fail to complement each other and have similar maternal effect neurogenic and ovarian phenotypes.

tissues. Confocal microscopy was used to obtain a complete z-series at 1 μm intervals for each egg chamber analyzed. Since egg chambers have an ellipsoid shape, each z-section is an ellipse (Fig. 1), with A/P length of each ellipse decreasing away from the egg chamber's center. To determine the position of dividing cells from the posterior pole, the position of each cell from the posterior in a given z-section, as well as the length of the z-section, was established. All measurements were obtained at 1 μm resolution using a scale bar tool (Biorad software package).

The center of each egg chamber is the z-section(s) with the longest A/P axis. For dividing cells in this plane, the distance from the posterior is equal to the measured distance. For dividing cells above and below the center plane, the distance from the posterior must be adjusted to take account of the ellipsoid's curvature. The following formula was used: actual distance from posterior = measured distance + (A/P length of center z-section – A/P length of the z-section including the dividing cell)/2.

Molecular biology

Two *egh* cDNAs were cloned from the 0- to 4-hour embryonic cDNA library described by Brown and Kafatos (1988) using a 2.5 kb *Eco*RI fragment as probe. These cDNAs and the 18XS genomic fragment were sequenced by standard methods and the sequence analyzed using the GCG package (Genetics Computer Group, Wisconsin, 1994). Kyte-Doolittle hydropathy analysis was completed with the DNASTAR program.

A transformation construct with the *egh* open reading frame behind the heat shock promoter was made by introducing *Bgl*II and *Xba*I restriction sites at the 5′ and 3′ ends of the *egh* ORF, respectively, using PCR. This product was then cloned into pCaSpeR-hs (Thummel et al., 1988). P-element transformations were completed by standard protocols (Rubin and Spradling, 1982).

In situ hybridization

In situ hybridizations were completed as described by Goode et al. (1996), using an antisense RNA probe generated from the T7 promoter in *egh* cDNA 4e (described in the Results).

RESULTS

egghead phenotypes are similar to *brainiac*

To account for both the neurogenic and ovarian defects associated with *zw4* mutations (Table 1; Robbins, 1983; Perrimon et al., 1989), we have renamed the gene *egghead* (*egh*). Females with germ line clones (GLCs) of *egh* mutations have phenotypes similar to *brn*. They lay a few eggs with fused dorsal appendages and the embryos have an expanded nervous system (this study; Perrimon et al., 1989; Goode et al., 1992). Only a few eggs are laid because early oogenesis is blocked due to the development of egg chambers containing multiple oocyte-nurse cell complexes.

egh and *brn* do not appear to be required for regulating germ cell division

Wild-type (WT) oogenesis is described in detail in Fig. 1. Since previous results indicated that *brn* is required in the germline for formation of the follicular epithelium, we characterized markers that would distinguish germ cells from follicle cells, and reveal the epithelial characteristics of the follicular epithelium. Phosphotyrosine (PT) substrates have been shown to be enriched in epithelial cell junctions and sites of cell contact in vertebrate epithelial cells (Maher et al., 1985; Takata and Singer, 1988), and we find a similar distribution of these substrates in follicular epithelial cells (Fig. 1, turquoise). In addition, PT substrates are localized in the cortex of germ cells (this study), and in specialized structures that interconnect germ cells, called ring canals (Robinson et al., 1994). In contrast, membrane protein 4.1 is specifically localized in follicle cells (Fig. 1).

GLCs of mutations in either *brn* or *egh* produce egg chambers containing more than one oocyte-nurse cell complex (Fig. 2A,B; Table 2). To rule out the possibility that *egh* mutant egg chambers result from supernumerary germ cell divisions, we stained egg chambers with PT and analyzed the ring canal pattern of oocytes as previously described for *brn* (Goode et al., 1992). As for WT and *brn*, oocytes in *egh* egg chambers have only 4 ring canals (Fig. 2B), indicating that *egh* does not inhibit germ cell divisions.

A second class of egg chambers observed less frequently in *brn* and *egh* mutant ovaries contain one oocyte with less than 15 nurse cells, or contain more than one oocyte, but without the normal complement of nurse cells (Fig. 2C; Table 2). These egg chambers may result from a deficit of germ cell division, but this does not seem likely since *brn* and *egh* transcripts do

Table 2. Supernumerary oocyte-nurse cell complexes

Genotype	Number scored	no OO*	1 OO	1 OO, detached†	1 OO, anterior	2 OO	3 OO	≥ 4 OO
$brn^{fs.107}$	>500	0	98	0	0	2	0	0
$brn^{l.6P6}$	256	6	60	NS‡	NS	25	9	0
egh^{DF944}	322	4	62 (68§)	2	4	24	4	0
egh^{GA107}	319	3	57 (68)	8	3	23	5	1
egh^{DF944} $brn^{fs.107}$	130	4	67	NS	NS	23	5	1
egh^{GA107} $brn^{fs.107}$	205	3	64	NS	NS	26	7	1
egh^{DF944} $brn^{l.6P6}$	125	4	64	NS	NS	25	6	1

The numbers in the table are percentages based on the total number of egg chambers scored (number scored). *OO, oocyte. †Oocyte detached or misplaced, but not found at anterior pole. ‡NS, not scored. §The number in parenthesis is the actual number with 1 oocyte, which accounts for the percentages in the next two columns.

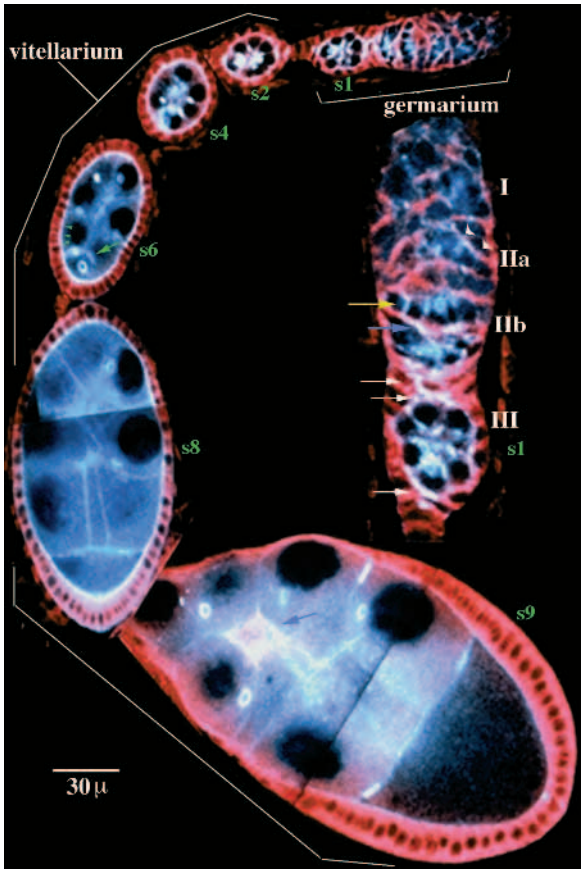


Fig. 1. Morphogenesis of the wild-type follicular epithelium is organized along the anterior-posterior axis of egg chambers starting early in oogenesis. The figure shows a composite of confocal images from an ovariole of an adult *Drosophila* female. The ovariole is double-stained with sera to phosphotyrosine (PT), which recognizes both germ and follicle cell tissue (white/turquoise), and protein 4.1 (Fehon et al., 1994), which is specifically localized in follicle cells (pink/red). The exclusion of PT and 4.1 staining from germ and follicle cell nuclei and late stage oocytes is evident as false black color.

As seen in the stage 6 (s6) follicle, the germline is composed of 15 polyploid nurse cells located anteriorly (large nuclei, only 6 revealed in this section), and a diploid oocyte at the posterior (arrow). PT substrates, but not 4.1, are localized to the cortex of all 16 germ cells (turquoise), and in specialized donut-shaped structures, called ring canals (obvious on the oocyte and some nurse cells), that interconnect the germ cells to form a syncitium. 4.1 is specifically localized in follicle cells (red). PT colocalizes with actin on the apical surface of follicle cells that abut the germ line, in zonulae adherens that interconnect apical membranes of follicle cells (green arrowheads), and in lateral follicle cell membranes.

Follicle cells (red) are associated with dividing germ cells (turquoise) as cysts are produced in germarium region I (cf. Mahowald and Strassheim, 1970). Once cysts have matured to 16 cells at the posterior of region I, follicle cells ensheathe the egg chamber at the beginning of region IIa (see also Fig. 3A). PT begins to accumulate in follicle cells at the posterior of cysts (white, arrowheads; this study), and in ring canals (Robinson et al., 1994). PT staining in posterior follicle cells is the first manifestation of anterior-posterior polarity, and is dramatic by the time egg chambers span the width of the germarium in region IIb (blue arrow), when ring canals are aligned at the anterior of the egg chamber (yellow arrow), and the oocyte begins to differentiate.

Following these primary organizational events along the A/P axis, germ cells elongate first posteriorly, then anteriorly, to produce a spherical s1 egg chamber. The oocyte is positioned at the posterior. Simultaneous follicle cell division produces an epithelium of approximately 80 cells. Apical accumulation of PT substrates in all follicle cells marks the establishment of the follicular epithelium, while strong PT staining persists in polar and intercalating follicle cells that will form a stalk to connect successive egg chambers (white arrow). The egg chamber is ready to begin growth in the vitellarium.

The spheroidal s2 egg chamber has a simple, monolayer, cuboidal follicular epithelium. The egg chamber elongates to an ellipsoid between s3 and s4. At s6, when follicle cells stop dividing, the epithelium has grown to approximately 1200 cells. Between s6 and s8, oocyte-associated nurse cells protract anteriorly, as the oocyte flattens across posterior follicle cells. By s8, columnar follicle cells associated with the oocyte show a clear posterior to anterior height gradient. At s9, most main-body epithelial cells migrate towards the oocyte in concert with a few mesenchymal-like, PT-rich, border cells that break from the epithelium to move through the center of the egg chamber (blue arrow).

not appear to be expressed in germ cells when they are dividing (Figs 1, 11A,B; Goode et al., 1996). Further, *hu-li tai shao* (*hts*) mutations specifically block germ cell divisions, but in contrast to *brn* and *egh* GLC females, *hts* females never produce egg chambers with more than one oocyte-nurse cell complex (Yue and Spradling, 1992), indicating that completion of germ cell division is not requisite for individuating germ cell cysts. Rather, egg chambers from *egh* and *brn* GLCs that contain too few germ cells probably result from splitting of cysts in the germarium, since these egg chambers are frequently arranged in complementary patterns, with sister cells of comparable size (Fig. 2C). We provide direct evidence for this hypothesis in the next section.

egh and *brn* are required for formation of the follicular epithelium

The analysis of *brn* and *egh* GLC egg chambers presented in the previous section indicated that these genes are not required for regulating germ cell divisions. To determine the primary defect in these mutants, we directly analyzed the process of egg chamber formation using confocal microscopy to visualize the distribution of α-spectrin (Byers et al., 1987). α-spectrin has previously been localized in a branching structure that runs throughout germ cell cysts, called the fusome (Lin et al., 1994; Fig. 3A). In addition, we find that α-spectrin recognizes follicle cell processes as follicle cells begin to surround and envelop newly formed 16-cell cysts (Fig. 3A). Follicle cell processes extend from cell bodies of prefollicle cells that line the germarium at the junction of regions IIa/b. These cells may be epithelial stem cells, or stem cell daughters, as stem cells have been localized to the IIa/b junction (Margolis and Spradling, 1995; Fig. 3A). Germ cells are absolutely essential for the extension of follicle cell processes and formation of a monolayer follicular epithelium, since these processes fail to occur in germaria that lack germ cells (Fig. 3A,B).

