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cut interacts with *Notch* and Protein kinase A to regulate egg chamber formation and to maintain germline cyst integrity during *Drosophila* oogenesis

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SUMMARY

Communications between the germline and the soma during *Drosophila* oogenesis have been previously shown to be essential for the formation of egg chambers and to establish polarity in the developing oocyte. In this report, we demonstrate that the function of a somatically expressed gene, *cut*, is critical for maintaining the structural integrity of germline-derived cells and their arrangement within an egg chamber. Genetic manipulations of *cut* activity resulted in defective packaging of germline-derived cysts into egg chambers and disintegration of the structural organization of oocyte-nurse cell complexes to generate multinucleate germline-derived cells. We also found that *cut* interacts genetically with the *Notch* gene and with the catalytic subunit of Protein kinase A gene during egg chamber morphogenesis. Since *cut* expression is restricted to the somatic follicle cells and *cut* mutant germline clones are phenotypically normal, we propose that the defects in the assembly of egg chambers and the changes in germline cell morphology observed in *cut* mutant egg chambers are the result of altered interactions between follicle cells and germline cells. *cut* encodes a nuclear protein containing DNA-binding motifs, and we suggest that it participates in intercellular communications by regulating the expression of molecules that directly participate in this process.

Key words: *cut*, *Notch*, Protein kinase A, oogenesis, *Drosophila*, germline, egg chamber

INTRODUCTION

Oogenesis in *Drosophila* occurs in ovarioles, composed of a series of increasingly mature egg chambers surrounded by a muscular sheath (reviewed in Spradling, 1993). At the proximal tip of each ovariole is a germarium, which contains the stem cells for both the germline-derived cells and the somatic follicle cells. The germarium has been divided into four regions. In regions 1 and 2a, germline stem cells divide four times to produce a 16-cell syncycial cyst and oocyte specification initiates in one of these cells. The other 15 germline-derived cells differentiate into trophic nurse cells. As a result of incomplete cytokinesis, the cells in each cyst are interconnected by cytoplasmic bridges known as ring canals. In region 2b, somatic follicle cells migrate to surround a single germline cyst and form an egg chamber. A few of these somatic cells become precursors to pairs of specialized follicle cells found at the anterior and posterior poles of each follicle (polar follicle cells). The remaining somatic cells produce a continuous epithelial sheet of follicle cells surrounding the germline cyst. In region 3 of the germarium, five to eight interfollicular stalk cells are specified, generating distinct boundaries between adjacent egg chambers. The egg chamber subsequently buds from the germarium and enters the vitellarium, where it progresses through 14 additional developmental stages.

Genetic analysis has identified several genes that participate in the process of egg chamber formation and the maintenance of egg chamber morphology through later stages of oogenesis. Mutations in *Notch* (*N*, Ruohola et al., 1991; Xu et al., 1992), *Delta* (*Dl*, Bender et al., 1993), *daughterless* (*da*, Cummings and Cronmiller, 1994), *hedgehog* (*hh*, Forbes et al., 1996), *fs(1)Yb* (Johnson et al., 1995), *gurken* (*grk*, Goode et al., 1996b), *brainiac* (*brn*, Goode et al., 1992, 1996a), *Egfr*^{torpedo} (*Egfr*^{top}, Goode et al., 1996) and *egghead* (*egh*, Goode et al., 1996a) result in fused egg chambers (compound follicles) containing multiple cysts. These have been interpreted to arise due to the absence of interfollicular stalk cells and/or the failure of follicle cells to migrate and enclose 16-cell cysts. *brn*, *grk* and *egh* are required in the germline; all the other genes are required in the follicle cells, suggesting that the correct encapsulation of cysts involves communications between the somatic and the germline-derived cells.

Another set of genes is involved in maintaining the structural integrity of the germline-derived cells during egg chamber morphogenesis. Mutations in the catalytic subunit of Protein kinase A (*Pka-C1*, also known as *DCO*, Lane and Kalderon, 1993) or *armadillo* (*arm*, Peifer et al., 1993), or altered

expression of members of the Rho subfamily of GTPases (Murphy and Montell, 1996), result in the production of multinucleate nurse cells, presumably as a result of fusions between germline-derived cells.

Previous work has shown that *cut* acts as a switch between alternate cell fates in the peripheral nervous system (Blochlinger et al., 1993; Bodmer et al., 1987). It encodes a nuclear protein (Cut) containing a homeodomain and three copies of a DNA-binding domain (*cut* repeat) that is unique to Cut and its relatives in other species (Andrés et al., 1992; Blochlinger et al., 1988; Neufeld et al., 1992; Valarché et al., 1993). In addition, *cut* has a role in the development of other embryonic and adult tissues, including the wing margin (Blochlinger et al., 1993; Jack and DeLotto, 1992; Jack et al., 1991). Cut is expressed in ovarian follicle cells (Blochlinger et al., 1993; this work), and we show here that *cut* is required both for egg chamber formation and for regulating germline cyst integrity.

Perturbations in *cut* activity resulted in defects during egg chamber formation, and we demonstrate a genetic interaction between *cut* and *Notch* in this process. In addition, reduction of *cut* activity produced multinucleate germline-derived cells and this phenotype is enhanced by a heterozygous mutation in *Pka-C1*. In contrast to *Pka-C1* and *armadillo*, which are required in germline-derived cells, *cut* is required in the follicle cells to preserve the structural integrity of the germline-derived cells. This demonstrates that the maintenance of germline cell morphology is regulated at least in part by the somatic follicle cells. Results from previous studies have emphasized the importance of intercellular signaling processes in the assembly of egg chambers and for the establishment of polarity in the developing egg and embryo (reviewed in Ray and Schüpbach, 1996). Our results provide evidence for the existence of an additional pathway of intercellular communication in which follicle cells regulate the organization of the oocyte-nurse cell complex during egg chamber morphogenesis.

MATERIALS AND METHODS

Stocks

The following fly stocks were maintained on standard yeast-cornmeal-molasses-agar food at 20°C.

y w (used as wild type), *y w* ct^{L188} (Liu et al., 1991), *y w* ct^{C145}/FM6 (Lindsley and Zimm, 1992), *y w* ct^{DB10}/FM7c (Blochlinger et al., 1990), *wimp*/TM3 (a gift from S. Parkhurst, Parkhurst and Ish-Horowicz, 1991) and *Pka-C1*$^{l(2)01272}$/CyO (Spradling et al., 1995; a gift from S. Parkhurst).

N^{ts1} (Lindsley and Zimm, 1992 a gift from E. Giniger). The temperature-sensitive phenotype of the N^{ts1} mutation was induced by placing adult flies in a 30°C incubator for 64-72 hours before ovary dissection.

y w; C2/CyO (Cut coding sequences inserted between hsp70 regulatory sequences, Blochlinger et al., 1990). Cut was ectopically expressed by placing *y w*, C2/CyO adults in a 37°C waterbath for 1 hour. Ectopic production of Cut protein in all follicle cells was verified by staining ovaries from these females 3 hours after the heat-shock with rat anti-Cut F2 antibody (data not shown).

