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A central role for microtubules in the differentiation of *Drosophila* oocytes

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SUMMARY

Drosophila **oocytes develop within cysts containing 16 cells that are interconnected by cytoplasmic bridges. Although the cysts are syncytial, the 16 cells differentiate to form a single oocyte and 15 nurse cells, and several mRNAs that are synthesized in the nurse cells accumulate specifically in the oocyte. To gain insight into the mechanisms that generate the cytoplasmic asymmetry within these cysts, we have examined cytoskeletal organization during oocyte differentiation. Shortly after formation of the 16 cell cysts, a prominent microtubule organizing center (MTOC) is established within the syncytial cytoplasm, and at the time the oocyte is determined, a single microtubule cytoskeleton connects the oocyte with the remaining 15 cells of each cyst. Recessive mutations at the *Bicaudal-D (Bic-D)* and *egalitarian (egl)* loci, which block oocyte differentiation, disrupt formation and maintenance of this polarized microtubule cytoskeleton. Microtubule assembly-inhibitors phenocopy these mutations, and prevent oocyte-specific accumulation of oskar, cyclin B and 65F mRNAs. We propose that formation of the polarized microtubule cytoskeleton is required for oocyte differentiation, and that this structure mediates the asymmetric accumulation of mRNAs within the syncytial cysts.**

Key words: mRNA localization, cytoskeleton, egalitarian, Bicaudal-D, *Drosophila*

INTRODUCTION

The role of cytoplasmic asymmetry in cellular determination and differentiation has been elegantly demonstrated through genetic and molecular analysis of axis specification in *Drosophila* (reviewed by St. Johnston and Nüsslein-Volhard, 1992; Nüsslein-Volhard and Roth, 1989; Manseau and Schüpbach, 1989). It is now clear that the anterior-posterior axis of the embryo is established through the asymmetric positioning of key morphogens (proteins and mRNAs) within the cortical cytoplasm of the oocyte. However, an earlier process also requires the generation of cytoplasmic asymmetry. Oogenesis in *Drosophila* begins with four divisions of a cystoblast. Because cytokinesis is incomplete at each of these divisions, the resulting 16 cells are connected by intercellular bridges (ring canals) and thus share a common cytoplasm (for review see Mahowald and Kambysellis, 1980). Nonetheless, only a single cell in each cyst differentiates to form an oocyte, while the remaining 15 cells become polyploid nurse cells. The nurse cells contribute cytoplasmic components to the oocyte, including a number of mRNAs that accumulate specifically in the oocyte (Wharton and Struhl, 1989; Suter and Steward, 1991; Ephrussi et al., 1991; Kim-Ha et al., 1991; Dalby and Glover, 1992). Through most of oogenesis, therefore, the oocyte is a specialized region within the cytoplasm of a syncytium, rather than a discrete cell.

The mechanisms that generate and maintain cytoplasmic asymmetry within the syncytial cysts during oocyte differentiation, and within the oocyte during axis specification, are not understood. The cytoskeleton is believed to organize the cytoplasm in eucaryotic cells, however, and is likely to play significant roles in generating these developmentally important cytoplasmic asymmetries. Consistent with this hypothesis, microtubule assembly inhibitors disrupt the anterior localization of *bicoid* mRNA, the primary anterior morphogen in the *Drosophila* egg (Pokrywka and Stephenson, 1991). Similarly, movement of the *Xenopus* Vg1 mRNA to the vegetal cortex of the oocyte appears to require microtubules, while maintaining the vegetal localization of this mRNA is dependent on actin filaments (Yisraeli et al., 1990). Two previous observations suggest that microtubules play an important role in oocyte differentiation. First, ultrastructural analyses indicate that centrioles dissociate from the germline nuclei and migrate through the cytoplasmic bridges to the pro-oocyte, and this process is initiated before the oocyte can be unambiguously identified by nuclear morphology (Mahowald and Strassheim, 1970; Carpenter, 1975). Because centrioles are central structural elements of centrosomes, which are the primary microtubule organizing

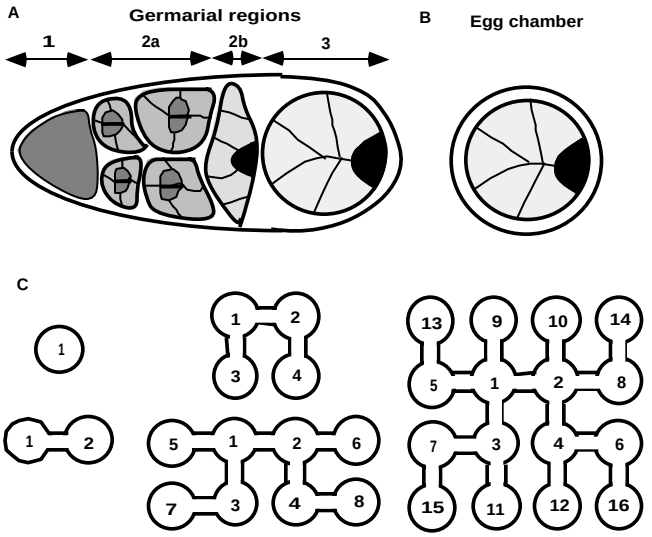

Fig. 1. Schematic diagram of the germarium and early egg chambers of *Drosophila*. (A) Cellular organization of the germarium. Germarial region 1 contains germ line stem cells and the mitotic cystoblasts. Germarial region 2a contains early 16 cell cysts. Cysts in region 2a span approximately half the width of the germarium. At this time, synaptonemal complexes form in the two cells with four ring canals, which thus initiate oocyte development. The later region 2b cysts are lens shaped and span the entire width of the germarium. In this region, synaptonemal complexes breakdown in one of the cells with four ring canals, which will form one of the 15 nurse cells. The second cell with four ring canals will form the oocyte. The oocyte occupies a characteristic central position in region 2b cysts, which are surround by somatic follicle cells. The round region 3 cysts are also referred to as stage 1 egg chambers. The oocyte is positioned at the posterior pole of these egg chambers. (B) Organization of stage 2 through 5 egg chambers. Stage 2 egg chambers are formed as the germ cell-follicle cell complex pinches off of the germarium. The egg chambers retain the same shape but grow in size until stage 5. The oocyte is positioned at the posterior pole of the egg chamber. The cell types in panels a and b are indicated as follows: the germ cell cysts are shaded; the two pro-oocytes in region 2a are striped; the single oocytes in regions 2b and 3 are black; the follicle cells are unshaded. (C) Pattern of intercellular bridges (ring canals) that result from the four incomplete mitotic divisions that give rise to the 16 germ cell cysts.