Likewise, in *brn* and *egh* GLC animals, follicle cells fail to extend processes, leading to a failure of germ cells to rearrange like WT cyst cells in region IIa (Fig. 3C). Thus, as previously suggested, *brn* and *egh* appear to disrupt a germline to follicle

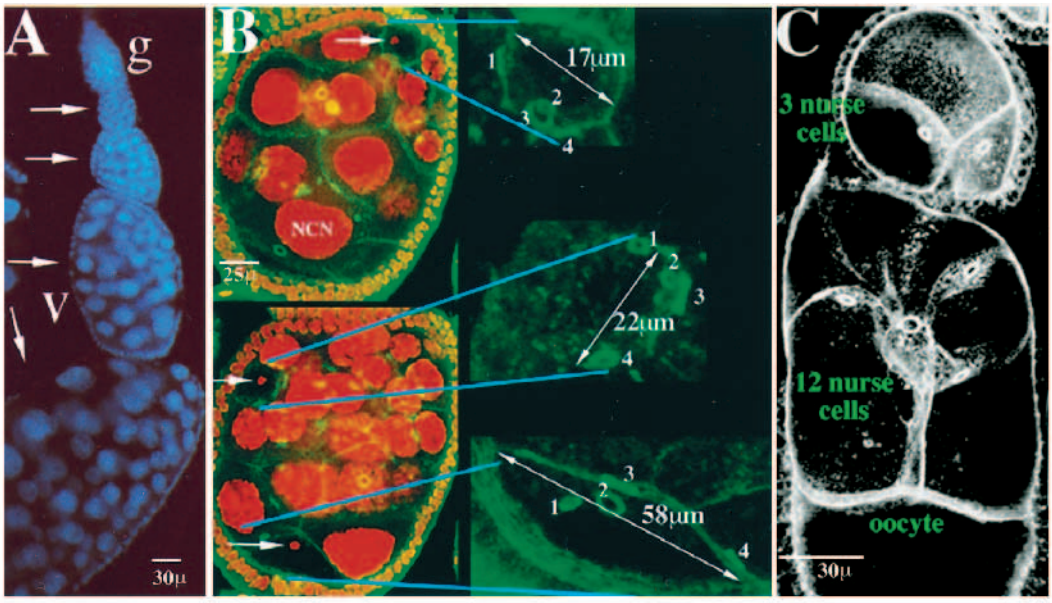


Fig. 2. *brn* and *egh* do not regulate germ cell division. (A-C) Analysis germ cells in *egh* mutant egg chambers. (A) An ovariole from an egh^{GA107}GLC female stained with DAPI reveals nuclei of germ and follicle cells. Each egg chamber in the vitellarium (arrows, v) has supernumerary nurse cells (large nuclei) and oocytes (not visible); (g) germarium. (B) An egg chamber from an egh^{GA107} female stained with propidium iodide and PT to reveal nuclei (red), and ring canals (green). On the left, two sections through the same egg chamber reveal all three oocytes (diploid nuclei, arrows). A complete z-series through each oocyte is shown on the right. As for WT, each oocyte contains four ring canals (numbered). (C) Complementing egg chambers from an egh^{DF944}GLC female stained with PT. The posterior egg chamber has one oocyte and 12 nurse cells, while the anterior egg chamber contains 3 complementary nurse cells, giving the WT oocyte to nurse cell ratio of 1:15.

cell signaling process essential for individuating germ cell cysts. Another possibility is that *brn* and *egh* are required in germ cells for cyst cell rearrangement, and that cyst cell reorganization drives follicle cell extension. We do not feel this is likely since (1) mutant germ cells undergo a large degree of normal differentiation (Figs. 2, 4), and (2) both genes are required in the germ line at later stages for the maintenance of the follicular epithelium (described below). Our results do not rule out the possibility that cyst cell reorganization and follicle cell extension is a cooperative process requiring continual signaling between these tissues.

In addition to a failure to extend processes, follicle cells in *brn* or *egh* GLC germaria display a number of unusual behaviors. Follicle cells sometimes appear organized as an epithelial sheet within the germarium (Fig. 3F), perhaps resulting from the aberrant movement of pre-follicle epithelial cells. This interpretation could explain how egg chambers containing multiple oocyte-nurse cell complexes emerge from the germarium. Further, follicle cells sometimes appear to have migrated in between germ cells, not around them, perhaps splitting them in two (Fig. 3D,E). Alternatively, inappropriate interactions between follicle cells and germ cells may stretch cysts, pulling them apart (Fig. 3C). Both of these mechanisms may contribute to the production of complementary cysts (Fig. 2C).

egh and *brn* are required for maintaining the follicular epithelium

After egg chambers exit the germarium, *brn* and *egh* are required in the germline for maintaining the follicular epithelium. In *brn* and *egh* GLC egg chambers, follicle cells frequently lose their cuboidal or columnar shape and exit the epithelium around the oocyte and its adjacent nurse cells (Table 3, Fig. 4A-D). Follicle cells have clearly lost polarity, since α-spectrin is localized on lateral membranes of follicular cells in WT egg chambers (Fig. 4A), whereas in *brn* and *egh* GLC egg chambers, α-spectrin accumulates ectopically or around the circumference of follicle cells (Fig. 4B). A surface view of mutant epithelia reveals that whereas WT follicle cells have hexagonal shapes arranged as an isotropic honeycomb array, follicle cells in *brn* and *egh* GLC egg chambers can be quadrate or pentagonal and arranged anisotropically (Fig. 4C). Further, follicle cells which are exiting the follicular epithelium have a rounded, apolar, mesenchymal-like shape compared to the polygonal architecture of follicle cells in the main body follicular epithelium (Fig. 4C).

Exiting follicle cells are observed in *brn* and *egh* GLC egg chambers containing a single oocyte and 15 nurse cells, suggesting that the phenotype is not linked to a problem in epithelium formation (see previous section). This hypothesis is further supported by determination of the earliest stage at which follicle cells exit the epithelium. Follicle cells exit the

Table 3. Stage specificity of dislocated cells

		% egg chambers with dislocated cells At indicated developmental stage*				
Genotype	Number	2-3	4-5	6-7	8-9	Total
$brn^{fs.107}$ (25°C)	126	0	0	1†	12	13
$brn^{fs.107}$ (18°C)	139	0	0	21†	28	49
$brn^{l.6P6}$	99	0	15	19	51	85
egh^{62d18}	103	0	12	13	39	64
egh^{GA107}	85	0	20	26	37	83
egh^{DF944} $brn^{fs.107}$	215	0	19	24	42	85

*Only egg chambers having a single nurse cell-oocyte complex were scored. For $brn^{fs.107}$, this was essentially every egg chamber (see text). For all other mutations, dislocated cells associated with egg chambers having multiple oocyte-nurse cell complexes are not included in this table. An egg chamber was scored positive if at least one dislocated cell was observed.

†All of these egg chambers were s7 (none were s6).

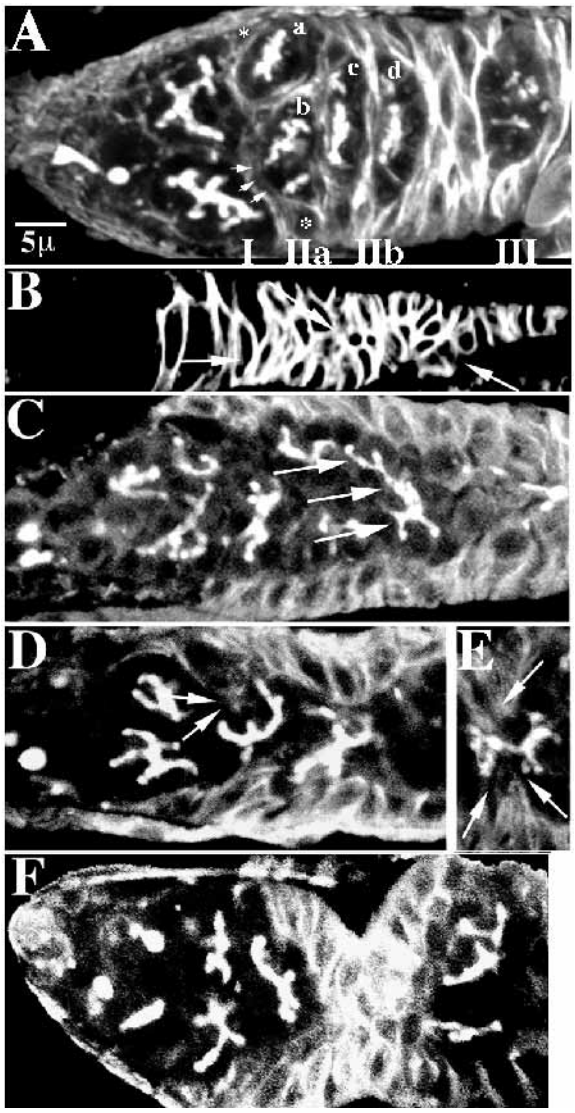


Fig. 3. *egh* and *brn* are required for the formation of the follicular epithelium. (A-F) α-spectrin staining and confocal microscopy of WT, *brn*, and *egh* germaria. (A) WT germarium. Germarial regions are numbered as in Mahowald and Strassheim (1970). In region I of the germarium two 16-cell cysts are recognized by the accumulation of α-spectrin in a branching structure that runs throughout the cysts, called a fusome (Lin et al., 1994). In regions IIa and IIb, 4 egg chambers are lettered by consecutive stage of maturation. Egg chamber formation is initiated at the junction of region I/IIa when the germline cyst is surrounded by follicle cell processes (cyst a, arrows) that extend from the cell bodies of presumptive stem follicle cells along the sides of the germarium (*). Ensheathing follicle cells rearrange cysts from a pancake-like structure to a ball-like configuration. The cyst then begins to spread across the width of the germarium as ensheathing follicle cells become more pronounced (cyst b). In region IIb, the cyst has completed the flattening process. (B) A germ cell-less germarium from a female homozygous for the null mutation ovo^{D1rs1}. In ovo^{D1rs1} females, germ cells do not populate the germarium (Oliver et al., 1987). Follicle cells do not extend processes or achieve a simple cuboidal epithelial phenotype characteristic of s1 egg chambers. (C) egh^{GA107}GLC germarium. Follicle cells fail to extend processes, yet line the walls of the germarium, leading to a failure to organize cysts as in WT. Interaction with follicle cells on the walls of the germarium appears to cause some cysts to be stretched (arrows). (D,E) $brn^{l.6P6}$GLC germarium. Follicle cell processes sometimes extend between germ cells (arrows), presumably resulting in split cysts like that shown in (Fig. 5C). (F) Germarium from an egh^{9PP4} female. Follicle cells arranged as a sheet encapsulate germ cells, pinching an egg chamber containing three cysts.

epithelium no earlier than stage 4 (s4) for the strongest *brn* and *egh* alleles (Table 3), when *egh* transcripts differentially accumulate in the oocyte (Fig. 11C), 16 hours after the epithelium has formed in the germarium.