Flies of genotype *w P[mini-*w^+*; hs-NM](8A) P[*ry^+*;* neo^R*; FRT](18A)* (18 NM) and *w P[*ry^+*;* neo^R*; FRT](18A);* MKRS *P[mini-*w^+*; hsp70-FLP]* (18-1F) were a gift from G. Rubin (Xu and Rubin, 1993). *y w* ct^{C145} was recombined onto the 18-1NM chromosome using standard genetic methods to produce the strain *y w* ct^{C145} NM/FM6.

Mosaic analysis

Recombination was induced in *y w* ct^{C145} *P[mini-*w^+*; hs-NM](8A) P[*ry^+*;* neo^R *FRT](18A)/w P[*ry^+*;* neo^R*; FRT](18A)*; +/MKRS *P[mini-*w^+*; hsp70-FLP]* adult females, from a cross between 18-1 F males and *y w* ct^{C145} NM/FM6 females, by incubating them in a 37°C waterbath for 1 hour on three successive days. 5-10 days after the final heat shock, the expression of the c-myc epitope tag was induced by incubating the females at 37°C for 1 hour followed by 30-60 minutes at room temperature before dissection.

Immunohistochemistry

Ovaries were dissected in phosphate-buffered saline containing 1 mM EDTA (PBSE), fixed in PBSE with 4% formaldehyde at room temperature for 20 minutes and washed once with PBSE. Ovaries were then permeablized in PBSE with 1% TRITON X-100 (TX-100) at room temperature for 1-3 hours, washed once with PBSE with 0.05% TX-100 (PBTE) and incubated for 1-24 hours in PBTE with 5% BSA and 0.02% sodium azide (blocking solution). Primary antibody was added in blocking solution, incubated overnight at 4°C and washed 5× 10 minutes with PBTE. Secondary antibody was diluted in PBTE, incubated with ovaries for 1-2 hours at room temperature and washed 5× 10 minutes with PBTE. DAPI (final concentration 2 μg/ml) and rhodamine-conjugated phalloidin (final concentration 2×10^{-3} units/μl, Molecular Probes) were added together with the secondary antibody, where appropriate. Ovaries were equilibrated in 50% glycerol in PBSE and mounted in glycerol/PBSE and Vectashield antiphotobleaching reagent (Vector laboratories). Biotinylated secondary antibodies followed by Vector Elite horseradish peroxidase (HRP)-diaminobenzidine (DAB) visualization were used to detect Cut, Reaper and FasIII immunoreactivity. The antibodies and concentrations used were: polyclonal rat anti-Cut F2 (1:100, Blochlinger et al., 1990), monoclonal anti-myc Ab-1 (1:25, Oncogene Science), monoclonal anti-FasIII 2D5 (1:100, a gift of Nipam Patel), polyclonal rabbit anti-Reaper (1:100, a gift of G. Pronk), biotinylated anti-rat IgG (1:1000, Vector), biotinylated anti-mouse IgG (1:1000, Vector), fluoroscein-conjugated anti-mouse IgG (1:100, Molecular Probes).

Images were examined on either a Zeiss Axioplan, a Biorad MRC-600 Confocal microscope, or an Applied Precision DeltaVision optical sectioning deconvolution microscope followed by corrections using nearest neighbor algorithms.

RESULTS

Cut protein expression is dynamic during oogenesis

Previous observations have demonstrated that Cut protein is expressed in ovaries (Blochlinger et al., 1993). Here, we provide a detailed description of the biphasic Cut protein expression pattern during oogenesis (Fig. 1). There is no detectable Cut expression in germline-derived cells at any stage of oogenesis. Cut expression initiates in somatic follicle cells in region 2b of the germarium (Fig. 1A), at about the time that follicle cells interleave and surround germline cysts. It persists in all follicle cells, including the polar follicle cells and the interfollicular stalk cells (Fig. 1B), until about stage 5. Between stages 6 and 9, Cut expression ceases in all the follicle cells except the polar follicle cells. At about stage 10, Cut expression resumes, first in the anterodorsal follicle cells (data not shown), then throughout the layer of columnar follicle cells which surround the oocyte, and continues in these

cells until stage 14 (Fig. 1C). No Cut immunoreactivity is detected in the squamous follicle cell layer overlying the nurse cells. The same temporal and spatial pattern of expression is detected by in situ hybridization with *cut* cDNA probes (data not shown).

Egg chamber defects in *cut* null mutant mosaics

We have not detected any Cut expression in germline-derived cells, suggesting that the phenotypes described here are the result of altered *cut* activity in the follicle cells. Consistent with this, previous analyses of *cut* germline clones indicated that there is no maternal contribution of *cut* during embryogenesis (Bodmer et al., 1987). Finally, we did not observe any mutant phenotypes in 315 germline clones homozygous for the *cut* null allele ct^{C145} (data not shown), confirming that *cut* is not required in the germline during oogenesis.

To address the role of *cut* in the somatic follicle cells, we used FLP-mediated recombination to produce clones homozygous for the null allele ct^{C145} and marked with a c-myc epitope tag (Xu and Rubin, 1993). ct^{C145} homozygous clones have two copies of the epitope tag and are most brightly labeled with myc antibodies (Fig. 2A); conversely, a 'twin' clone homozygous wild-type for *cut* (ct^{+}) does not react with the myc antibodies. Cells that remain heterozygous stain with intermediate intensity. Mitotic recombination was induced in adult females, followed by a 4-14 day recovery period. This treatment results in recombination in all dividing follicle cells, including the follicle cell precursors, and allowed the analysis of ct^{C145} clones during many stages of oogenesis.

Wild-type egg chambers contain 15 nurse cells with polyploid nuclei and an oocyte with a single diploid nucleus (Fig. 3A). The oocyte is connected to the nurse cells by four ring canals and each egg chamber contains a total of 15 ring canals. Filamentous actin (F-actin) is present at the cortices of all cells, but is more abundant in the oocyte. F-actin is also prominent at the interface between follicle cells and germline-derived cells, and at adherens-type junctions between follicle cells (Peifer et al., 1993).

Egg chambers containing somatic clones of ct^{C145} null follicle cells exhibited three phenotypes: one phenotype was observed in the follicle cells themselves, and two other phenotypes were associated with the germline cyst.

Homozygous *cut* mutant clones within the follicular epithelium contained 2- to 5-fold fewer cells than their corresponding wild-type twin clones. Also, mutant cells and their nuclei were larger than surrounding heterozygous or wild-type cells (Fig. 2A-C). This phenotype was present in all clones observed 4 days, but not 7 days or more after recombination. In addition, 44% of the ovarioles examined 7 days after recombination contained germaria that were either much reduced in size or completely absent (Fig. 2D-F). These results suggest that (1) *cut* mutant cells stop dividing before the surrounding wild-type cells and (2) *cut* mutant cells are eventually lost. To test whether this loss is a consequence of apoptosis, we labelled mosaic ovarioles with anti-Reaper antibodies; however, we did not find any evidence for apoptosis at any time after recombination, nor did we ever observe degenerating follicle cell nuclei (data not shown).