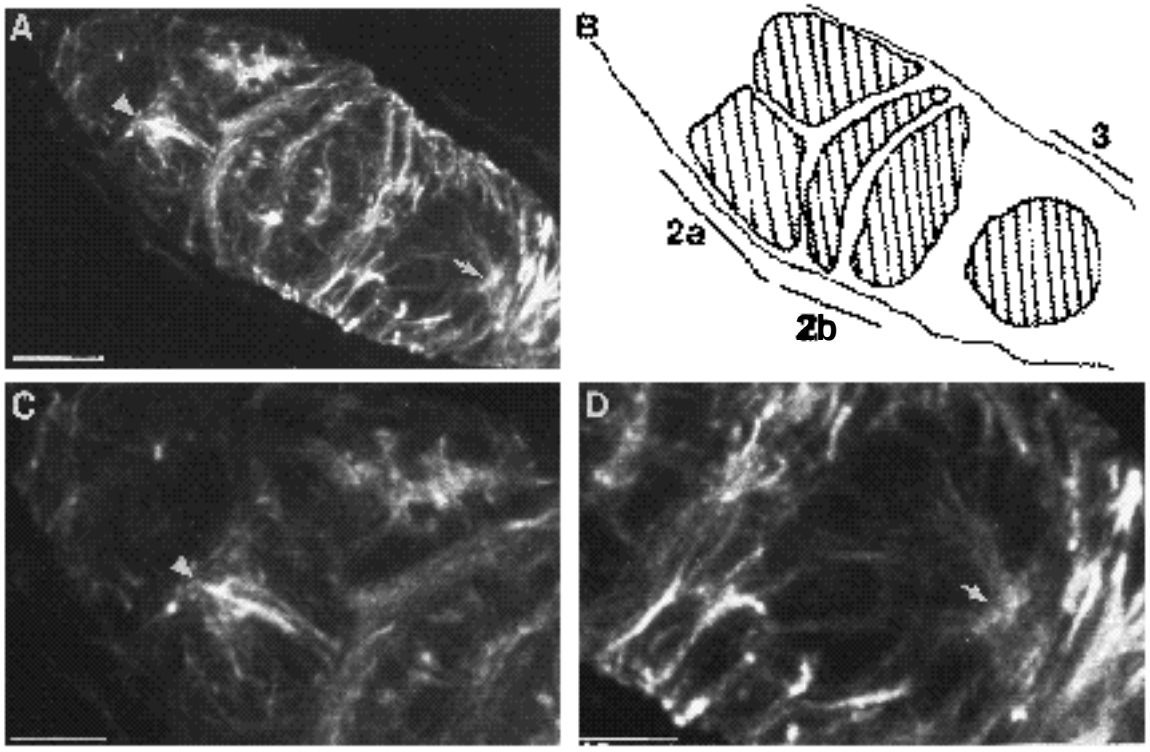

Fig. 2. Formation of a single MTOC in each 16 cell cyst. (A) Microtubule organization in the germarium. This image is a linear projection of 6 optical sections taken at 1 μm intervals with a laser scanning confocal microscope. The arrowhead indicates the focus of microtubule organization in one of the region 2a cysts. In this example the MTOC is toward the anterior of the cyst. See Results for a discussion of pro-oocyte position in region 2a cysts. The single prominent MTOC, within the oocyte in the region 3 cyst, is indicated by the arrow. (B) Schematic diagram of the germarium shown in A. The germ cell cysts are striped, and the somatic cells are unshaded. The positions of region 2a, 2b, and 3 cysts are indicated. This germarium is oriented with posterior towards the lower right and anterior towards the upper left. (C) Details of microtubule organization in the germarial region 2a cyst shown in A. The apparent MTOC is indicated by an arrowhead. (D) Details of microtubule organization in the germarial region 3 cyst shown in A. The position of the MTOC is indicated by the arrow. Bars: A, 10 μm; C,D, 5 μm.