To characterize in greater detail the function of *egh* and *brn* in maintaining the monolayer follicular epithelium, we analyzed their involvement in inhibiting follicle cell divisions (described below), in determining follicle cell fates (described below), and in maintaining the apical membrane domain of follicle cells. We focused on the apical membrane domain of follicle cells since *brn* and *egh* are required in the germline, which abuts the apical surface of the follicular epithelium. We analyzed $brn^{fs.107}$ because relatively few follicle cells exit the follicular epithelium, but not before they have stopped dividing at s7 (Table 3). This allowed us to detect morphogenetic defects associated with single follicle cells, independent of the morphogenetic transitions of follicle cell division. In s7 $brn^{fs.107}$ egg chambers, apical PT staining frequently appears to be decreased, or absent, in some follicle cells adjacent to the oocyte (Fig. 4G). Observation of the lateral membrane domain in these defective cells using α-spectrin suggested that follicle cells can be distinguished according to the degree to which they have exited the epithelium. Follicle cells that have left the epithelium may have completely lost apical PT staining, or residual staining may not be distributed apically, confirming a loss of polarity and orientation to the germ line (Fig. 4G).

Exited follicle cells do retain, or regain, some orientation to the germline, since by vitellogenesis they secrete vitelline membrane towards the oocyte (not shown). Further, PT and actin, which colocalize in the apical membrane domain follicle cells in WT egg chambers (not shown), are localized apically, yet disorganized, in exited follicle cells (Fig. 4H). Thus, *brn* and *egh* do not appear to be absolutely required for maintaining apical-basal polarity in follicle cells, suggesting that redundant mechanisms may provide polarizing information.

Role of *brn* and *egh* in inhibiting follicle cell division

Since follicle cells accumulate in multiple layers around the oocyte of mutant egg chambers, we sought to determine if follicle cell divisions occur more frequently in this region of the epithelium. We compared numbers of mitotic follicle cells as a function of position along the anterior-posterior axis of WT and egh^{GA107} GLC egg chambers. We found approximately 80% more mitotic follicle cells around the oocyte of mutant egg chambers compared to WT, correlating with the dramatic propensity of follicle cells to exit in this region (Figs. 4B, 5D). This analysis does not distinguish between two alternatives for how *brn* and *egh* might inhibit follicle cell division: (1) these genes might be required for inhibiting follicle cell division as well as maintaining the polarity of follicle cells, or (2) these genes might only be required for maintaining follicle cell polarity, with increased follicle cell divisions reflecting a

requirement to replace exiting cells. Based on the following observations, we favor the second hypothesis.

As in WT, we never observe follicle cells dividing in s7 to s10 *brn* and *egh* GLC egg chambers, suggesting that these genes do not play a role in prohibiting follicle cell divisions during the late phases of oogenesis. In contrast, *discslarge* does appear to play such a role, since in *discslarge* mutant animals mitotic follicle cells are observed after s6 (S. G., unpublished). Further, in animals harboring the weak mutation $brn^{fs.107}$, follicle cells never exit the epithelium before s7, when follicle cells have normally stopped dividing (Table 3). Likewise, in strong *egh* and *brn* mutant backgrounds follicle cells rarely accumulate in more than a single layer before s7, yet cells can accumulate in multiple layers by s10 (Fig. 4D). The most discernible requirement for *brn* and *egh* in regulating follicle cell divisions correlates with their role in maintaining follicle cell polarity. In *egh* and *brn* GLC egg chambers, follicle cells sometimes divide perpendicular to, instead of within the plane of the follicular epithelium, resulting in at least one sister cell ending up outside the epithelium (Fig. 5A-C).

egh and *brn* are required for induction of dorsal follicle cell fates, but not specification of polar/stalk cell fates

N signals participate in a lateral specification mechanism that regulates the ratio of polar precursor to stalk cells as egg chambers exit the germarium, as well as the ratio of polar to 'flanking cells' during later stages of oogenesis (Ruohola et al., 1991). Using FasIII as a marker for polar cells (Ruohola et al., 1991), and a new marker, α-PS1-integrin, as a marker for stalk cells (Fig. 6A,B), we confirmed the misappropriate development of these cells in N^{ts1} animals (Fig. 6C-E). In contrast, we do not observe supernumerary polar cells in *brn* and *egh* GLC egg chambers (Fig. 6H,J). Further, although stalks separate *brn* and *egh* GLC egg chambers, they are typically disorganized because the cells making up the stalks are rounded compared to WT disc-shaped cells (Fig. 6F,G,I).

brn signals have been shown to be essential in the germline at stages 6-7 of oogenesis for establishing dorsal follicle cells (Goode et al., 1992, 1996), and *egh* is differentially expressed in the oocyte at this time (see below, Fig. 11). As for *brn* animals, we observe that *egh* GLC animals lay eggs with fused dorsal appendages, suggesting a shift of dorsal follicle cell fates to more dorsal-lateral fates (Fig. 7A,B). We confirmed this by analyzing the expression of an enhancer trap inserted in the *kekkon-1* gene (*kek*-1; Musacchio and Perrimon, 1996), which is expressed in a broad dorsal to ventral gradient in dorsal follicle cells at s10 (Fig. 7C). In *egh* GLC egg chambers, *kek-1* expression is significantly eliminated in dorsal-lateral follicle cells, consistent with a shift of ventral-lateral cells to more dorsal positions (Fig. 7D). This alteration in gene expression occurs independently of whether follicle cells have exited the epithelium (not shown).

egh and *brn* are required for concerted border cell-main body epithelium migration

Polar cells at the anterior of the egg chamber are part of a larger group of cells, termed border cells, that break from the main body epithelium (MBE, i.e. that portion of the epithelium that maintains a cuboidal/columnar phenotype and ends up covering the oocyte), become mesenchymal-like, and migrate through the center of the egg chamber at s9 of oogenesis (Fig. 1). *slow border cells* (*slbo*) is specifically expressed in border cells starting at s8 of oogenesis (Fig. 8A; Montell et al., 1992). Although follicle cells at the posterior of *brn* and *egh* mutant egg chambers become mesenchymal-like (Fig. 4C), they do not express *slbo*, indicating that they have not adopted a border cell fate (Fig. 8C).

Instead, analysis of *slbo* expression at s8-s10 in these mutants reveals that the migration of the MBE and border cells is not always concerted. The MBE frequently move ahead of, and completes its migration well before, the migration of the border cells (Fig. 8B,D,E). The shape of the migrating epithelium is sometimes unusual (Figs 3, 8D). Further, border cells frequently split apart before or during their migration (Fig. 8E,F), and sometimes remain with the thin squamous follicle cell population that covers nurse cells (Fig. 8F). Once the MBE has completed its migration, *slbo* expression initiates at the anterior and posterior of the oocyte, but expression appears weaker compared to that in WT (Fig. 8B,F). All of these defects are independent of whether multiple layers of follicle cells accumulate around the posterior of the oocyte, and are consistent with a requirement for *brn*, *egh*, and *N* in regulating the relative epithelial state of the follicular epithelium during a dramatic morphogenetic reorganization.

N is also required for the development and maintenance of the follicular epithelium

The results presented thus far indicate that *egh* and *brn* are essential for the development and maintenance of epithelial structure, a function previously assigned to *N* and other neurogenic genes based on embryonic patterning defects (Hartenstein et al., 1992). Like *brn* and *egh* mutant animals, animals mutant for *N* frequently develop egg chambers containing multiple oocyte-nurse cell complexes (Xu et al., 1992), and *N* is required in the germarium for formation of the follicular epithelium (S. G., data not shown). By s1 of oogenesis, *N* is localized on the apical and lateral surface of all follicular epithelial cells in a pattern indistinguishable from PT and f-actin (Fig. 4E; Xu et al., 1992). We analyzed s5 to 10 N^{ts1} mutant egg chambers and found epithelial defects similar to those described for *brn* and *egh* (Fig. 4F). While Notch is expressed at low levels in the germline, these epithelial defects are not found in *N* GLC animals, indicating that as for its role in specifying stalk cells versus polar cells (Fig. 6), *N* is required in follicle cells for maintenance of the follicular epithelium. Further, N^{ts1} egg chambers having multilayered epithelia may or may not have supernumerary polar cells (described above, not shown), indicating that *N* has separable functions in both cell fate specification and epithelial maintenance (see Discussion).