Two other phenotypes were observed which involved the morphology of the germline cyst. 4 days after recombination, greater than 50% of stage 6 and later egg chambers contained nurse cells with multiple nuclei (Fig. 2G-I). In these egg chambers, at least 30% of the follicle cells were mutant. The multinucleate cells contained abnormal intercellular actin fibers and actin-rich spots, possibly the remnants of ring canals (Fig. 2C,D). Multinucleate cells were still observed 7 days after recombination; however, in most of these egg chambers, we could not detect *cut* mutant follicle cells, although a corresponding twin spot was always present. Since the mutant cells were presumably lost, it was not possible to correlate the phenotype with a particular size or distribution of the *cut* mutant cells.

7-14 days after recombination, egg chambers containing aberrant numbers of germline-derived nuclei were observed (Fig. 2J,K). This phenotype was observed in less than 1% of the egg chambers examined. In most of these a mutant clone could be detected, but sometimes we inferred that a mutant clone had existed from the presence of the wild-type twin spot. Whenever a mutant clone was observed in these egg chambers, it was found in region 2b of the germarium or at the anterior of early staged follicles. These phenotypes are consistent with cyst encapsulation defects.

Since follicle cells move throughout oogenesis and *cut* mutant clones tend to disappear, it was difficult to temporally and spatially correlate the presence of *cut* mutant clones with the phenotypes observed. In order to thoroughly characterize the role of *cut* during oogenesis using a more tractable experimental protocol, we looked for other ways of altering *cut* activity in the ovary. We found that subsets of the phenotypes observed in *cut* null clones were reproduced by different genetic manipulations, as described below.

Hypomorphic *cut* alleles affect cyst organization

The allele ct^{L188} was isolated by hybrid dysgenesis (see Liu et al., 1991). It is homozygous viable, but is cold-sensitive lethal in combination with null alleles of *cut* (Blochlinger et al., 1990, S. M. J. and K. B., unpublished observations). At 24°C about 40% of flies containing this combination of alleles, referred to here as *cut* hypomorphs, eclose.

Most of the egg chambers produced by the *cut* hypomorphs were morphologically normal (Fig. 3A). However, 11% of stage 4 or older egg chambers from *cut* hypomorphs (n=1996 total egg chambers scored) contained multinucleate germline-derived cells. Nurse cells containing multiple polyploid nuclei (Fig. 3E) or oocytes containing two diploid nuclei were observed (Fig. 3B,C). Although these egg chambers invariably contained 16 germline-derived nuclei, the total number of ring canals was frequently decreased to 14 or 13. Abnormal or degenerating ring canals, similar to those seen in the mosaic ovarioles, could be observed (data not shown); presumably, degeneration accounted for the decrease in total ring canal number. The distribution of ring canals was also abnormal in affected egg chambers: dinucleate oocytes sometimes contained five or six ring canals (Fig. 3C,D). About half of the egg chambers containing multinucleate cells had 16 germline nuclei but did not contain a recognizable oocyte (Fig. 3E). Occasionally, the multinucleate cells in these egg chambers were centrally located and contained at least six ring canals. No egg chambers with multinucleate cells were observed prior to stage 4 in the *cut* hypomorphs. Given that these abnormalities were only seen in older egg chambers, it appears that the multinucleate phenotype resulted from degeneration of

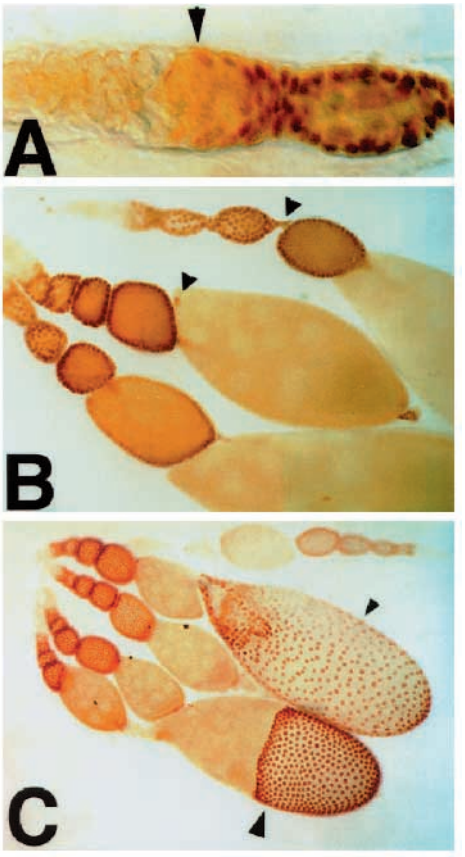

Fig. 1. Cut protein expression is dynamic during oogenesis. (A) Cut immunoreactivity initiates in the somatic follicle cells in region 2b of the germarium (arrow). (B) Expression continues in all follicle cells through stage 6, when it becomes restricted to the polar follicle cells and interfollicular stalk cells (arrowheads) during these stages. Cut expression persists in the anterior polar follicle cells once they begin migrations through the nurse cells and become border cells (data not shown). (C) Cut immunoreactivity resumes at stage 10 (large arrowhead) in the follicle cells that cover the oocyte and persists in these cells until oogenesis is completed (small arrowhead). At no time is Cut expression observed in the germline-derived cells. All panels are bright-field micrographs of DAB-HRP-stained ovarioles.