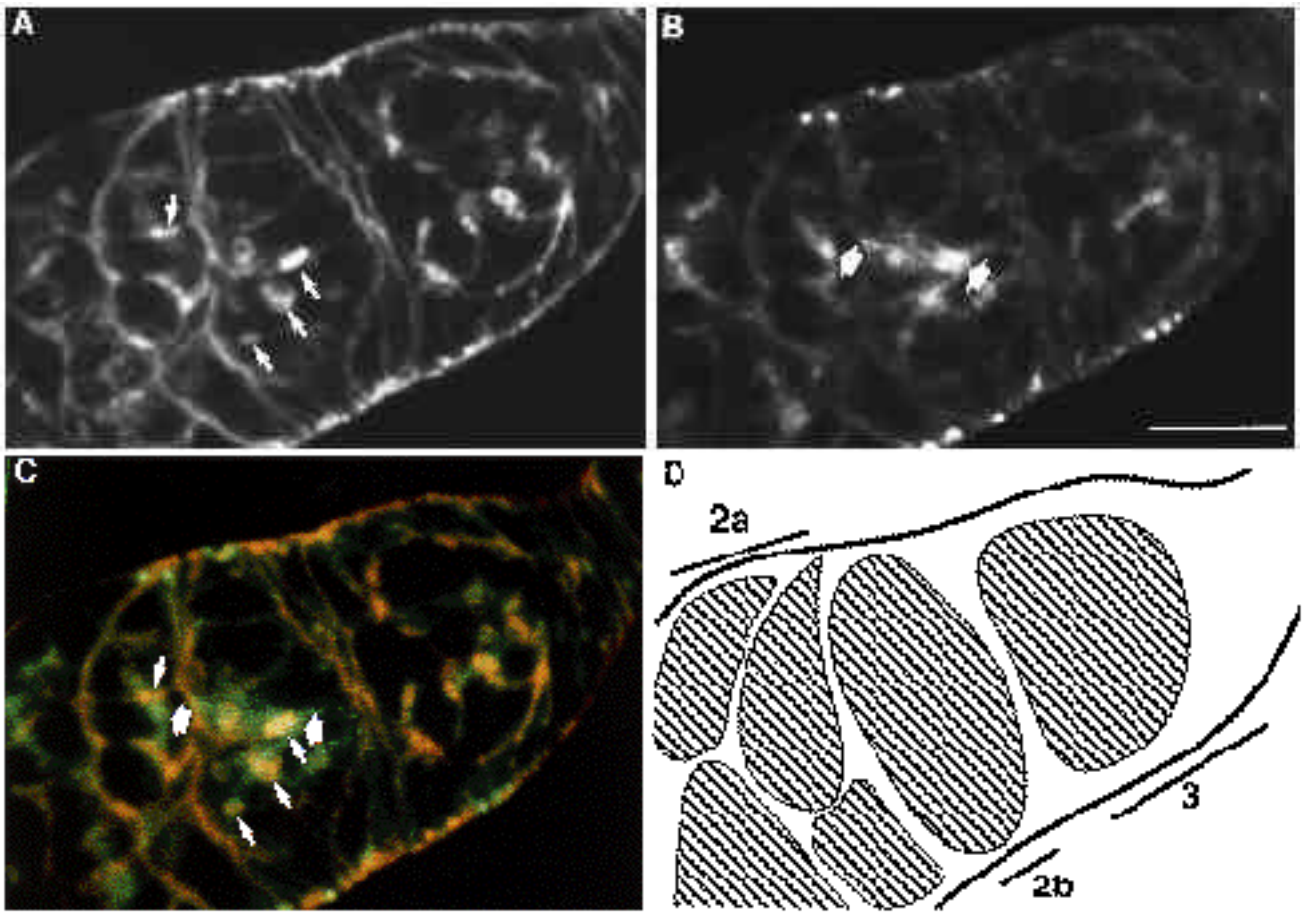

Fig. 3. Microtubules link the pro-oocyte with the remaining cells in each cyst. Actin and microtubule distributions are shown separately in A and B, respectively, and superimposed in C (actin filaments are red and microtubules are green). The organization of the germarium is diagrammed in D. The images are linear projections of 7 optical sections, obtained as described in Fig. 2. The narrow arrows(A,C) indicate actin-staining ring canals, through which microtubules pass. The wide arrows (B,C) indicate the positions of MTOCs in a late region 2a and a region 2b cyst. The germarium is oriented with posterior towards the upper right, and anterior towards the lower left. Bar, 10 μm.

centers in animal cells, this observation implies that microtubule reorganization is closely associated with pro-oocyte specification. Second, flies fed the microtubule-depolymerizing drug colchicine frequently produce germ cell cysts that appear to contain 16 nurse cells (Koch and Spitzer, 1983).

To address more directly the functions of the cytoskeleton in oocyte differentiation, we have analyzed the organization of actin filaments and microtubules during early oogenesis, determined the effects of mutations that disrupt oocyte differentiation on cytoskeletal organization, and evaluated oocyte differentiation and oocyte-specific mRNA localization in ovaries treated with a variety of drugs that inhibit microtubule polymerization. In a previous report, the microtubule cytoskeleton of the oocyte was shown to become axially polarized at the time when mRNAs are first localized to the anterior and posterior poles (Theurkauf et al., 1992). This observation, along with the inhibitor studies described above (Pokrywka and Stephenson, 1991), suggests an important role for microtubules in axis specification (Theurkauf et al., 1992). The present study indicates a similarly central role for microtubules during the earlier process of oocyte differentiation.

Review of early oogenesis in *Drosophila*

Drosophila ovaries are composed of a series of ovarioles, which are divided into anterior and posterior compartments. Oogenesis is initiated within the anterior compartment, or germarium (for a review of oogenesis, see Mahowald and Kambysellis, 1980). The germarium has been subdivided into four cytologically distinct regions (Fig. 1A). Region 1 contains the stem cells and the mitotically dividing cystoblasts, which are derived from the stem cells. As described above, the cystoblast divisions are incomplete, and the 16 cells that result are interconnected by cytoplasmic bridges. The pattern of interconnections that result from these incomplete divisions is diagrammed in Fig. 1C. The newly formed 16 cell cysts are found in region 2a, and these cysts span approximately one half the width of the germarium. In region 2a, synaptonemal complexes form in the two germ cells with four cytoplasmic bridges (Koch et al., 1967; Day and Grell, 1976; Carpenter, 1975). Both of these cells, therefore, begin oocyte differentiation and are referred to as pro-oocytes.

The oocyte becomes morphologically distinct from the remaining 15 germline cells in region 2b (Koch et al., 1967;

Day and Grell, 1976). At the transition between region 2a and 2b, cysts contain two cells with synaptonemal complexes (Carpenter, 1975). Within region 2b, synaptonemal complexes begin to break down in one of the pro-oocytes, and synaptonemal complexes are present in a single cell in region 3 cysts. Therefore, the cell which will form the oocyte appears to be specified within region 2b.

The future oocyte occupies a characteristic position within region 2b and 3 cysts. This cell is in the center of the lens shaped region 2b cysts, and at the posterior pole of region 3 cysts (Fig. 1A). Several mRNAs begin to accumulate in the oocyte during these stages (Wharton and Struhl, 1989; Kim-Ha et al., 1991; Suter and Steward, 1991; Ephrussi et al., 1991; Dalby and Glover, 1992).