Neurogenesis in *egh* and *brn* mutant animals

We obtained additional evidence that *egh* and *brn* are not identical to *N* by comparing the neurogenic defects associated with these mutants. Although several *egh* alleles cause lethality as pharate adults (Table 1), like $brn^{l.6P6}$, these animals never have ectopic bristles like other neurogenic mutants (Goode, 1994; not shown). Further, as shown above, N^{ts1} animals develop extreme hyperplasia of polar cells during oogenesis, yet only develop a weak embryonic neurogenic phenotype (not shown). In contrast, *egh* and *brn* do not develop hyperplasia of

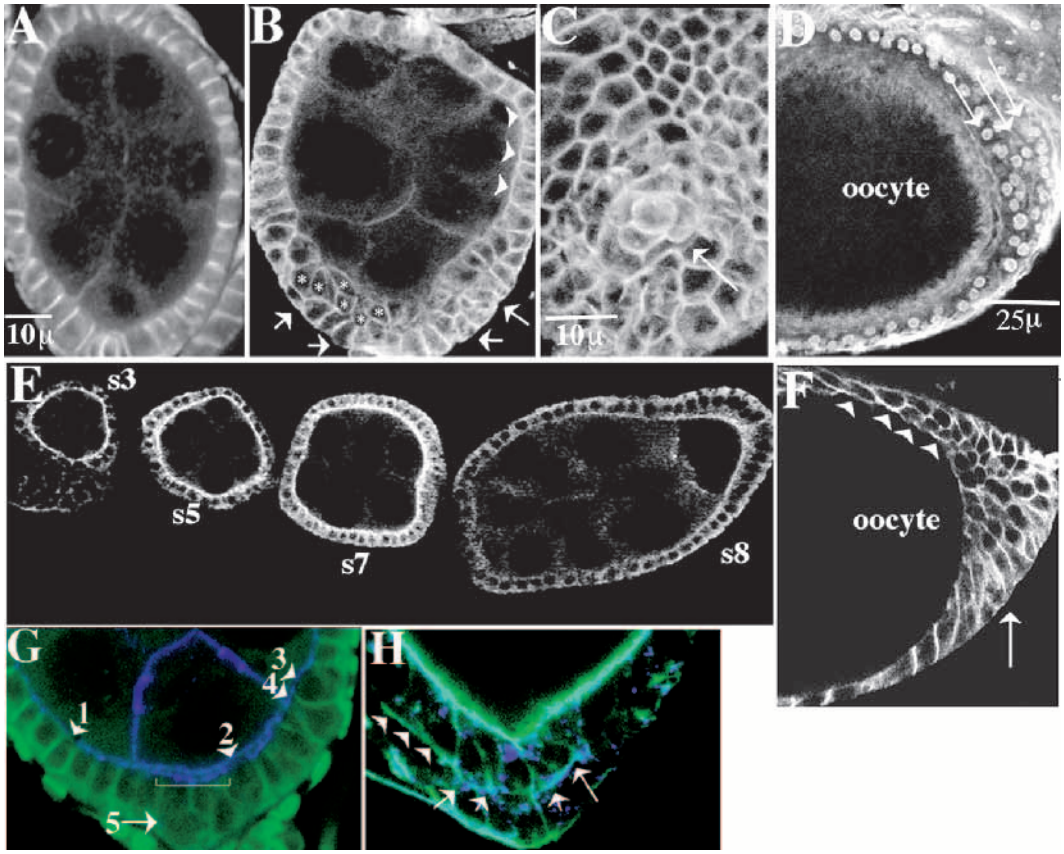


Fig. 4. *egh*, *brn*, and *N* are required for maintenance of the follicular epithelium. (A-D) α-spectrin staining in WT, *egh*, and *brn* mutant egg chambers. (A) A WT s6 egg chamber. α-spectrin accumulates at high levels on lateral follicle cell membranes, and weakly in the cortex of germ cells. (B) A s6 egh^{DF944} GLC egg chamber. α-spectrin staining reveals that follicle cells accumulate in a bilayer around the oocyte and adjacent nurse cells (arrows). α-spectrin staining is reduced in some multi-layered follicle cells (**), perhaps resulting from the spread of α-spectrin around the cellular circumference. In anterior regions of the epithelium, α-spectrin is mislocalized to the apical surface of follicle cells (arrowheads). α-spectrin accumulates at normal levels in germ cell membranes. (C) Surface view of a s5 $brn^{l.6P6}$ egg chamber. Follicle cells within the epithelium are disorganized; follicle cells exiting the monolayer follicular epithelium have an apolar-mesenchymal-like appearance. (D) Posterior of a s10 egh^{GA107} egg chamber simultaneously stained with propidium iodide to recognize follicle cell nuclei and with phalloidin to recognize actin. Follicle cells have accumulated in several layers at the posterior of the oocyte (arrows). (E-F) *N* mutations cause epithelial defects during oogenesis. (E) A WT ovariole stained with Notch serum. Notch is expressed at low levels in germ cells, at intermediate levels in lateral follicle cell membranes, and at high levels on the apical surface of follicle cells. Follicle cell expression is essentially indistinguishable from PT (Fig. 3) and actin (not shown). (F) A s10 N^{ts1} mutant egg chamber stained with α-spectrin antiserum. Follicle cells accumulate in multiple layers at the posterior of the oocyte. (G) s7 $brn^{fs.107}$ egg chambers stained with α-spectrin (green) and PT (blue) sera. Follicle cells do exit the epithelium of $brn^{fs.107}$ egg chambers before s7, after follicle cells have stopped dividing. Single follicle cells with decreased or lost apical PT staining (compare to WT, Fig. 1) are numbered according to the degree to which they appear to have exited the epithelium. Cell 1 retains a WT columnar morphology; cells 2 and 3 are losing shape and have a rounded morphology; cell 4 has lost shape and is exiting the epithelium, cell 5 has exited the epithelium and has very weak PT staining on its lateral surface (arrow). (H) Polarity information retained by exited follicle cells. (H) Posterior of a s8 egh^{DF944} egg chamber stained with sera to PT (blue) and phalloidin (green). While actin and PT substrates colocalize at the presumptive apical surface of many exited follicle cells (light blue, arrows), some cells contain apical actin, but not PT substrates (green, arrowheads).

polar cells (Fig. 6), yet strong alleles of both mutants produce a differentiated maternal effect neurogenic phenotype that is intermediate in strength, indistinguishable from intermediate strength mutants of the neurogenic genes *N*, *Dl*, *E(spl)*, or *neu* (Fig. 9; Goode et al., 1992; Lehmann et al., 1983). The phenotype is characterized by the absence of ventral and cephalic epidermal cells (Fig. 9A-B), a hypertrophy of the nervous system in corresponding territories (Fig. 9D-F), and a failure of the midgut epithelium to achieve a folded pattern like WT (Fig. 9B,E). In contrast, animals mutant for strong *N* alleles have a severe embryonic phenotype characterized by a very small patch of dorsal cuticle and extremely hypertrophied nervous system (Lehmann et al., 1983).

egh brn double mutants display phenotypes no more severe than either mutant alone

One explanation for the difference in the severity of the neurogenic phenotypes between *brn*, *egh*, and *N*, consistent with our results, is that *N* is required for both cell fate specification as well as epithelial development, whereas *brn* and *egh* are strictly required for epithelial development. Alternatively, *egh* and *brn* may be functionally redundant for cell fate specification. We constructed *egh brn* double mutant animals to distinguish these possibilities. We found that double mutant animals do not display ovarian or embryonic phenotypes any more severe than either mutant alone (Fig. 9C, Table 4; data not shown), suggesting that these genes are not functionally redundant, but function in a common pathway.

egh encodes a novel, putative secreted or transmembrane protein

A genomic walk across the *egh* region, and characterization of a rearrangement in the putative *egh* gene, has been reported (Williams et al., 1995). We established that sequences in the

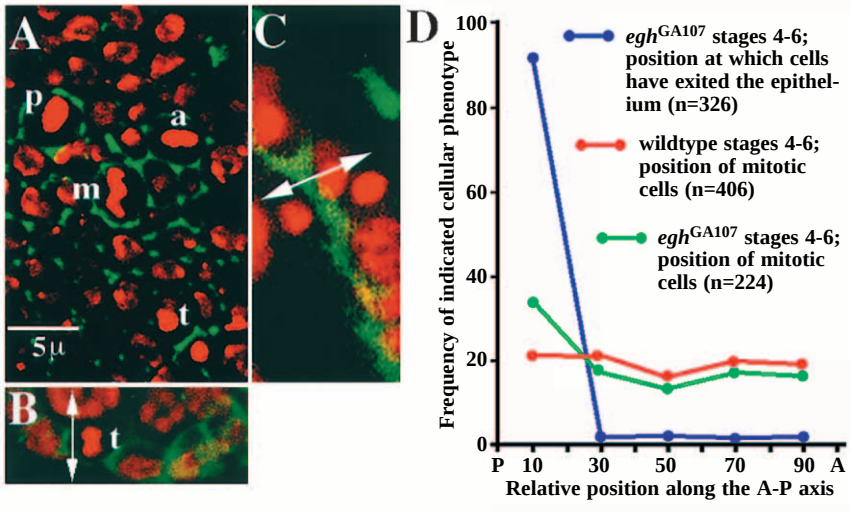


Fig. 5. Follicle cell division in WT and mutant egg chambers. (A) Cross section through a WT follicular epithelium stained with phalloidin (green), which recognizes actin cortices, and with propidium iodide (red), which recognizes follicle cell DNA. Follicle cell divisions take place in the plane of the epithelium. Dividing cells are distinguished by enriched membranous f-actin and heightened propidium iodide staining (due to DNA condensation). Four dividing cells at four cell cycle stages are evident (p, prophase; m, metaphase; a, anaphase; t, telophase). (B) A dividing follicle cell in telophase at the anterior pole of a s4 egg chamber. Curvature of the follicular epithelium at the poles of the egg chamber results in a component of the follicle cell division axis being perpendicular to the plane of the epithelium. (C) Occasionally, in contrast to WT, follicle cells divide perpendicular to the plane of the follicular epithelium in *brn* and *egh* mutant egg chambers. (D) Comparison of cell division frequency along the A/P axis of WT and mutant egg chambers with the position at which cells exit the monolayer follicular epithelium. The points plotted on the graphs were obtained by totaling the number of dividing cells between positions 0-20, 21-40, 41-60, 61-80, and 81-100, and plotting the total for each interval at positions 10, 30, 50, 70, and 90 on the graph, respectively. Red line; cell divisions along the A/P axis of WT s4-6 egg chambers occur at about equal frequency. Green line: the relative frequency of cell divisions in *egh* mutant egg chambers is approximately 80% greater around the oocyte compared to WT. Purple line: almost all follicle cells exit the epithelium of *egh* mutant egg chambers around the oocyte.

putative *egh* region (Fig. 10A) hybridize to a 3.7 kb transcript from 0-2 hour embryos, consistent with this region coding for a maternally expressed gene (not shown). To determine if this transcript corresponds to *egh*, we attempted to rescue *egh* mutations by transformation with a 7 kb genomic fragment, 18XS (Fig. 10A). 18XS rescues *egh* mutations, but does not complement mutations in a gene immediately proximal, *KLP 3A* (Fig. 10A).

We sequenced the genomic rescue fragment 18XS and isolated and sequenced *egh* cDNAs. cDNA 7 appears to be derived from a 2.2 kb *egh* transcript because it starts immediately downstream of a TATA box in genomic sequence and contains a complete ORF with good codon usage (Fig. 10B). cDNA 4e is collinear with cDNA 7 starting at 4e base pair 771, contains a complete ORF, and appears to correspond to a 3.7 kb *egh* transcript. Although the first 930 base pairs of this cDNA are not found in 18XS genomic rescue DNA, cDNA 4e is probably derived from an authentic *egh* transcript (see Fig. 10B).

Considering the complexity of the *egh* gene, we wanted to determine if we had isolated all protein coding sequences essential for *egh* function. We constructed a chimeric *HSegh* transgene by placing the entire putative *egh* ORF of cDNA 7 (also present in cDNA 4e) under the control of the heat-inducible *hsp70* promoter (see Materials and Methods). The basal expression level of *HSegh* is sufficient to rescue the lethality and fertility of partial loss-of-function *egh* mutations, confirming that these sequences correspond to *egh*. Additionally, we sequenced the egh^{63e4} mutation and found that it deletes *egh* protein coding sequences starting in an intron at the 3′ end of the *egh* gene (Fig. 10A,B).