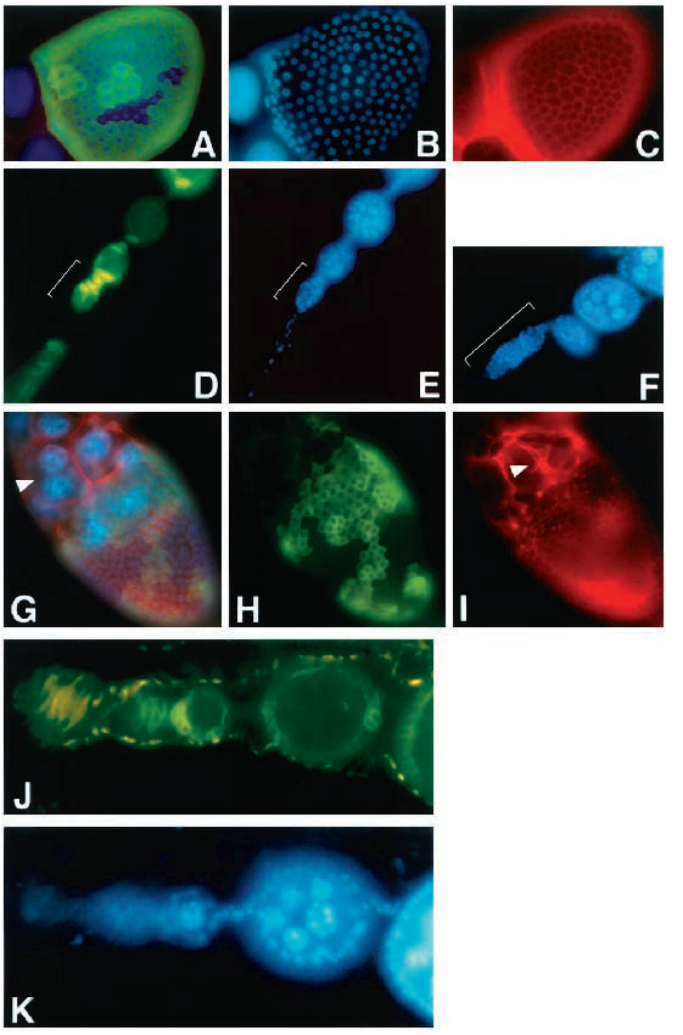

Fig. 2. Mosaic analysis reveals multiple requirements for *cut* during oogenesis. (A-C) Stage 10 egg chamber dissected 4 days following recombination, showing (A) homozygous ct^{C145} follicle cell clones (2× myc immunoreactivity), the wild-type twin spots (no myc immunoreactivity) and heterozygous cells (1× immunoreactivity), (B) larger size of nuclei in *cut* mutant clone, (C) subcortical actin in follicle cells stained with rhodamine-phalloidin. (D,E) Ovariole dissected 7 days following recombination showing (D) position of a mutant clone and (E) reduction in size of germarium. For comparison, a normal germarium is shown in (F). Brackets indicate germaria. (G-I) Late stage 9 egg chamber dissected 7 days following recombination, showing (G) multinucleate nurse cell (arrowhead), (H) position of homozygous mutant and wild-type clones and (I) subcortical actin staining and ectopic actin structures within a nurse cell (arrowhead). (J,K) Ovariole dissected 7 days following recombination, showing (J) position of homozygous mutant clone and (K) two egg chambers with inappropriate numbers of nurse cell nuclei (one and five, respectively). All panels are epifluorescent micrographs of ovaries stained with anti-myc antibodies (green), DAPI (blue) and rhodamine-phalloidin (red).

membranes, or from fusion of cells in an initially normal 16-cell germline cluster.

Multinucleate nurse cells have previously been observed in ovaries of females carrying a mutation in the Protein kinase A catalytic subunit (*Pka-C1*) (Lane and Kalderon, 1993, 1995). We tested the possibility that *cut* and *Pka-C1* interact genetically during oogenesis. No phenotype was observed in ovaries of flies doubly heterozygous for null mutations in *cut* and in the catalytic subunit of *Pka* ($Pka\text{-}C1^{l(2)01272}$). However, the heterozygous $Pka\text{-}C1^{l(2)01272}$ mutation enhanced the frequency and severity of the multinucleate cell phenotype observed in *cut* hypomorphs (Fig. 3F). Although 16 germline-derived nuclei were still present, the majority of egg chambers (68%, n=582) did not contain a recognizable oocyte, and most of the ring canals were found in the center of the follicle. The number of ring canals in an egg chamber was reduced significantly; in the examples shown, only eight or nine ring canals were present. The subcortical actin layer demarcating individual nurse cells was not detectable in these follicles. Finally, the abnormal organization of the follicles was apparent at an earlier stage (stages 2 and 3) than in the *cut* hypomorphs alone. Thus, *Pka-C1* is a dominant enhancer of the multinucleate cell phenotype produced by *cut* hypomorphs.

cut null alleles in combination with *wimp* result in egg chamber assembly defects

Null alleles of *cut* are embryonic lethal and no *cut* mutations are known that selectively affect *cut* activity in the ovary. However, it has been shown that expression levels of a specific set of genes can be reduced maternally in females containing *wimp*, a mutation in the gene encoding the RNA polymerase II 140 kD subunit, resulting in pseudodominant maternal effects (Parkhurst and Ish-Horowicz, 1991). We tested for interactions between *cut* and *wimp* and observed a mutant phenotype in ovaries from females doubly heterozygous for a *cut* null mutation (ct^{C145}) and *wimp* (see below), suggesting that *wimp* reduces *cut* expression levels during oogenesis.

Ovaries from ct^{C145}/+; *wimp*/+ females contained egg chambers with aberrant numbers of germline cells and demonstrated cyst encapsulation defects, similar to those observed using mosaic analysis. Most frequently (15% of egg chambers, n=1393), affected egg chambers had fewer than 15 nurse cells (Fig. 4A), containing anywhere from 1-14 nurse cells. In contrast to the phenotype observed with *cut* hypomorphs, however, the number of germline nuclei always equaled the number of germline cells and no multinucleate cells were observed. Partially fused egg chambers were seen that appeared to be the result of defective and incomplete encapsulation by follicle cells (Fig. 4B). 5% of the egg chambers had supernumerary germline-derived cells, containing either polyploid nuclei (data not shown) or non-polyploid nuclei (Fig. 4C). In 1% of the egg chambers, the polarity of the cyst was reversed such that the oocyte was found at the anterior (Fig. 4D). Similar defects were observed in double heterozygotes of *wimp* and other *cut* null alleles (data not shown); however, no phenotype was observed in ovaries from females doubly heterozygous for *wimp* and ct^{L188}, or in heterozygous *wimp* females.

These data suggest that germline cyst encapsulation is affected in ct^{C145}/+; *wimp*/+ females. To analyze the behavior of migrating follicle cells that surround the germline cysts (interleaving follicle cells), ovaries were stained with antibodies against Fasciclin III (FasIII). This antigen is expressed in the interleaving follicle cells and in the polar follicle cells in region 2b of the germarium (Ruohola et al., 1991). In wild-type ovarioles, a single band of FasIII-expressing cells forms perpendicular to the anterior/posterior axis of the germarium (Fig. 4E). In ct^{C145}/+; *wimp*/+ germaria, several bands of FasIII expressing cells were present (Fig. 4F). Some of these bands of cells did not extend along the entire width of the germarium and were at odd angles relative to the germarial axis. FasIII-expressing cells or groups of cells appeared to be between cells within a germline cyst (Fig. 4F). Thus, loss of *cut* activity results in improper cyst encapsulation by follicle cells.

Ectopic Cut expression produces compound egg chambers

To determine the effect of ectopic Cut expression during oogenesis, a heat-shock-inducible Cut transgene (hsCut, Blochlinger et al., 1991) was used to induce Cut expression in all somatic cells of the ovariole, resulting in Cut expression in some cell types that do not normally express Cut (e.g. the non-follicle somatic cells of the germarium) and Cut overexpression in follicle cells that express endogenous Cut (data not shown). We will refer to the expression of the Cut transgene