The germ cell-follicle cell complex in region 3 is referred to as a stage 1 egg chamber.Stage 2 egg chambers are formed when the germ cell-follicle cell complex pinches off from the germarium (Fig. 1B) and enters the posterior compartment of the ovariole, which is referred to as the vitellarium.

Egg chambers mature as they move through the ovariole, and have been divided into 14 morphologically distinct stages (Cummings and King, 1969). The present study addresses cytoskeletal organization and function through stage 5. During stages 2 through 5 the oocyte remains approximately the same size as a single nurse cell, and cytological differences between the oocyte and nurse cell nuclei become apparent at the light microscopic level. The nurse cell nuclei increase in size through multiple rounds of endomitotic DNA replication, and the nuclear surface becomes irregular. In contrast, the oocyte nucleus proceeds through meiotic prophase and remains compact and smooth-surfaced. The nurse cells and oocyte can also be distinguished using molecular markers. During stages 1 through 5, the vasa antigen is present at the surface of the nurse cell nuclei, but is excluded from the oocyte (Lasko and Ashburner, 1990; Hay et al., 1990; see below), and the mRNAs which accumulate in region 2b pro-oocytes remain concentrated in the oocyte (Wharton and Struhl, 1989; Kim-Ha et al., 1991; Suter and Steward, 1991; Ephrussi et al., 1991; Dalby and Glover, 1992). The oocyte and nurse cells are therefore easily distinguishable in early egg chambers.

METHODS

Ovariole isolation, fixation and immunofluorescence

Portions of individual ovarioles containing the germarium and stage 1 through 5 egg chambers were mass isolated from wild-type (Oregon R) flies by a modification of the procedure of Mahowald et al. (1983), as described previously (Theurkauf and Hawley, 1992; Theurkauf et al., 1992).

Whole ovaries were dissected from *Bic-D* and *egl* mutant females in either modified Robb's medium (see Theurkauf and Hawley, 1992), or Grace's insect medium (GibCo). Whole ovaries were then fixed as described for dissociated ovaries prepared by the mass isolation method (Theurkauf et al., 1992). Prior to immunolabeling, fixed mutant ovaries were mechanically teased apart with fine point (no. 5) dissecting forceps, to reveal individual ovarioles. Ovarioles were then incubated in PBS containing 1% Triton X-100 for 1-2 hours, and transferred to PBS containing 0.05% Triton X-100. Egg chambers were immunolabeled for microtubules using a rhodamine conjugated anti-α-tubulin antibody, nuclei were labeled with 4,6-diamidino-2-phenylindole, and actin filaments were labeled using fluorescein-conjugated phalloidin (Sigma, St. Louis MO), as described previously (Theurkauf et al., 1992). Fluorescently labeled ovaries were resuspended in mounting medium (Theurkauf, 1992), transferred to glass slides, and sealed under a cover glass with nail polish.

Laser scanning confocal microscopy was performed using a Nikon optiphot fluorescence microscope equipped with the BioRad MRC 600 laser scanning confocal attachment. All images were collected using a Nikon 60X Plan Apo lens with a numerical aperture of 1.4.

For both wild-type and mutant germaria, at least five independent fixation and immunolabeling reactions were performed and analyzed. Initial screening was performed on a Nikon FXA microscope equipped with an epifluorescence attachment. Typical samples contained 20-50 well separated germaria. Antibody penetration into the germline cells varied somewhat from preparation to preparation, and between individual germaria. The images shown here are typical of germaria showing strong germline microtubule staining, which varied from approximately 20-80% of each sample pool. In the other germaria, microtubule were not visible, or were weakly stained and could not be effectively imaged. We assume that this weak labeling was due to poor antibody penetration or insufficient microtubule fixation, rather than variability in the actual microtubule content of the cysts.

Inhibitor treatment

To determine the effects of microtubule assembly inhibitors on oogenesis, adult female flies were fed yeast paste supplemented with drug (Koch and Spitzer, 1983). To prepare the yeast paste, inhibitor stocks, in ethanol, were diluted to the desired concentration with distilled water. Dry bakers yeast was then added to form a smooth paste. Flies were fed inhibitor supplemented yeast paste continuously for 6-48 hours before egg chambers were isolated and fixed (Theurkauf et al., 1992).

To determine the effect of microtubule depolymerization on mRNA localization, flies were continuously fed 50 μg/ml colchicine for 6, 12, 18, or 24 hours before ovaries were isolated and fixed. Half of the material from each time point was then stained for microtubules, to access the effectiveness of the inhibitor treatment, and the remaining material was subjected to in situ hybridization using specific probes.

Whole-mount in situ hybridization with *osk*, 65F and *cyclin B* probes was performed as described by Tautz and Pfeiffe (1989), with the following modifications. Oocytes were either hand dissected or mass isolated and fixed as described previously (Theurkauf and Hawley, 1992). Fixed egg chambers were digested with 50 μg/ml proteinase K in PBS/1.0 mM Na_3EDTA at room temperature for 5-10 minutes. The protease treatment was stopped and the oocytes were taken through the rest of the procedure as described by Tautz and Pfieffe (1989).

Strains

Bic-D^{PA66}, *Bic-D*^{R26}, *egl*^{WU50} and *egl*^{RC12} are described elsewhere (Schüpbach and Wieschaus, 1991; Suter et al., 1989), and *Df(2)119* is described by Lindsley and Zimm (1992). For cytological analysis of the *Bic-D* alleles, hemizygous mutants were examined *(Bic-D/Df(2)119)*. Transheterozygotes of *egl*^{RC12}/*egl*^{WU50} were examined to determine the effect of *egl* mutations on cytoskeletal organization.