The only open reading frame with good codon usage in either *egh* cDNAs 7 or 4e, or in the corresponding 4.5 kb of genomic DNA, predicts a 52×10^3 M_r protein product (Fig. 10B). The protein is 60% identical to a predicted *C. elegans* protein of identical length, but unknown function (Fig. 10C; Wilson et al., 1994). Examination of both sequences reveals two notable features: a putative signal sequence and at least one putative transmembrane domain (Fig. 10B-D). Our best prediction of the primary structure of the *egh* protein is indicated in Fig. 10E. Without further characterization, we cannot rule out other structures for the predicted *egh* protein product, but the likelihood that *egh* encodes for a secreted or transmembrane molecule is in accordance with the non-autonomy of *egh* function during oogenesis (described above).

Table 4. Penetrance of *egh*, *brn*, and *egh brn* embryonic neurogenic phenotypes

Genotype*	Number scored	% Weak neurogenic	% Intermediate neurogenic	% Strong neurogenic
$brn^{fs.107}$	252	16	83	1
$brn^{l.6P6}$	116	12	87	1
egh^{DF944}	102	15	83	2
egh^{GA107}	99	11	88	1
$egh^{DF944}brn^{fs.107}$	108	10	89	1
$egh^{GA107}brn^{fs.107}$	125	14	82	4
$egh^{DF944}brn^{l.6P6}$	91	12	87	1

*All embryos were derived from germline clones of the indicated mutation, except for $brn^{fs.107}$, for which embryos were derived from homozygous females. For analysis of embryonic neurogenic phenotypes (Table 4), all females were crossed to OrR/Y males, and hatching, zygotically rescued embryos were discarded. Hoyer's mounts of brown eggs were scored for the strength of the neurogenic phenotype according to the following designations: weak, at least two denticle belts on the ventral surface, intermediate, dorsal cuticle without ventral denticle belts, filzkörper and pharyngeal cuticle intact, strong, dorsal patch of cuticle, no pharyngeal cuticle, filzkörper absent. Most embryos in the weak class represent partial zygotically rescued embryos. Essentially no embryos with a strong *N*-like phenotype are observed for any of the indicated genotypes. Embryos scored in the strong class meet the criteria described above, but do not look exactly like strong *N* mutants (not shown), and may result from secondary development defects such as failed germband retraction.

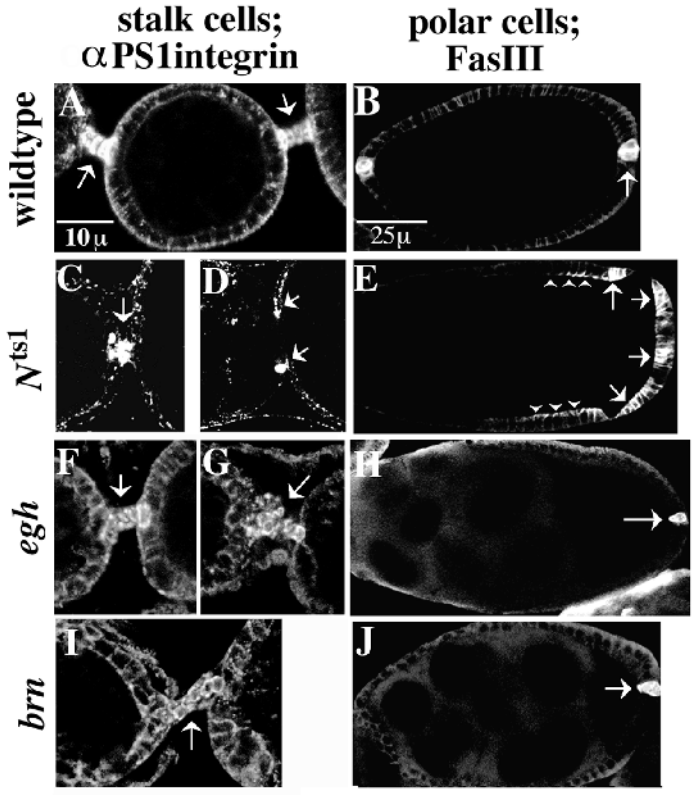


Fig. 6. Expression of α-PS1 integrin and FasIII in WT, *N*, *brn* and *egh* egg chambers. (A) α-PS1 integrin is highly expressed in stalk cells that interconnect WT egg chambers (arrows). (C,D) In N^{ts1} egg chambers shifted to restrictive temperature for 36 hours, stalk cells are not established. Only a few α-PS1 integrin-positive cells remain between egg chambers that fail to separate (C,D, arrows). (F,G,I) In *egh* and *brn* egg chambers, stalk cells are determined correctly (arrows), but stalks are usually disorganized compared to WT (G,F, arrows). (B) FasIII expression in 4 polar cells of a s8 WT egg chamber (arrow, posterior polar cells). (E) FasIII expression in a s9 N^{ts1} egg chamber that developed at the restrictive temperature for 36 hours. Supernumerary polar cells have developed (arrows) and FasIII expression becomes enriched in apical membranes of some follicle cells (arrowheads). (H,J) In *egh* and *brn* egg chambers, there is typically no increase in the number of FasIII-positive polar cells (arrows).

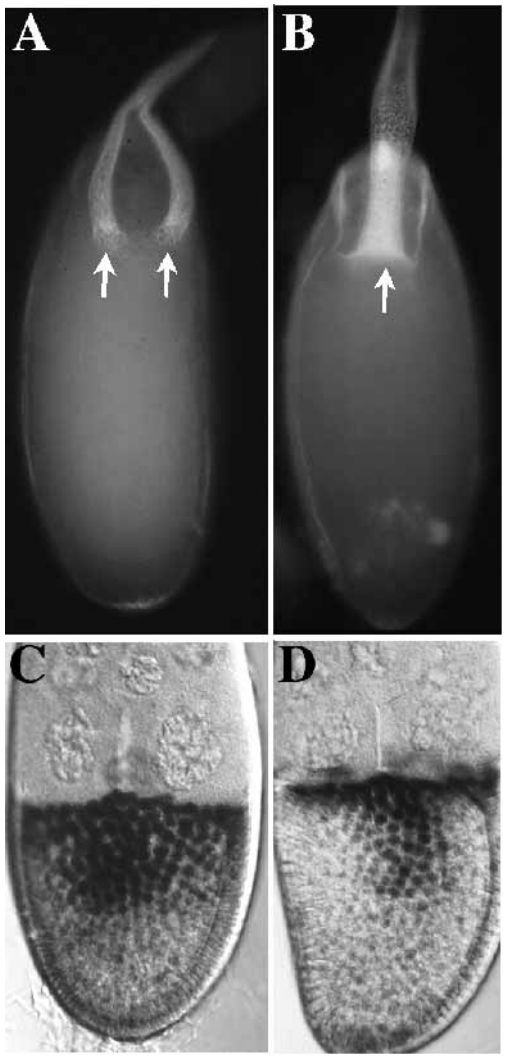


Fig. 7. *egh* is required for specification of dorsal follicle cell fates. (A,B) Eggs visualized with pseudoepifluorescence from a UV filter. (A) A WT egg has two dorsal appendages located in dorsal-lateral positions (arrows). (B) *egh* GLC eggs have a single appendage located on the dorsal midline. (C, D) *kek*-1 expression in follicle cells. (C) *kek*-1 is expressed in dorsal follicle cells of WT s10 egg chambers. (D) In *egh* GLC egg chambers, *kek*-1 expression is reduced laterally, consistent with the fused dorsal appendage phenotype (B).

Accumulation of *egh* transcripts in germ tissue correlates with *egh* and *brn* requirements in the morphogenesis of the follicular epithelium

Accumulation of *egh* transcripts in germ tissue correlates with *brn* and *egh* requirements in the morphogenesis of the follicular epithelium. Like *brn*, *egh* is first expressed in germ cells as prefollicle cells ensheathe newly formed cysts at the junction of germarium region IIa/b, when both genes are essential for forming the follicular epithelium (Figs. 11A,B; 1; 3A; Goode et al., 1996). *egh* expression appears to increase in germ cells as egg chambers span the width of the germarium (Figs. 11B; 1; 3A), and expression continues at uniform levels in all germ cells as the egg chamber assumes a spheroidal shape through s3 of oogenesis (Figs. 11; 1). At s4, when egg chambers transform from a spheroidal to an ellipsoidal shape, *egh* transcripts differentially accumulate in the oocyte (Figs. 11C; 1), corresponding to the time that *egh* and *brn* become essential for maintaining the follicular epithelium around the oocyte. Differential *egh* expression persists through s9 of oogenesis, as (1) the egg chamber undergoes further extension along the A/P axis, (2) follicle cells begin to accumulate and achieve a columnar shape around the oocyte (Figs. 11D; 1), and (3) the follicular epithelium migrates towards the oocyte (Figs. 11E; 1). We have shown that *brn* and *egh* are required throughout these stages for maintaining and helping to achieve normal migration and activation of gene expression within the follicular epithelium. At s10a of oogenesis, follicle cells have completed their migration, and *egh* expression decreases dramatically in the

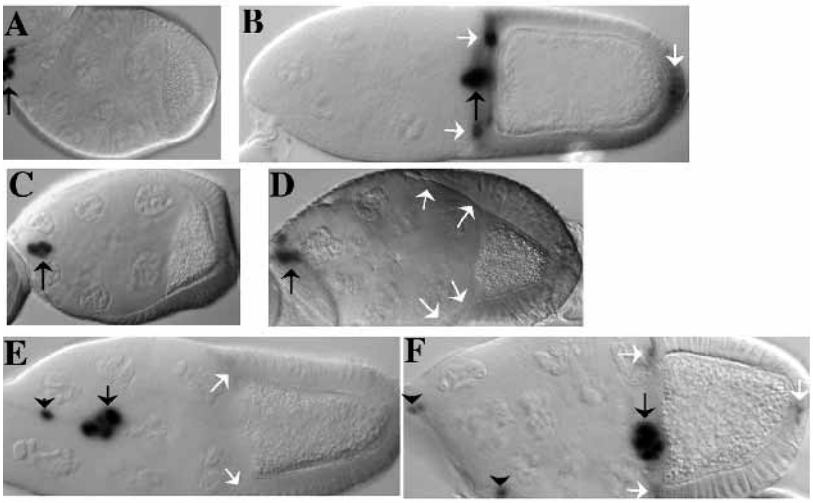


Fig. 8. Expression of *slbo* in *brn* and *egh* mutant egg chambers. The relative size of the egg chambers is not to scale. (A) At s8, a small cluster of cells, termed border cells, which include anterior polar cells, express *slow border cells* (*slbo*) (arrow). Border cells become mesenchymal-like, break form the main body epithelium (MBE), and migrate in concert with the MBE towards the oocyte during s9 (Fig. 1). (B) At s10, both the border cells (black arrow) and the MBE have completed their migration. *slbo* is expressed in follicle cells at the posterior of the oocyte (white arrow), and in circumferentially migrating follicle cells (white arrows). (C) Early s9 *egh* GLC egg chamber. The border cells (black arrow) and MBE have started to migrate towards the oocyte. No border cells are observed at the posterior of *brn* or *egh* egg chambers. (D) Border cell (black arrow) migration lags behind the migration of the MBE (white arrows) in this s9 *egh* GLC egg chamber. (E) The MBE has completed its migration in this s10 $brn^{l.6P6}$ egg chamber (white arrows), while the border cells (black arrow) have only migrated about half the distance to the oocyte. A border cell has separated from the main cluster (arrowhead). (F) s10 $brn^{l.6P6}$ egg chamber. Border cells and the MBE have completed their migration. A few border cells have failed to exit the MBE, and are included in the thin squamous layer of follicle cells that surround the nurse cells (black arrowheads).

oocyte, but increases in nurse cells (Fig. 11F). At subsequent stages, nurse cell transcripts will be 'dumped' into the oocyte (not shown), which is presumably essential for *egh*'s contribution to neurogenesis.