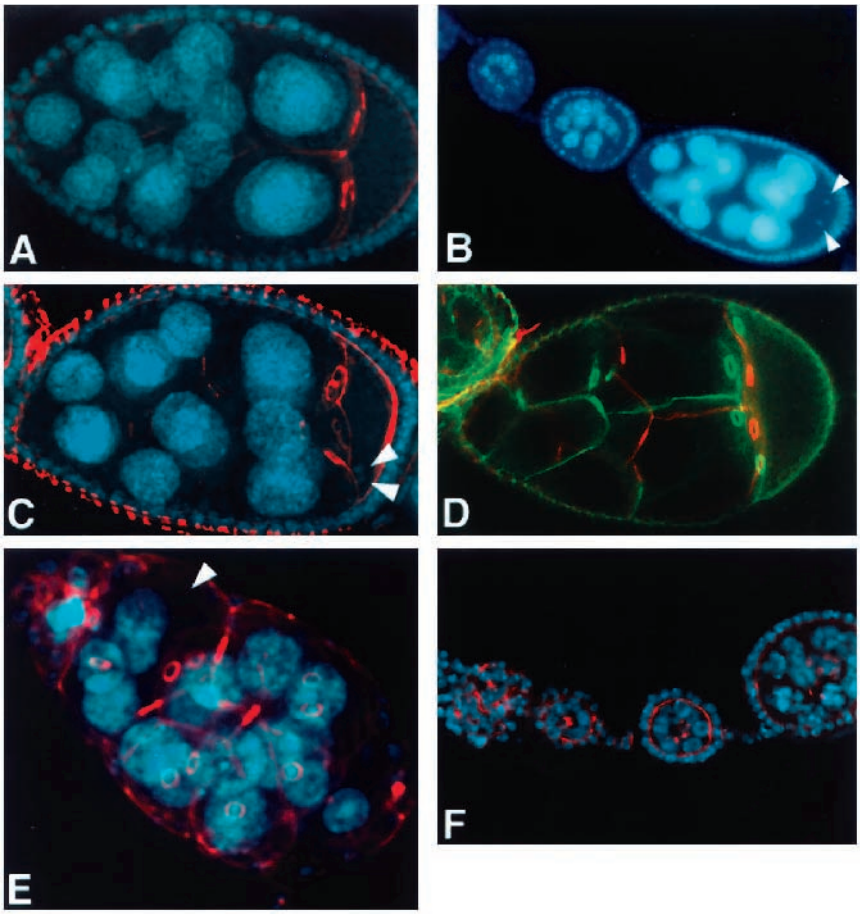

Fig. 3. Multinucleate cell phenotypes produced by *cut* hypomorphs are enhanced by a *Pka-C1* mutation. (A) Wild-type stage 10 follicle, the oocyte containing four ring canals (edges only are visible). (B) Egg chamber from a ct^{C145}/ct^{L188} female containing 14 polyploid nurse cells and two diploid oocyte nuclei (arrowheads). (C) Egg chamber from a ct^{DB10}/ct^{L188} female containing a dinucleate oocyte with five ring canals (arrowheads indicate two oocyte nuclei). (D) Egg chamber from ct^{C145}/ct^{L188} female with an oocyte containing six ring canals. Two different focal planes taken through single egg chamber are shown in red and green. (E) Egg chamber from a ct^{DB10}/ct^{L188} female containing 16 nurse cell nuclei and a multinucleate nurse cell (arrowhead), but no detectable oocyte. Occasionally, entire ovaries exhibited this defect. (F) Ovariole from a ct^{DB10}/ct^{L188}; $Pka^{l(2)01272}$/+ female, each egg chamber contains 16 nurse cell nuclei, centrally located ring canals, no oocyte and no discernible subcortical actin layer in the germline-derived cells. A, C, E and F are Deltavision projections of 2-4 optical sections stained with DAPI (blue) and rhodamine-phalloidin (red); D consists of two confocal sections through a rhodamine-phalloidin-stained egg chamber, B is an epifluorescent micrograph of a DAPI-stained ovariole.

Table 1. *cut* rescue of *Notch^{ts1}* defects during oogenesis

Genotype	Normal	Compound
w N^{ts1}/FM6	93% (*n*=951)	0.9% (*n*=10)
w N^{ts1}/w N^{ts1}	11% (*n*=117)	84% (*n*=875)
w N^{ts1} ct^{C145}/w N^{ts1}	83% (*n*=1223)	3.5% (*n*=43)
w N^{ts1} ct^{DB10}/w N^{ts1}	63% (*n*=1464)	23% (*n*=326)

Egg chamber morphology was scored as either wild-type (normal), compound or degenerating after 64 hours at the *N^{ts1}* restrictive temperature. Both numbers and percentage of follicles with a given phenotype are provided. The remainder of the egg chambers examined in each class was degenerating.

as ectopic Cut expression. Approximately 75% of stage 2 or 3 egg chambers that emerged from the germarium 24-30 hours after the pulse of ectopic Cut expression contained 30 polyploid nurse cell nuclei and two diploid oocyte nuclei (Fig. 5A,B). The two oocytes were always found on opposite poles of the chamber. No cell present in affected egg chambers contained more than four ring canals, indicating that the extra cells did not arise from extra germline cell divisions. In addition, the presence of two populations of differently sized nurse cell nuclei in some egg chambers suggests that they contained two adjacent germline cysts (compound egg chambers), as first described in *Notch*-mutant egg chambers (Ruohola et al., 1991; see below). Multiple pulses of ectopic Cut expression increased the incidence of compound egg chambers to more than 90% and produced compound follicles containing even larger numbers of cysts (Fig. 5H). For the most part, these ovarioles did not have interfollicular stalks, even between egg chambers that appeared morphologically normal.

These results are consistent with the hypothesis that ectopic Cut expression causes defective encapsulation and separation of individual germline cysts in the germarium. Interleaving follicle cells and polar follicle cells were therefore examined by immunocytochemistry with FasIII antibodies in either *y w* controls or hsCut females 24 hours after heat-shock. This treatment did not alter the FasIII expression pattern in *y w* controls (Fig. 5E). In hsCut females, however, the FasIII-expressing cells did not span the width of the germarium (Fig. 5F). FasIII immunoreactivity was also used to examine the polar follicle cells in compound follicles 48-72 hours after the heat-shock. The compound egg chambers produced by ectopic Cut expression contained two polar follicle cells each at the anterior and posterior of the chamber (Fig. 5G). Four extra polar follicle cells were also found grouped between anterior and posterior poles of these compound egg chambers, presumably overlying the junction of two germline cysts. The number and position of the polar follicle cells was identical when a *neuralized-lacZ* enhancer trap line (A101, Grossniklaus et al., 1989), which also stains polar follicle cells, was used (data not shown). Thus, ectopic Cut expression affects cyst encapsulation in a manner reciprocal to that observed when *cut* activity is reduced.

cut null mutations suppress loss of *Notch* function during oogenesis

Reducing *Notch* activity using a temperature-sensitive mutation (*N^{ts1}*) results in large compound egg chambers that are similar to those seen after multiple pulses of ectopic Cut expression (Ruohola et al., 1991; Xu et al., 1992). This result suggests that *Notch* and *cut* act antagonistically during the early stages of oogenesis. We tested if heterozygous mutations in *cut* affected the *N^{ts1}* mutant phenotype. After 64 hours at the restrictive temperature for *N^{ts1}*, 84% of the egg chambers scored were compound (Table 1). We found that two different *cut* null mutations (*ct^{C145}* and *ct^{DB10}*) reduced the incidence of compound egg chambers found in *N^{ts1}* ovaries after 64 hours at the restrictive temperature (Table 1) so that most of the early stage egg chambers examined appeared morphologically normal when labeled with DAPI, rhodamine-conjugated phalloidin and FasIII antibodies (data not shown).