RESULTS

Reorganization of the germline cytoskeleton

Cytoskeletal asymmetry develops within the 16 cell cysts as

they pass through the germarium (Fig. 2). Microtubule and actin organization appear uniform in the dividing cystoblasts of region 1, and in some region 2a cysts. However, microtubules are clearly organized around a single focus within a subset of region 2a cysts (Fig. 2A,C, arrowheads). Because the region 2a cysts are arranged randomly with respect to each other and the axis of the germarium (Mahowald and Strassheim, 1970), the positions of the pro-oocytes within these early cysts cannot be predicted. Therefore, it is not possible to determine if the microtubule foci in region 2a cysts are within one of the two pro-oocytes, or within a pro-nurse cell. However, the cysts rearrange during germarial development, and the oocyte occupies a characteristic position in the center of the lens shaped region 2b cysts, and is at the posterior of region 3 cysts. The MTOC is similarly located during these later stages, indicating that it lies within the oocyte (Figs 2, 3). In early egg chambers the oocyte can be identified morphologically, and the MTOC is clearly within the oocyte during these stages (Theurkauf et al., 1992).

Actin begins to accumulate in the cytoplasmic bridges, or ring canals, in late region 2a cysts. Double labeling germaria for actin and microtubules indicates that microtubules originating at the MTOC pass through the actin-containing ring canals and into the adjacent cells. Fig. 3 shows a germarium double labeled for actin and microtubules. Within the region 2b cyst in this germarium, a prominent MTOC is located at the expected position of the oocyte (Fig. 3B, thick arrow). Microtubules that appear to originate at this MTOC pass through ring canals and into the adjacent cells (Fig. 3C, arrows). A similar organization is also apparent in the somewhat elongated cyst at the boarder between region 2a and 2b. Ultrastructural analysis of the germarium reveals midbody microtubules in the ring canals during the cystoblast divisions (Mahowald and Strashiem, 1970; Carpenter, 1975). The ring canals are cleared of midbody microtubules shortly after completion of the cystoblast divisions, and microtubules then reappear in the ring canals of region 2a cysts (Mahowald, 1971). We believe that the microtubules revealed by immunofluorescence correspond to the microtubules found to repopulate the ring canals in earlier ultrastructural studies. These data indicate that a polarized microtubule network begins to form in region 2a, and that within region 2b this network interconnects a cell at the position of the future oocyte with the remaining cells in the cyst.

Actin filament accumulation within developing germarial cysts is also asymmetric (Fig. 3A). The largest ring canal in each cyst links the two cells with four ring canals, one of which will form the oocyte (Koch and King, 1969). Ring canal size and actin accumulation decrease as a function of distance from the pro-oocyte, so that a size gradient of ring canals forms within each cyst. In region 3 cysts and later egg chambers, where the oocyte is always found at the posterior pole, a posterior to anterior size gradient in the actin staining ring canals is apparent (Warn et al., 1985; Theurkauf et al., 1992; Fig. 3A). During early oogenesis the size and amount of filamentous actin in the ring canals increase, and the differences in actin staining between the ring canals associated with the oocyte and ring canals associated with other cells become more pronounced. This differential actin accumulation in the ring canals therefore appears to respond to positional information within each cyst.

Microtubule assembly inhibitors block oocyte differentiation

The organization of the cytoskeleton during early oogenesis is consistent with an important role for the microtubule cytoskeleton in oocyte differentiation. The developmental consequences of disrupting germline microtubule organization were therefore of interest. Koch and Spitzer (1983) have reported that feeding adult females the microtubule depolymerizing drug colchicine leads to formation of egg chambers that appear to contain 16 nurse cells. We have extended the results of Koch and Spitzer (1983) by determining the effects on oogenesis of microtubule-depolymerizing drugs other than colchicine, and by showing that inhibitor treatment induces the expected microtubule depolymerization in the ovary. In addition, we used molecular markers for the oocyte and nurse cells that have only recently become available.

Interpretation of biological defects induced by microtubule assembly inhibitors can be complicated by toxic effects of the drugs that are not directly related to microtubule depolymerization. To help determine if the developmental defects observed with colchicine are due to microtubule depolymerization, we evaluated the effects on microtubule morphology and oocyte differentiation of the additional microtubule assembly inhibitors colcemid, podophyllotoxin, and vinblastin. Podophyllotoxin is structurally related to colchicine and colcemid, but produces distinct side effects from these compounds. Vinblastin is structurally and pharmacologically distinct (see Dustin, 1978). All of these inhibitors produce the same developmental and cytological defects as described below with colchicine. As a further control, microtubule morphology and oocyte differentiation were examined in flies fed lumicolchicine, an inactive isomer of colchicine. This compound had no effect on either microtubule morphology or oocyte formation (data not shown). We therefore conclude that the developmental defects observed with all of these inhibitors are the result of microtubule depolymerization.

Analysis of microtubule organization in ovarioles isolated from adult females fed varying concentration of colchicine revealed that germline and somatic cell microtubules differ in their stability. Feeding flies 20-50 μg/ml colchicine for up to 48 hours disrupted germline microtubules in the majority of ovarioles, but had little effect on microtubules in the somatic follicle cells (Fig. 4A). Feeding females 2 μg/ml colchicine for up to 24 hours failed to consistently disrupt germline microtubules, whereas microtubules in both the follicle cells and germline cells were disrupted in flies fed 1 mg/ml colchicine for 24 hours (Fig. 4C).

When flies are fed colchicine at 20-50 μg/ml, germaria decrease in size with increasing length of inhibitor treatment (data not shown). This observation suggests that the mitotic divisions that initiate oogenesis are arrested by this treatment, but that new egg chambers continue to be produced from the germarial cysts present at the time inhibitor treatment was initiated. The microtubules that remain in the follicle cells of colchicine-fed females,