DISCUSSION

egh and *brn* appear to be components of a novel signaling pathway essential for epithelial development and maintenance

In this study we describe the molecular characterization of *egh* and extend the functional analysis of *brn*, *egh*, and *N* during oogenesis. We find that *egh* encodes a novel, putative secreted or transmembrane protein. The spatiotemporal pattern of increasing *egh* expression in germ cells suggests Egghead is a critical component of a germline signaling pathway, organized as a cascade of morphogenetic activity, which helps to coordinate the growth and differentiation of germ cells with morphogenesis of follicular epithelium. Several observations suggest that *egh* and *brn* function in a common pathway specific for the formation and maintenance of epithelial structure: (1) the expression of both genes is restricted to the germline (this study, Goode et al., 1996); (2) they are required

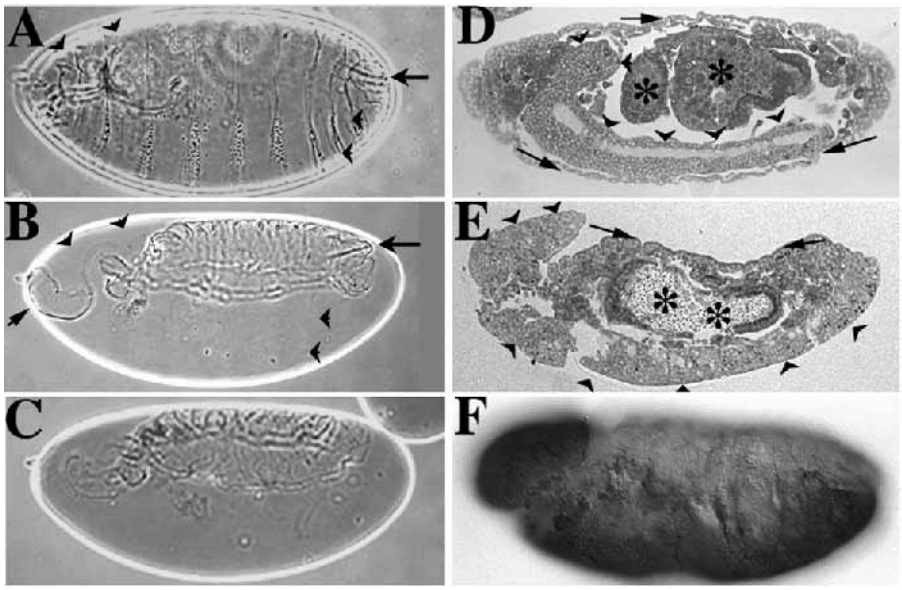
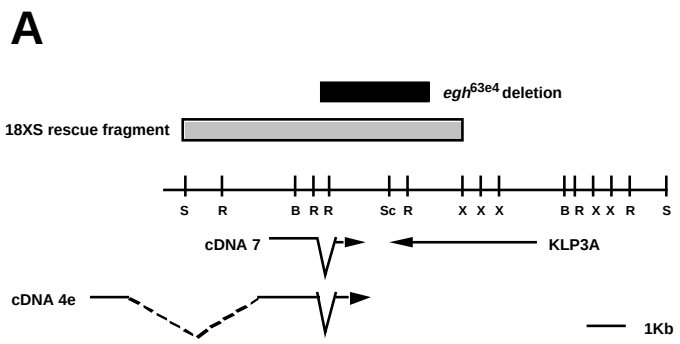
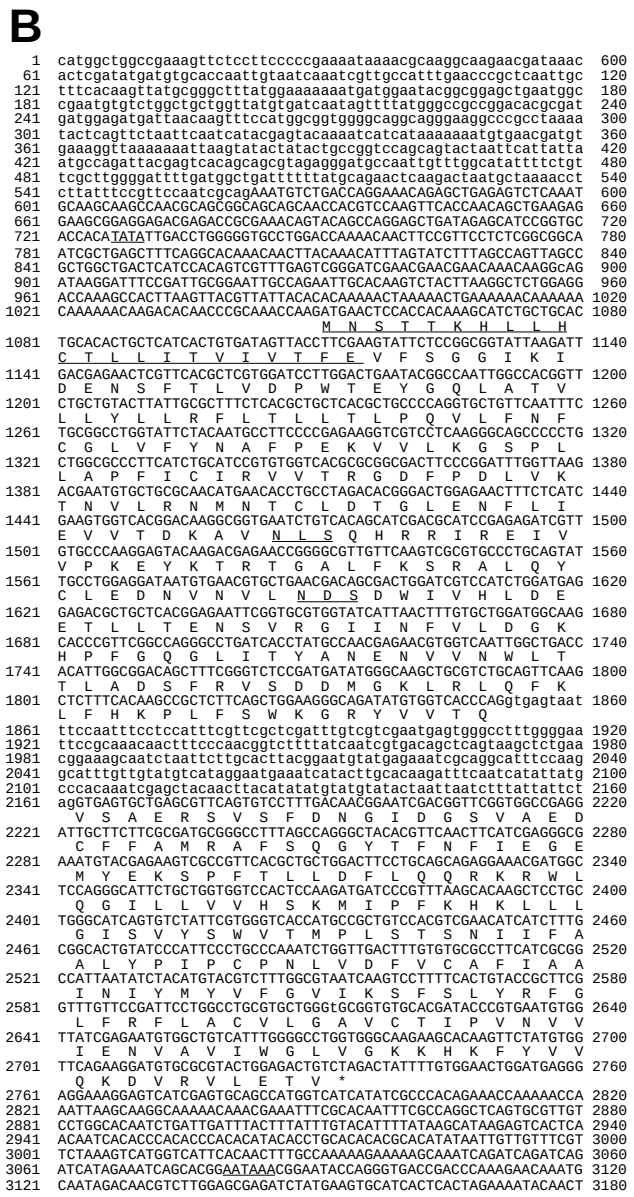
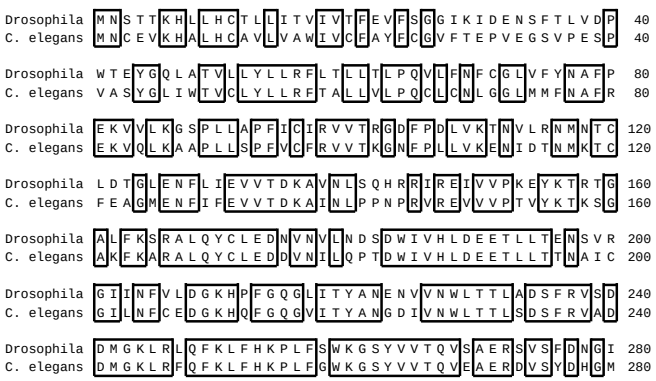
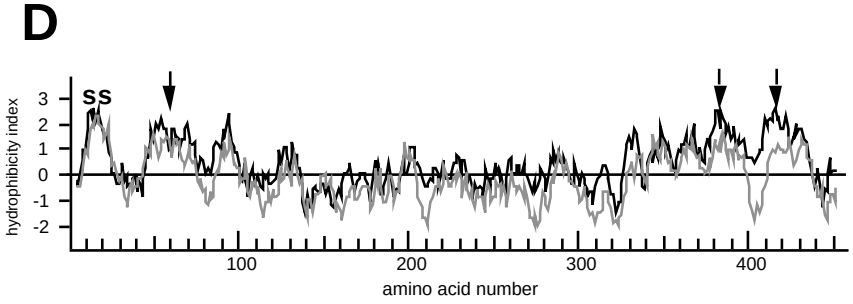


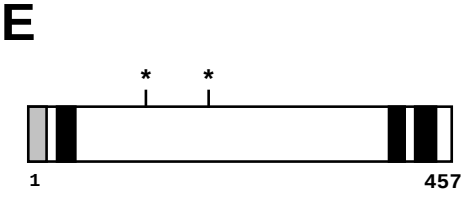
Fig. 9. *egh* neurogenic phenotype. Embryos oriented anterior left, dorsal up. Mutant embryos were derived from *egh* or *egh brn* GLC females crossed to wild type (+/Y) males. Embryos are presumably of genotype mutant(s)/Y, lacking both maternal and zygotic expression of the mutant gene(s), since the phenotypes shown are the most severe observed, and since mutant/+ embryos, fertilized with WT sperm, show less severe, or completely rescued phenotypes. (A-C) Hoyer's mounts. (A) WT embryo. Arrowheads indicate ventral and cephalic cuticle. (B) egh^{GA107} embryo. Ventral and cephalic structures are missing. As for *brn* embryos, *egh* embryos have an intermediate neurogenic phenotype (cf. Lehmann et al., 1983). (C) egh^{DF944} $brn^{l.6P6}$ embryo. *egh brn* embryos do not have a phenotype more severe than either mutant alone. (D,E) Sections of s14 embryos. (D) WT embryo. The embryo is ensheathed in an epidermal layer that secretes the larval cuticle (arrows, compare with A). The nervous system is fully developed (arrowheads). The gut is convoluted (*). (E) egh^{DF944} embryo. The nervous system is expanded in regions where the epidermal layer is missing (arrowheads, compare with B). The gut has not differentiated the WT folded pattern (*, compare with D). (F) An egh^{DF944} embryo stained with the neural specific antigen horseradish peroxidase demonstrates that hypertrophied ventral and cephalic tissue is neural.