DISCUSSION

Somatic requirement for *cut* in multiple processes during oogenesis

In this report, we describe three roles for *cut* during oogenesis. We show that *cut* is required to maintain the follicle cell population, consistent with the expression of Cut in the majority of follicle cells throughout most of oogenesis. We also demonstrate that Cut is necessary for the partitioning of individual germline cysts into egg chambers and show that *cut* genetically interacts with *Notch* during cyst encapsulation. It is probable that this requirement correlates with the expression of Cut in the germarial follicle cells, because in the mosaic analysis the defects in follicle formation were mostly associated with a mutant follicle cell clone in region 2b of the germarium. Finally, *cut* is required for maintaining the structural organization of oocyte-nurse cell complex, perhaps reflecting a specific role for Cut expression in the follicle cells of the vitellarium. The organization of germline-derived cells also depends on *Pka-C1* activity, and we document a genetic interaction between *cut* and *Pka-C1*. Our results provide experimental evidence for a previously proposed model (Peifer et al., 1993) in which the arrangement of the germline-derived cells within an egg chamber depends on cell contacts with the surrounding follicle cells.

Three lines of evidence indicate that the requirement for *cut* in these processes is confined to the somatic follicle cells. First, *cut* is expressed continuously in all follicle cells subsequent to region 2b of the germarium until stage 6 in the vitellarium, and no expression is observed in germline-derived cells at any stage of oogenesis. Second, the same phenotypes obtained by reducing *cut* activity were observed when *cut* expression was eliminated exclusively in follicle cells in somatic cell mosaics. Third, no phenotype was observed in *cut* mutant germline clones.

Mosaic analysis demonstrated that *cut* function is required in the follicle cells themselves, since fewer, and frequently larger, follicle cells were present in clones homozygous for the *cut* null mutation than the corresponding wild-type twin clone, and the germaria were reduced in size. At longer intervals after the induction of recombination often only the wild-type twin clone could be found. This absence of *cut* mutant cells could be the result of death of these cells. Loss of *cut* activity has been previously linked to cell death in the developing wing disc, leading to scalloping of the wing margin (Fristrom, 1969). Since we did not observe any evidence of apoptosis, other mechanisms explaining the apparent loss of *cut* mutant follicle cells must be considered.

The loss of *cut* null mutant cells and the continual movement of follicle cells during oogenesis impeded the detailed characterization of ovarian *cut* mutant phenotypes using mosaic analysis. These difficulties were circumvented by using hypomorphic *cut* alleles and *wimp* to alter levels of *cut* activity. These genetic combinations accurately reproduced the cyst encapsulation defect and the multinucleate germline cell phenotype observed by mosaic analysis. Interestingly, different *cut* mutant phenotypes were observed depending on the genetic combination used to manipulate Cut expression levels. One possible explanation for this difference is that the mutant protein produced by the ct^{L188} allele, present in the hypomorphs, might affect only a subset of the wild-type Cut activities. The molecular nature of the ct^{L188} mutations is not known. However, the altered ratio of the two Cut-specific species detected on protein gels (Blochlinger et al., 1990) suggests that post-transcriptional processing might be compromised. Notably, the function of the human Cut analog has recently been shown to be regulated by phosphorylation (Coqueret et al., 1996). Another possibility is that the activity of another gene required for follicle assembly may additionally be lowered in ct^{C145}/+; *wimp*/+ females. Nevertheless, since both multinucleate germline-derived cells or cyst encapsulation defects were produced by somatic clones of *cut* null mutant cells, we firmly conclude that both these phenotypes are due to decreased *cut* activity in follicle cells.

Reduced *cut* activity produces multinucleate germline cells

Reducing *cut* activity in flies using a hypomorphic combination of *cut* alleles resulted in egg chambers in which at least one of the germline-derived cells contained more than one nucleus. In principle, multinucleate cells could result from defective cytokinesis in the germarium or alternatively from fusions between cells. Multinucleate nurse cells have been previously shown to be caused by mutations in several other genes. Some of these genes, including *chickadee*, the *Drosophila* homolog of the actin-binding protein profilin (Verheyen and Cooley, 1994), *cappuccino* and *spaghetti-squash*, which respectively encode a protein related to vertebrate formins (Emmons et al., 1995; Manseau et al., 1996) and the regulatory light chain of nonmuscle myosin II (Edwards and Kiehart, 1996; Wheatly et al., 1995), regulate cytoskeletal organization. In *spaghetti-squash*, *chickadee* and *cappuccino* mutants, the multinucleate nurse cells have been interpreted to be caused by defective cytokinesis. In contrast, adjacent nurse cells have been shown to fuse in *Pka-C1*-deficient females through an apparent reversal of cytokinesis (Lane and Kalderon, 1995). Mutations in *armadillo*, a β-catenin homolog, disrupt the cortical actin cytoskeleton of germline-derived cells, and this has been suggested to lead to the breakdown of nurse cell membranes (Peifer et al., 1993). A similar disruption of the subcortical actin cytoskeleton producing apparent nurse cell collapse and fusion was observed after expression of mutant forms of Cdc42 and RhoL, two members of the rho subfamily of GTPases involved in actin filament reorganization (Murphy and Montell, 1996).

The cause of the multinucleate cells produced in *cut* mutant females is more consistent with fusion events than with a defect during cytokinesis, because no multinucleate cells have been observed in egg chambers prior to stage 4, and the phenotype appears to be progressively more severe in older egg chambers. Moreover, we have shown that a heterozygous mutation in *Pka-C1* enhances both the penetrance and the severity of the phenotype in *cut* hypomorphs, suggesting that *cut* and *Pka-C1* are involved in the same process. Interestingly, however, both multinucleate nurse cells and binucleate oocytes are observed in *cut* hypomorphs, whereas the phenotype produced by mutations in *Pka-C1* or any other of the above-mentioned genes appears to be restricted to nurse cells. In addition, since *cut* is required in the follicle cells, it is the only gene whose role in this process has been shown to be non-cell-autonomous. This demonstrates that communication between the somatic follicle cells and the germline-derived cells are important to regulate the morphological integrity of the nurse cells and oocyte. Contacts between the somatic follicle cells and the germline-derived cells have been previously proposed to be necessary to preserve the normal organization of nurse cells and oocyte within the follicle (Peifer et al., 1993).

The genetic interaction that we have observed between *cut* and *Pka-C1* and the requirement of *Pka-C1* and *cut* in distinct cell types (*Pka-C1* in the germline and *cut* in the follicle cells) suggest that *cut* and *Pka-C1* participate in a novel signaling pathway between the follicle cells and germline cells, which is necessary to maintain the cytoarchitecture of the growing egg chamber. Since *cut* encodes a nuclear protein with DNA-binding motifs and *Pka-C1* expression is associated with the germline cell plasma membranes (Lane and Kalderon, 1995), it is possible that *cut* influences the expression in follicle cells of a secreted or membrane-bound ligand which regulates *Pka-C1* activity in germline-derived cells, ultimately leading to alterations in the cytoskeleton in the oocyte-nurse cell complex. In fact, we have found that *cut* interacts specifically with *cappuccino*, a mutation known to regulate the activity of the germline cytoskeleton (Manseau et al., 1996), but not with other mutations which affect the actin cytoskeleton (S. M. J. and C. Berg, unpublished data).

cut mutants affect the assembly of egg chambers

cut is also required in the somatic cells of the germarium for the encapsulation of germline cysts into egg chambers. Reduction or loss of *cut* activity leads to apparent mispackaging of germline cysts and fragmentation of 16-cell cysts into several egg chambers. Ectopic Cut expression produces compound egg chambers in which adjacent cysts have failed to separate or are incompletely separated. Compound egg chambers are also observed in ovaries mutant for a number of other genes as a consequence of follicle cell fate changes, abnormal follicle cell migrations and/or morphological alterations in the germline cyst. We will discuss these phenotypes in relation to the possible roles of *cut* in egg chamber formation below.