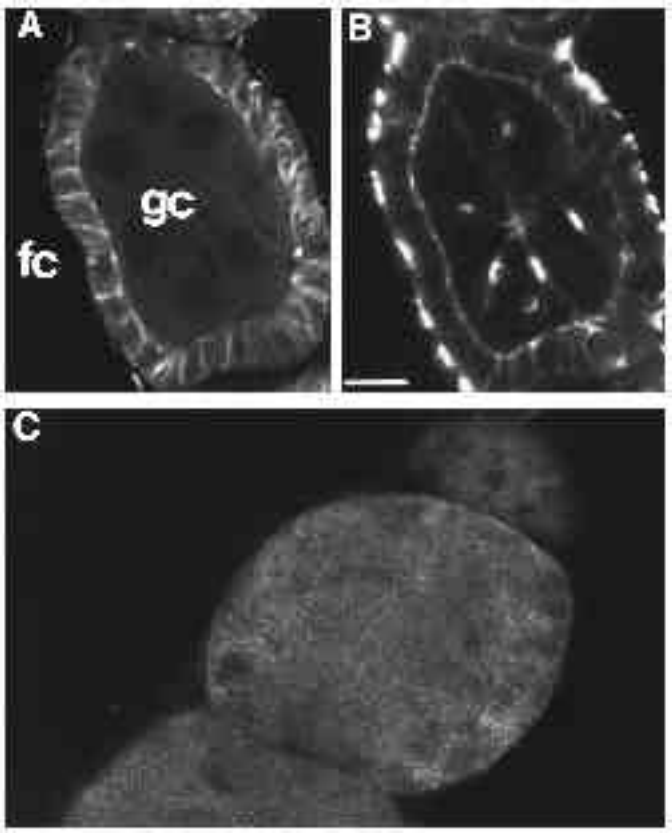

Fig. 4. Microtubules in germline and somatic cells of the ovary are differentially sensitive to colchicine treatment. Microtubules (A) and actin filaments (B) in a stage 3 egg chamber isolated from a female that was fed 20 μg/ml colchicine for 48 hours. No microtubules are observed in the germline cells (gc), while microtubules are abundant in the monolayer of somatic follicle cells surrounding the germline cells (fc). Actin organization is unaffected by colchicine treatment (B). The egg chamber shown in A and B is oriented with anterior down. (C) Microtubules in a stage 2 or 3 egg chamber isolated from a female fed 1 mg/ml colchicine for 24 hr. No microtubule staining is observed in either germline or somatic cells. Bar, 10 μm.

therefore, appear to be sufficient to support the follicle cell movements needed for egg chamber morphogenesis.

To determine the effect of germline microtubule disassembly on oocyte differentiation, flies were fed 50 μg/ml colchicine for varying times before ovarioles were isolated, fixed, and double labeled either for microtubules and actin filaments, or for nuclei and vasa protein. Actin and microtubule markers were used to determine the effectiveness of the colchicine treatment, and to access egg chamber morphology. Nuclear size, nuclear morphology, and vasa protein distribution were used to distinguish nurse cells from oocytes. A control egg chamber stained to reveal nuclei and vasa protein is shown in Fig. 5A. During stages 2 through 5 the nurse cell nuclei are irregularly shaped and have a greater diameter than the oocyte nucleus. In addition, the nurse cell nuclei accumulate the vasa antigen, which is excluded from the oocyte.

We find that feeding females colchicine for 16-48 hours leads to the production of egg chambers containing 16 nurse cell nuclei. An example of such an egg chamber is shown in Fig. 5C-F. All 16 germline nuclei in this chamber labeled strongly with the anti-vasa antibody, had irregular surface morphology, and were of uniform size. Based on these morphological criteria, the 16 cells in this cyst are nurse cells.

The number of 16-nurse cell egg chambers present in an individual ovariole increased as the length of inhibitor treatment was increased. In control flies, and flies fed colchicine for less than 16 hours, all of the egg chambers in the vitellarium consistently contained an identifiable oocyte. After 16 hours of colchicine treatment, a single 16-nurse cell egg chamber was often located proximal to the germarium; at the position of a newly formed stage 2 egg chamber. After 48 hours of inhibitor treatment, individual ovarioles contained up to 4 16-nurse cell egg chambers. These egg chambers were always contiguous within the ovariole, and proximal to the germarium.

The position of the 16-nurse cell egg chambers in the ovariole, and the decrease in germarial size with increasing length of inhibitor treatment, suggest that germarial cysts give rise to the 16-nurse cell egg chambers observed in the vitellarium. Stage 1 egg chambers have been estimated to remain in region 3 of the germarium for approximately 10 hours before entering the vitellarium as stage 2 egg chambers (Mahowald and Kambysellis, 1980). Therefore, the single 16-nurse cell egg chambers observed at 16 hours were likely to be in germarial region 2b at the time inhibitor treatment was initiated, while the four 16-nurse cell egg chambers found in ovarioles after 48 hours were in germarial regions 2a and 2b when inhibitor treatment was initiated.

In these inhibitor experiments, the egg chambers posterior to those containing 16 nurse cells always have an identifiable oocyte nucleus. At the time inhibitor treatment was initiated, these egg chambers were likely to have been in germarial region 3, or to have budded off the germarium and formed egg chambers. Therefore, the differentiated morphology of the oocyte nucleus in stage 1 through 5 egg chambers is not affected by colchicine treatment. Differentiation of the oocyte therefore appears to be uniquely sensitive to microtubule depolymerization when cysts are within germarial region 2.

Within colchicine-treated stage 1 through 5 egg chambers, which had an identifiable oocyte nucleus, the oocyte compartment was consistently smaller than a single nurse cell. This is in contrast to control egg chambers, in which the cytoplasmic compartment containing the oocyte remains approximately the same size as a single nurse cell. This observation suggests that microtubules are required for oocyte growth during early egg chamber development in the vitellarium.

egl and recessive *Bic-D* mutations disrupt the MTOC

Recessive mutations at the *egl* and *Bic-D* loci disrupt oocyte differentiation, leading to production of egg chambers containing 16 nurse cells (Schüpbach and Wieschaus, 1991). This phenotype is essentially identical to that produced by microtubule assembly inhibitors, raising the possibility that the *Bic-D* and *egl* gene products are involved in germline