identically in the germline for differentiation of the follicular epithelium; (3) neither gene appears to be essential for *N* mediated cell fate specification during oogenesis, and (4) *egh brn* double mutant animals do not show phenotypes any more severe than either mutant alone.

egh and *brn* are clearly required for the formation of the follicular epithelium since in *egh* and *brn* GLC animals follicle cells fail to extend processes in region I/IIa of the germarium, where germline cysts are initially encapsulated. Significantly, we obtained evidence that mutant egg chambers containing

Fig. 10. Molecular characterization of *egh*. (A) Molecular map of the *egh* region. The position, relative size, direction of transcription, and intron structure of *egh* transcripts are represented by cDNAs 4e and 7 (arrows). *KLP3A* lies immediately proximal (Williams et al., 1995). DNA fragment 18XS (gray box) rescues *egh* but not *KLP3A* mutations. egh^{63e4} is a deletion that breaks in the indicated intron (Williams et al., 1995), and removes the 3′ end of the *egh* gene, leaving 3/5ths of *egh* protein coding sequences intact. Restriction sites: B, *Bam*HI; R, *Eco*RI; Sc, *Sca*I; S, *Sal*I and X, *Xho*I. (B) *egh* sequence. Upper case nucleotide sequence is found in both genomic DNA and cDNA 4e. Lower case sequence is restricted to genomic DNA. The first 930 base pairs (bps; not shown) of *egh* cDNA 4e originate outside the 18XS fragment and have not been mapped on the genomic walk. This sequence appears to be derived from an independent exon(s) based on the observations that, (1) bp 931 of cDNA 4e is adjacent to a putative CAG splice acceptor site at genomic position 563, and (2) cDNA 4e detects a transcript of equivalent size on northern blots (not shown). cDNA 7 is collinear with cDNA 4e starting at position 771, and appears to represent an independent transcript based on the presence of a 5′ TATA box in genomic sequence (underlined, position 727). All three sequences contain a presumptive AATAAA polyadenylation signal (underlined). The only open reading frame (ORF) with good codon usage in c- or genomic DNA is represented by the predicted Egghead sequence. These ORF sequences are sufficient for *egh* function (see text), which is consistent with the ability of genomic fragment 18XS to rescue *egh* mutations (see above). The open reading frame is interrupted by a single intron starting at position 1852. egh^{63e4} is a deletion which removes DNA starting within this intron at position 1963. Egghead is predicted to have 457 amino acids and a relative molecular mass of 52×10^3 without posttranslational modification. The first 22 amino acids (underlined) are extremely hydrophobic (see D) and conform to the rules established for signal sequences (von Heine, 1985). The protein has two potential N-linked glycosylation sites (NXS, underlined). cDNA 4e sequence can obtained via GenBank accession number U15602; genomic sequence, U21218. (C) The predicted Egghead protein sequence is 60% identical to a *C. elegans* protein of identical length, but unknown function (Wilson et al., 1994). (D) Hydropathy plots of the *Drosophila* (bold) and *C. elegans* (shaded) Egghead proteins. Both proteins have a conserved signal sequence (SS; B). Arrows indicate presumptive membrane spanning domains, inferred from the content of hydrophobic residues flanked by positively charged amino acids, and the conservation of hydrophobicity between the two proteins (threshold = 1.3). (E) Schematic representation of a potential Egghead protein. The presumptive signal sequence is indicated by a gray box, putative transmembrane domains are indicated by black boxes, and potential glycosylation sites are indicated by asterisks.

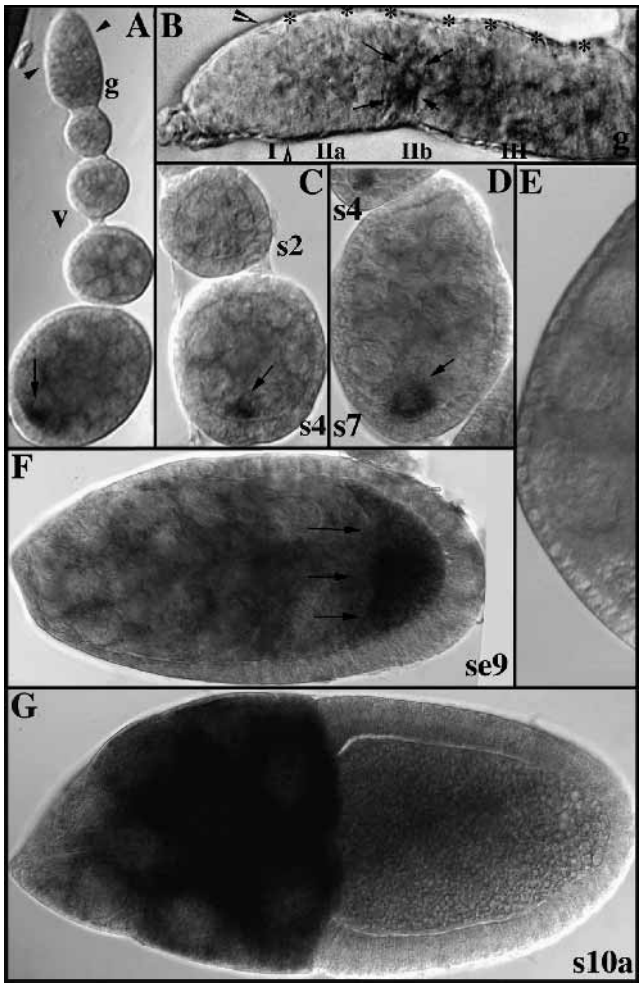


Fig. 11. mRNA expression of *egh* during oogenesis. WT ovarian tissue (g, germarium; v, vitellarium). Tissue in B was stained twice as long as tissue in other panels. (A,B) Germarium expression. *egh* expression appears to be initiated at the junction of germarium region I/IIa (arrowheads). Germ cell expression is evident by accumulation in germ cell cytoplasm (B, arrows); follicle cell bodies, located at the periphery, do not stain (B, **; see also Fig. 6A). (A,C,D,F) *egh* is expressed uniformly in all germ cells until s4 (C, s4), when transcripts differentially accumulate in the oocyte (arrow). *egh* continues to be expressed at uniform levels in nurse cells, but differentially in the oocyte until s9 of oogenesis. Restriction of *egh* transcripts to germ cells is clear in (A). Apparent faint follicle cell staining (C,D,F) results from optical diffraction in images captured at high magnification, as can be seen in (E), a s8 control egg chamber stained with *egh* sense strand probe. The absence of follicular phenotypes in follicle cell clones of *egh* mutations further supports that this staining is an artifactual (not shown). (G) *egh* expression is tremendously reduced in the oocyte while transcription increases dramatically in nurse cells.

germ cells diverging from a 15:1 nurse cell to oocyte ratio arise from (1) the inappropriate stretching and breaking of unencapsulated cysts, and/or (2) the inappropriate migration of follicle cells between, instead of along the outside of germ cell cysts (Fig. 3C-E). These observations are consistent with the complementarity observed between adjacent egg chambers in the vitellarium (Fig. 2C), but do not exclude the possibility that *brn* and *egh* may regulate germ cell divisions. The observation that *hts* mutants never produce egg chambers containing more than a single oocyte-nurse cell complex suggests that if *brn* and *egh* do play a role in organizing germ cell divisions, they do so as a cooperative process with cyst encapsulation.

Our results provide compelling evidence for a role of neurogenic genes in epithelial maintenance, distinct from epithelium formation and cell fate specification. We found that cells start to exit the follicular epithelium and lose apical-basal polarity well after the epithelium is formed, at s4 for the strongest *brn* and *egh* mutant alleles. In this context, it is worth noting that Notch has been suggested to act as a tumor suppressor gene in both vertebrates and invertebrates (Ellisen et al., 1991; Watson et al., 1994). Our results are consistent with a specific role for *egh*, *brn*, and *N* in tumor suppression, since