Notch (Ruohola et al., 1991; Xu et al., 1992), *Delta* (Bender et al., 1993), *daughterless* (Cummings and Cronmiller, 1994) and *fs(1)Yb* (Johnson et al., 1995) have been shown to interact genetically during follicle formation, and their mutant phenotypes have been attributed to defects in the specification of the interleaving follicle cell and stalk cell fates. It has recently been proposed that this set of genes act temporally downstream of *hedgehog*, which is required for both follicle cell proliferation and specification (Forbes et al., 1996). We have shown that a heterozygous *cut* null mutation suppresses the compound

follicles produced in *Notch*-deficient ovaries, suggesting that *cut* opposes the activity of *Notch* in the same pathway of cell fate specification. It should be noted, however, that there are several differences between the phenotypes obtained through ectopic Cut expression and through loss of *Notch* activity. Perhaps most importantly, loss of *Notch* activity results in an expansion of polar follicle cells and a concomitant reduction in the numbers of stalk and flanking follicle cells (Ruohola et al., 1991), whereas the number of polar follicle cells is unaffected after ectopic Cut expression. Moreover, no changes in cyst orientation have been observed in *Notch* mutant follicles, whereas adjacent cysts are in opposite orientations in compound follicles resulting from ectopic Cut expression (Fig. 5A). Finally, unlike loss of *Notch* function, ectopic Cut expression does not affect the morphology of the germarium. The interpretation of the significance of these phenotypic differences is complicated because it is not clear if the phenotypes observed after ectopic Cut expression are the result of altering the pattern or the level of Cut expression in the ovary. Interestingly, *Notch* and *cut* also interact, albeit synergistically, during the development of the wing margin, and the activation of *cut* expression along the prospective wing margin has been shown to depend on *Notch* activity (de Celis et al., 1996; Doherty et al., 1996; Jack and DeLotto, 1992; Micchelli et al., 1997; Neumann and Cohen, 1996).

Mutations in another set of interacting genes, *brainiac*, $Egfr^{top}$ and *gurken*, lead to the production of compound follicles (Goode et al., 1992, 1996a,b). It has been postulated that the phenotype in these mutants is a consequence of the failure of interleaving follicle cells to migrate and surround germline cysts. *brainiac* and *gurken*, which encode a putative secreted protein and a TGFα homolog, respectively, are required in the germline-derived cells and cooperate with $Egfr^{top}$, which encodes the *Drosophila* EGF receptor and is expressed in the follicle cells. This illustrates the importance of germline-soma interactions during the assembly of follicles. It has recently been shown that mutations in *egghead*, another gene required in the germline and encoding a putative secreted or transmembrane protein, cause phenotypes identical to *brainiac*, and that *egghead*, *brainiac* and *Notch* are all necessary to maintain the follicle cell epithelium (Goode et al., 1996a,b). We have observed no alterations in the follicle cell epithelium in *cut* mutant egg chambers; however, it is possible that *cut* has a role in regulating the migration of the interleaving follicle cells in the germarium in addition to, or instead of, a role in follicle cell fate specification. The finding that cyst orientation is affected in *brainiac* or *gurken* mutant follicles (Goode et al., 1992, 1996a,b), as it is after ectopic Cut expression, indicates that *cut* may participate in a process involving this set of genes. However, we have not detected genetic interactions between *cut* and $Egfr^{top}$ during oogenesis (data not shown).

It is conceivable that the primary defect leading to the *cut* mutant phenotype is not cell autonomous and that the disruption of a soma-to-germline communication pathway culminates in the loss of structural integrity of the germline-derived cells (perhaps via alterations in cytoskeletal elements), which would permit interleaving follicle cells to separate cells within a cyst. In support of this model, occasional defects in the packaging of cysts into follicles have been observed in *arm* mutant ovaries (Peifer et al., 1993). In addition, a mutation in cytoplasmic dynein produces compound egg chambers, further illustrating the importance of cytoskeletal elements during this process (Dick et al., 1996)