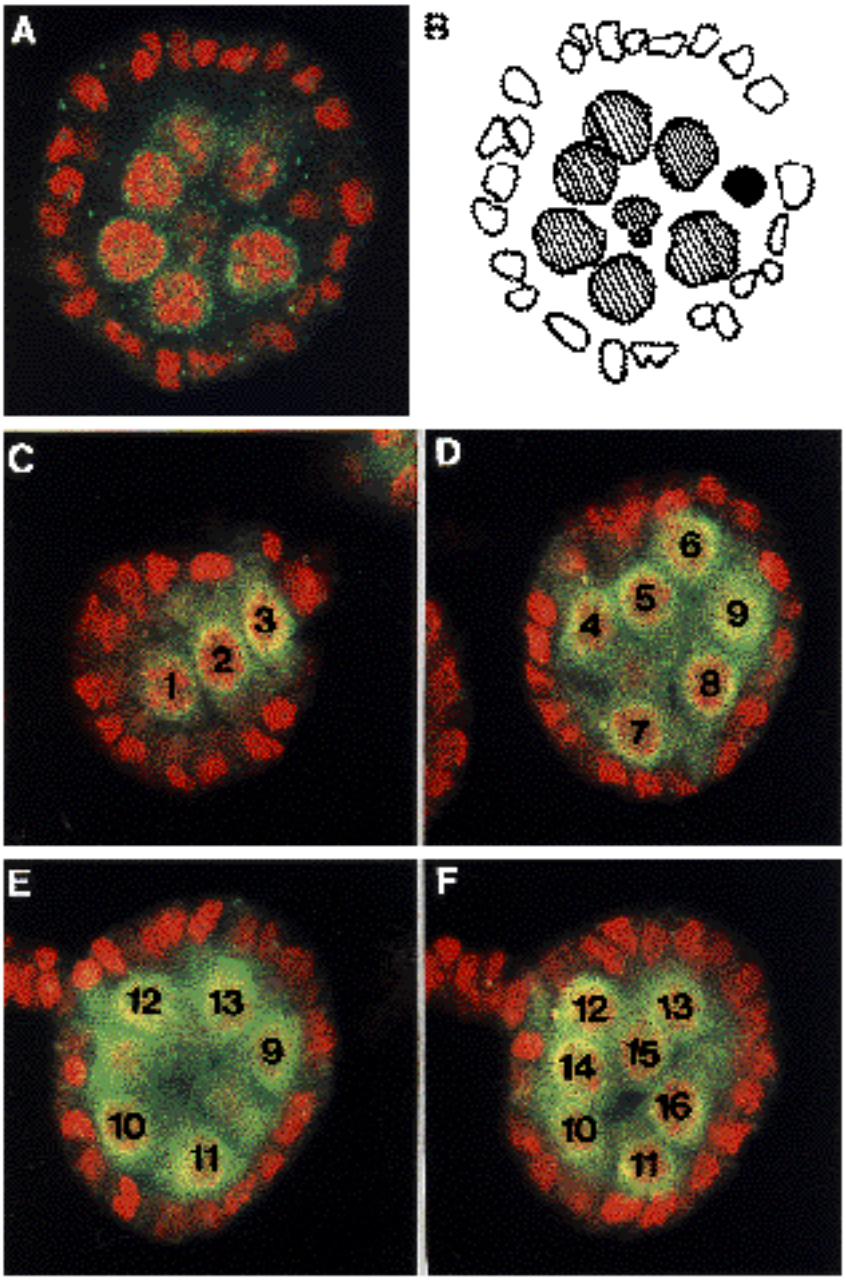

Fig. 5. Microtubule assembly inhibitors block oocyte differentiation. (A) Control stage 3 egg chamber, double labeled with propidium iodide to reveal nuclei (red) and anti-vasa protein antibody (green). The nurse cell nuclei are larger than the follicle cell nuclei, and label with the anti-vasa antibody. In contrast, the oocyte nucleus is small, smooth surfaced, and does not label with anti-vasa antibody. (B) Organization of the nuclei in the egg chamber shown in A. The oocyte is black, nurse cells are shaded, and the follicle cells are unshaded. (C-F) Optical sections revealing all 16 germline nuclei in an early egg chamber isolated from a female fed colchicine for 48 hours. All 16 nuclei are larger than the follicle cell nuclei and stain intensely with the anti-vasa antibody, indicating that they nurse cells. No oocyte is apparent.

microtubule function. We therefore examined the organization of microtubules and actin filaments in *egl* and *Bic-D* mutant ovaries.

Microtubule reorganization appears to be initiated normally in *egl* mutant germaria, but the MTOC that forms is unstable. Single microtubule foci are present in region 2a *egl* cysts. In region 2b and 3 cysts, however, microtubules are evenly distributed among the germline cells (Fig. 6A,C). *egl* function therefore appears to be required to maintain the polarized microtubule cytoskeleton in region 2b and later cysts.

Bic-D function, in contrast, appears to be required to initiate microtubule reorganization. Single microtubule foci were not apparent in germaria isolated from females hemizygous for either the *Bic-D*^{PA66} (Fig. 7A,C) or *Bic-D*^{R26} (Fig. 7E) mutations. Microtubules were instead dispersed throughout the germline cells of these mutant cysts, or several small foci were present.

While *Bic-D* function appears to be essential for polarization of the microtubule cytoskeleton, it is not required to establish the polarized organization of actin filaments. In both *Bic-D*^{PA66} and *Bic-D*^{R26} ovaries, the largest ring canal is associated with a single cell at the normal posterior position of the oocyte (Fig. 7D,F). As in wild-type cysts, the remaining ring canals decrease in size as a function of distance from the posterior pole (Fig. 7D). Polarization of the actin cytoskeleton and polarization of the microtubule cytoskeleton are therefore genetically separable processes.