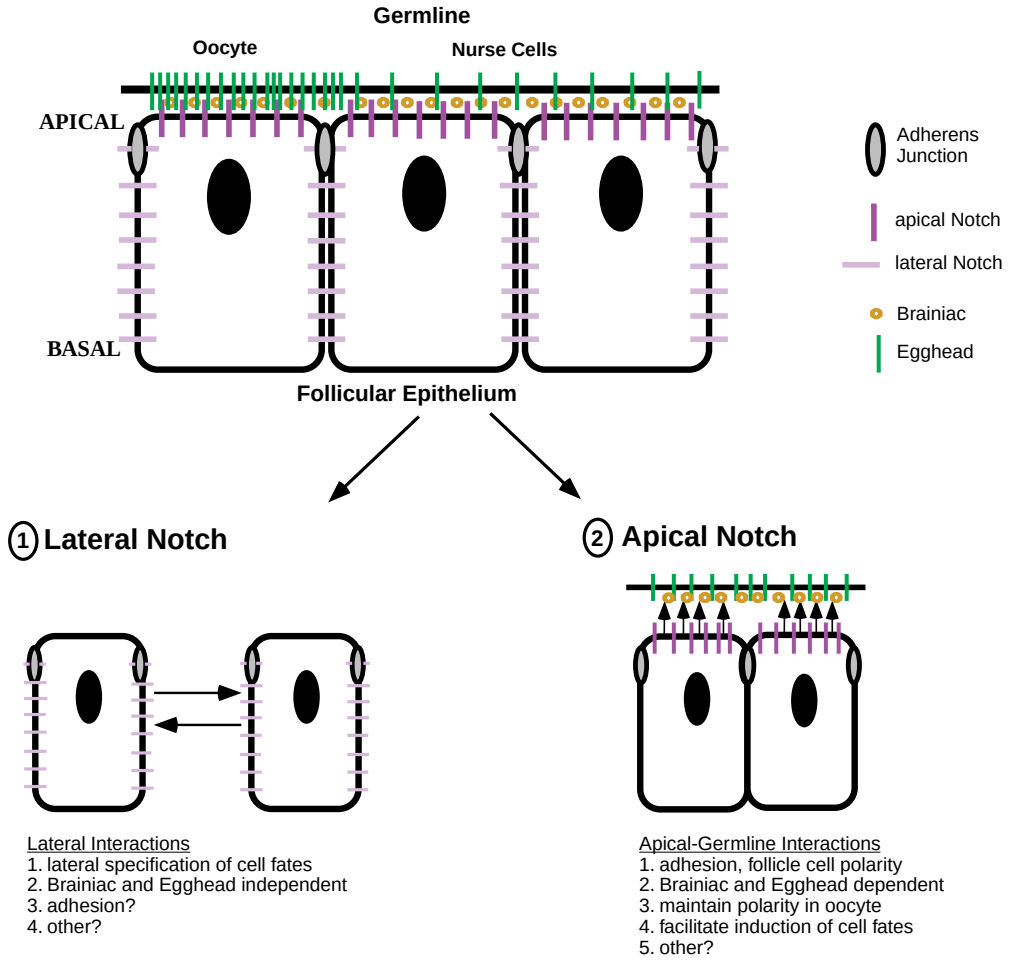


Fig. 12. A model for Brainiac, Egghead and Notch action during oogenesis. The upper portion of the figure shows the presumptive distributions of Brainiac and Egghead in germ tissue, and the distribution of Notch in lateral and apical follicle cell membranes (see Figs 4, 10, 11, and Goode et al., 1996). The distribution of Egghead and Brainiac are inferred from their primary structure; while it is possible that they are expressed in a different germ cell compartment, their requirement for maintaining the follicular epithelium indicates that they are components of a germ cell to follicle cell signaling pathway. The lighter shade of Notch in lateral membranes is meant to represent the lower levels of expression of the protein in this cellular domain compared to apical Notch. Egghead molecules are shown at greater density in oocyte membranes, consistent with the greater expression of transcripts in that cell, while Brainiac molecules are evenly distributed. As discussed in the text, we have obtained evidence that Egghead and Brainiac mediate adhesion of follicle cells to germ tissue, presumably in collaboration with Notch. While we believe that Brainiac and Egghead are components of the same pathway, we do not have data to order the action of these genes, nor do we have data which allows us to decide if Brainiac and/or Egghead bind to Notch or to another receptor. The crucial points of the model are that, (1) Notch expression in lateral membranes is likely to be required for mediating lateral specification of polar versus flanking cells, and perhaps adhesion between follicle cells, and (2) the restriction of Brainiac and Egghead action to the apical surface of follicle cells provides an explanation for how these genes and apical Notch may be specifically required for epithelial maintenance, how Brainiac, Egghead, and Notch might maintain polarity in the oocyte, and how Brainiac and Egghead facilitate inductive events mediated by the Egfr signaling process (see Discussion).

follicle cells appear to over proliferate in these mutants, and the follicular epithelium acquires an early onset adenoma-like phenotype with a spatial-temporal pattern that directly correlates with the differential expression of *egh* in the oocyte.

Most significantly, loss of structure in the follicular epithelium appears to be independent of a role for *brn*, *egh*, or *N* in cell fate specification. Neither *brn* or *egh* are required for regulating polar versus stalk cell fate decisions, and although cells which have exited the follicular epithelium are mesenchymal-like, they have not adopted a mesenchymal border cell fate. Further, while polar cell fates are shifted in only about half of N^{ts1} mutant egg chambers (Ruohola et al., 1991; this study), many *N* mutant egg chambers that suffer multilayered epithelia do not have ectopic polar cells (not shown), supporting the hypothesis that cell fate specification and epithelial maintenance are separate functions of *N*.

To account for the requirement of Notch in both cell fate specification and epithelial development, and the specificity of *brn* and *egh* in epithelial development, we suggest a model for Brainiac, Egghead, and Notch action during oogenesis (Fig. 12). Notch is distributed on both lateral and apical follicle cell membranes, and therefore has the potential to interact with molecules in the germline, as well as with signals from neighboring follicle cells. Lateral specification of stalk and polar cells appears to depend on Notch signals acting between follicle cells, and therefore presumably laterally expressed Notch (Ruohola et al., 1991). In contrast, we propose that *brn* and *egh* germ line functions mediate epithelial development in collaboration with apically expressed Notch.

Based on the following considerations, we propose that germline Brainiac and Egghead and apically expressed Notch function in epithelial development by mediating germline-follicle cell adhesion; (1) Notch acts as a multifunctional receptor and has been implicated in cell adhesion (Cagan and Ready, 1989; Fehon et al., 1990; Rebay et al., 1991), (2) Brainiac is a putative secreted protein, and Egghead is a putative secreted or transmembrane protein; secreted proteins have not been implicated in lateral specification processes, but

have extensively been implicated in cell adhesion and epithelial development, and (3) follicle cells appear to detach from the cell in which *egh* is expressed at highest levels, the oocyte.

One important issue is whether this model can be extended to other tissues. As we noted in the results, the intermediate strength of the *brn*, *egh*, and *brn egh* embryonic neurogenic phenotypes, and the apparent lack of a requirement for these genes in cell fate specifications in the pupal ectoderm is consistent with the specificity of action we have described during oogenesis. One puzzle is that *brn* and *egh* appear to be required for cell fate specification in the embryonic ectoderm (Fig. 9). We think that the apparent difference between *brn* and *egh* function in oogenesis versus early neurogenesis is likely to reflect the distinct structures of the simple monolayer follicular epithelium versus the more complex and dynamic arrangement of cells in the process of cell fate determination within the neurogenic ectoderm (cf. Uemura et al., 1996; Tepass et al., 1996). Thus, we suggest that a loss of epithelial structure within the neurogenic ectoderm, similar to what we have described for the follicular epithelium, would likely have dramatic consequences on signaling processes regulating cell fate specification. A more instructive model for *brn* and *egh* function in the neurogenic ectoderm is that they help to couple the dynamic rearrangement of neuroblasts and epidermoblasts with fate specification. Significantly, not all neurogenic genes have been implicated in epithelial development (Hartenstein et al., 1992), raising the possibility that some neurogenic genes are specifically required for lateral specification. Since *N* and other primary neurogenic genes are required for both epithelial development and cell fate specification, the severe embryonic neurogenic phenotype of these mutants could be explained by a requirement in both processes.

Neurogenic genes appear to encode components of a differential oocyte-follicle cell adhesion system

Disrupting the function of members of the Egfr signaling process leads to discontinuities of the follicular epithelium, in which follicle cells covering nurse cells, but not the oocyte, are missing due to failed migration and/or division (Goode et al., 1996). Based on these findings, it has been suggested that the oocyte has a specialized adhesive system that distinguishes it from the nurse cells. The observations that follicle cells show a propensity to detach from the oocyte rather than nurse cells in *brn*, *egh* and *N* egg chambers starting at s4 and continuing through s9 of oogenesis (Figs 4, 5; Table 3), and that *egh* is differentially expressed in the oocyte throughout this time, suggests that *brn*, *egh*, and *N* are components of at least one oocyte-follicle cell adhesive system during stages 4 to 9 of oogenesis, and that *egh* is a critical component.

Differential adhesion to the oocyte and maintenance of the follicular epithelium is likely to be crucial for the efficiency of at least two parallel, perhaps interconnected, sets of processes that depend on germline-follicle cell interactions. The first set of processes include the changes in follicle cell shapes that occur concomitantly with their gradual accumulation around the oocyte during mid-oogenesis, before the MBE migrate towards the oocyte in concert with border cells at s9 (Fig. 1). The observation of exiting, apolar follicle cells during mid oogenesis, and the failure of border cells to migrate in concert with the MBE indicates that *brn*, *egh* and *N* are essential for both processes. The breaking of border cells from the MBE, and the migration of the MBE towards the oocyte are both likely to require tight regulation of the relative epithelial state of follicle cells (see Introduction), and differential adhesion/attraction to the oocyte (Lee et al., 1996), and *brn* and *egh* appear to influence both of these processes (Fig. 8). Strikingly, the MBE appears to migrate too fast in *brn* and *egh* GLC egg chambers, suggesting that these genes may be needed to inhibit the migration process, a particularly intriguing possibility considering the role of the genes in inhibiting the segregation (migration) of neuroblasts during early neurogenesis.

A second set of processes that appear to depend on oocyte-follicle cell adhesion are inductive events mediated by the Egfr signaling process. Egfr signals on the apical surface of follicle cells are induced by germline *grk* TGFα signals to both form the follicular epithelium (Goode et al., 1996), and to establish anterior-posterior and dorsal-ventral pattern within the epithelium, which is used for localization of morphogens in and around the oocyte (Roth et al., 1995). Likewise, *N* neurogenic function has previously been shown to be required for localization of morphogens at the anterior and posterior poles of the egg chamber (Ruohola et al., 1991), and we observe delayed or mislocalized *oskar* at the posterior pole of *brn* and *egh* mutant egg chambers (not shown). Our current analysis does not allow us to decide the hierarchical relationship between neurogenic and Egfr signaling in controlling these processes (if one exists). One possibility is that the Egfr pathway is essential for establishing and maintaining posterior cell fates, while neurogenic gene function acts subsequently to mediate adhesion of the follicular epithelium to the oocyte. Adhesion appears to be essential for maintaining the apical-basal polarity of follicle cells, which in turn may be necessary for establishing polarity of the cytoskeleton in the oocyte. Alternatively, morphogen localization may primarily depend on the Egfr signaling pathway, which is facilitated by neurogenic adhesion function. Loss of *brn* or *egh* function during oogenesis results in a ventralized chorion phenotype that is more severe than eliminating half of *grk* TGFα molecules from the system (Goode et al., 1996; Fig. 7; unpublished observations). These hypotheses are not mutually exclusive.

Functional tests are consistent with the interdependence of the neurogenic and Egfr signaling processes. *brn*, *egh*, and *N* mutations show dominant and synergistic interactions with mutations in components of the Egfr signaling process, for both dorsal-ventral patterning and induction of cell proliferation and/or migration (Goode et al., 1992, 1996; Goode, 1994; not shown). In contrast, like *brn egh* animals, *N brn* double mutant animals do not show phenotypes any more severe than either mutant alone during oogenesis (Goode, 1994), consistent with the close functional relationship that we have documented between these genes in this study.

These observations are consistent with a plethora of studies linking adhesion, maintenance of cell polarity, and execution of inductive signals. For example, in budding yeast, proteins essential for the development and maintenance of cell polarity organize a large complex of proteins, including actin, cdc24, ste20 and Cla4 protein kinases, as well as MAP kinases at sites of polarized growth (reviewed by Drubin and Nelson, 1996). In *C. elegans*, *lin7* encodes a membrane associated DHR-containing protein that is essential for maintaining the localization of the Egfr, and loss of *lin-7* function results in inefficient Egfr signaling, and Egfr mutant phenotypes in most animals

(Simske et al., 1996). In vertebrate cells, integrins are essential for the organization of a protein scaffold for the assembly of a signaling network that includes focal adhesion kinase and components of the Ras pathway, and GTP-binding proteins (reviewed by Clark and Brugge, 1995). In these studies, we have shown a clear disruption in cell polarity, loss of apical membrane integrity, and presumably adhesion in *brn*, *N*, and *egh* mutants; interestingly, disruption of spectrin function results in the accumulation of follicle cells in multiple layers similar to that seen in *brn*, *egh*, and *N* mutants (Deng et al., 1995), suggesting a close functional relationship between Brn/Egh and Notch adhesive signals and regulated cytoskeletal processes. It will be interesting in future studies to determine if the apparent interdependence of neurogenic and Egfr signaling processes is at the level of organization and interdependence of intracellular signaling complexes within the apical cortex of follicle cells, synergistic action in the activation of transcription of new genes, a combination of these processes, or even more complex scenarios.

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