Cut proteins in development

In the developing peripheral nervous system, *cut* is both necessary

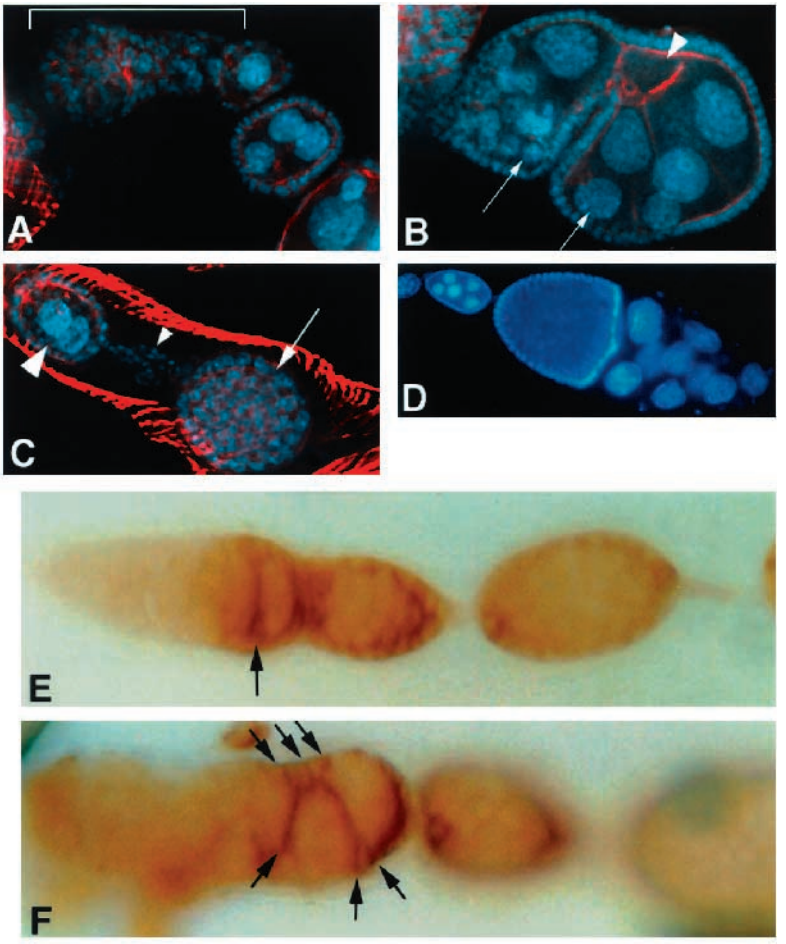

Fig. 4. Egg chambers from ct^{C145}/+; *wimp*/+ females show defects during egg chamber formation. (A) Egg chambers containing fewer than 15 nurse cells and no oocyte. Bracket indicates germarium. (B) Two partially fused egg chambers apparently resulting from incorrect and incomplete encapsulation by follicle cells; two groups of different-sized nurse cells are indicated by arrows, the oocyte by a large arrowhead. (C) Two egg chambers, one containing supernumerary germline-derived cells (arrow), the other egg chamber containing only two polyploid nurse cells (large arrowhead); extra interfollicular stalk cells appeared to be present (small arrowhead). (D) Egg chamber with reversed cyst polarity. (E,F) FasIII expression pattern in interleaving follicle cells (arrow) in a *y w* ; *wimp*/+ germarium (E) and in a ct^{C145}/+; *wimp*/+ germarium (F). Note that several FasIII-expressing cells were at abnormal angles relative to the A/P axis of the germarium (arrows). A-C are Deltavision projections of 1-3 optical sections of ovarioles stained with DAPI (blue) and rhodamine-phalloidin (red); D is an epifluorescent micrograph of DAPI-stained ovariole, E and F are bright-field micrographs of HRP-DAB-stained germaria.

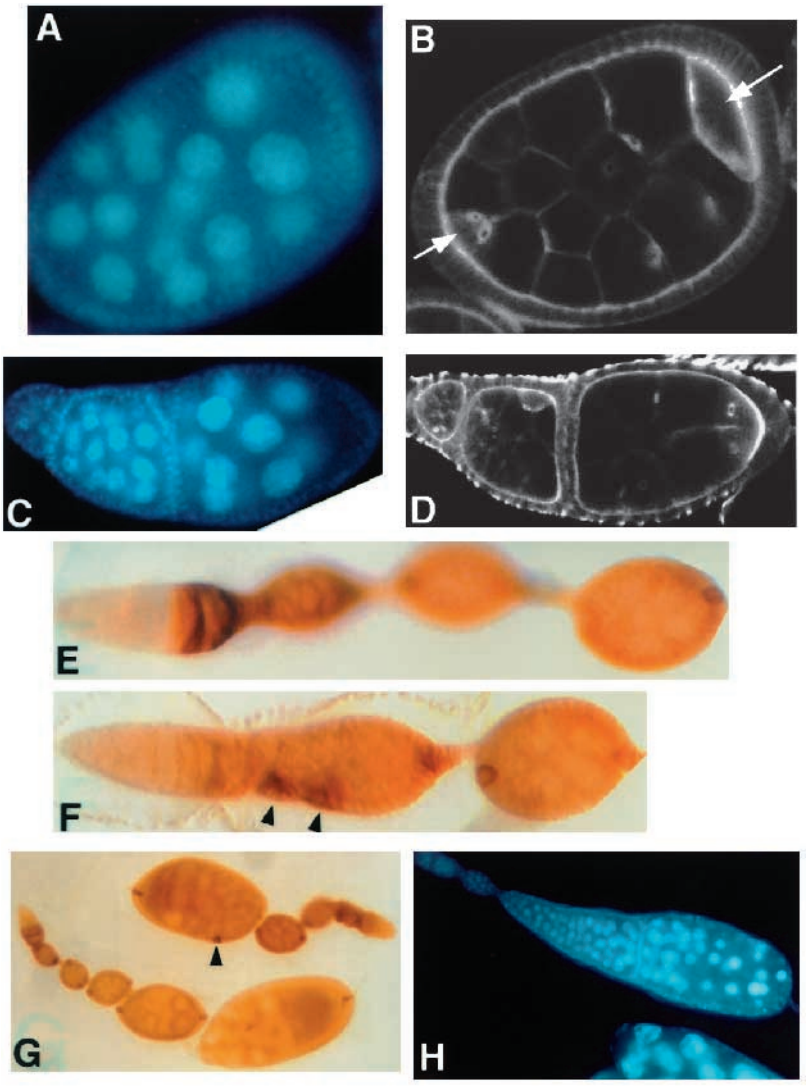

Fig. 5. Ectopic Cut expression produces compound egg chambers. (A,B) Compound egg chamber containing 30 nurse cells and two distinct oocytes, each with four ring canals and slightly more intense subcortical actin staining intensity, at opposite poles of the egg chamber (arrows). (C,D) Less than 20% of compound egg chambers contained greater numbers of nurse cells, always in multiples of 15 (C). Although a layer of follicle cells separated distinct compartments from each other (D), interfollicular stalks were not present. (E,F) FasIII expression pattern in *y w* ovariole (E) and in hsCut ovariole (F) following heat-shock. Arrowheads in F indicate a group of FasIII immunoreactive cells that do not span the width of the germarium. Note that FasIII staining in the polar follicle cells is unaffected in the adjacent stage 2 follicle. (G) FasIII expression pattern in a wild-type (bottom) and a compound (top) egg chamber. Note the group of 4-6 FasIII-immunoreactive cells midway between the poles of the compound chamber (arrowhead). (H). Ovariole from a hsCut ovariole after multiple pulses of ectopic Cut expression containing a giant compound egg chamber. A, C and H are epifluorescent micrographs of DAPI-stained ovarioles, B and D consist of confocal sections of rhodamine-phalloidin-stained follicles, E, F and G are bright-field micrographs of HRP-DAB-stained germaria.

and sufficient for the formation of external mechanosensory and chemosensory organs instead of internal proprioceptive chordotonal organs, and it has been proposed to function as a switch gene between morphologically and antigenically distinct cell fates (Blochlinger et al., 1993; Bodmer et al., 1987; Dick et al., 1996). The presence of a homeodomain in Cut, as well as three copies of a unique protein motif (*cut* repeat) that can bind DNA in vitro (Andrés et al., 1994; Aufiero et al., 1994; Harada et al., 1995; Valarché et al., 1993, Q. Fan and K. B., unpublished data) indicate that Cut may be involved in transcriptional regulation. In support of this, vertebrate Cut-like proteins have been isolated that apparently participate in developmentally regulated gene expression in a variety of mammalian tissues (Andrés et al., 1994; Aufiero et al., 1994; Harada et al., 1995; Valarché et al., 1993). At this point, it is not clear if *cut* is acting as a switch between alternate cell fates during oogenesis. It is, however, possible that *cut* regulates the expression of a common gene or set of genes in the ovary and the peripheral nervous system because mutations in genes encoding proteins associated with the cytoskeleton have been shown to produce defects in both egg chamber and sensory bristle morphology (Cant et al., 1994; Verheyen and Cooley, 1994). The phenotypes observed in *cut* mutant ovaries could also be a consequence of alterations in cell adhesive properties of the follicle cells. Interestingly, the murine Cut-like protein, Cux, regulates the expression of the neural cell adhesion molecule N-CAM in tissue culture (Valarché et al., 1993). We have recently shown that ectopic expression of two mammalian Cut-like proteins in flies can rescue a *cut* mutant wing phenotype to the same extent as ectopic expression of Cut in flies (Ludlow et al., 1996) and therefore it is conceivable that the regulatory targets of fly and vertebrate proteins are evolutionarily conserved.

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