Microtubules and asymmetric mRNA localization

A number of mRNAs appear to be synthesized in the nurse cells and preferentially accumulate in the developing oocyte,

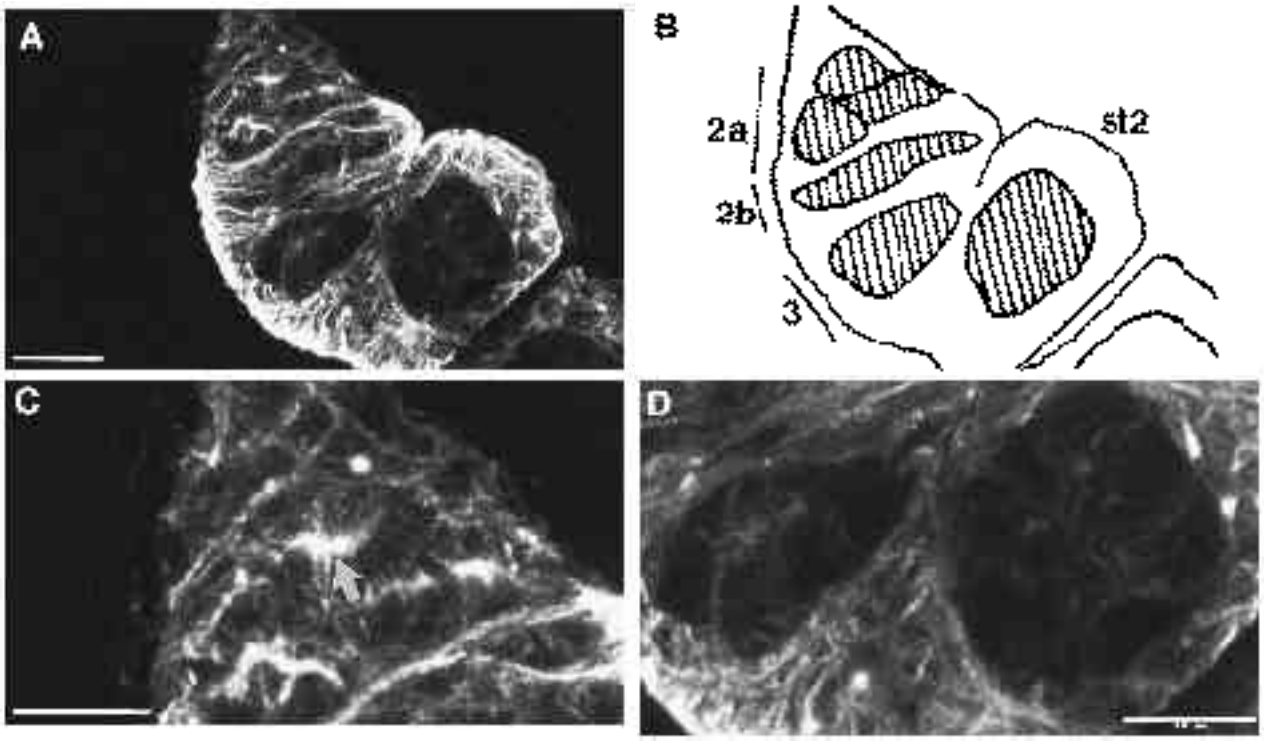

Fig. 6. Maintenance of the MTOC in germline cysts is disrupted in *egl* ovaries. (A) Microtubule organization in a egl^{wu50}/egl^{R26} germarium. (B) Organization of cysts in the germarium shown in A. Prominent MTOCs are present in region 2a cysts. In region 2b and 3 cysts, however, no clear MTOC is apparent. (C) Details of region 2a of the germarium shown in A. The MTOC in a region 2a cyst is indicated by the arrow. (D) Details of the region 3 cyst and the newly forming stage 2 egg chamber shown in A. Microtubules are dispersed throughout germline cells in both cysts. Bars, 10 μm.

suggesting that they are actively transported within the cysts. Because microtubules are known to mediate transport in other systems, we examined the effect of colchicine treatment on the location of *Bic-D*, *osk*, *cyclin B*, and *65F* mRNAs, all of which accumulate in the oocyte in later germarial cysts and early egg chambers. Oocyte-specific accumulation of *osk*, *Bic-D* and *cyclin B* mRNAs have been described previously (Kim-Ha et al., 1991; Ephrussi et al., 1991, Suter and Steward, 1991; Dalby and Glover, 1992), whereas 65F is a newly identified oocyte-specific mRNA (H. Ruohola-Baker and T. A. J., unpublished data).

Fig. 8 shows *osk*, *cyclin B*, and 65F mRNA distributions in control ovarioles, and in ovarioles isolated from flies fed colchicine for 12 hours. All three mRNAs were concentrated in the oocyte in the controls, but were uniformly distributed after inhibitor treatment (Fig. 8). In this experiment, microtubules in germline cells were disrupted in essentially all egg chambers (not shown). The mislocalization of these mRNAs in inhibitor-treated ovaries is highly reproducible. In 5 separate experiments we observed a loss of oocyte-specific mRNA localization in ovaries isolated from flies fed inhibitor for 12 hours or longer. In each experiment the time required to produce a consistent loss of localization correlated with the time required to consistently depolymerize germline microtubules. These observations strongly suggest that microtubules are required to maintain asymmetric localization of selected mRNAs within early egg chambers.

In ovarioles isolated from flies fed colchicine for 6 hr, microtubules were disrupted in some ovarioles, but microtubule organization was similar to controls in others. This variability is likely to be due to differences in the amount of yeast paste consumed, and the time when feeding was initiated, by individual flies. The *cyclin B* and 65F mRNA distributions were similarly variable at this time point. In contrast, osk mRNA localization was consistently oocyte-specific at this early time point. This observations suggests that oocyte-specific localization of *osk* mRNA may not be directly dependent on microtubule function.

DISCUSSION

Cytoskeletal asymmetry within germarial cysts

Cytological analysis of microtubule and actin reorganization during early oogenesis indicates that cytoskeletal asymmetry develops very early in germarial development. Within a subset of the newly formed 16 cell cysts in region 2a, and all region 2b cysts, a prominent MTOC is observed (Fig. 2). In later region 2b and 3 cysts, the single MTOC is located at the expected position of the oocyte, and microtubules originating at this MTOC pass through the ring canals that join the oocyte with the nurse cells, indicating that the oocyte is linked to the remaining cells in each cyst by a single microtubule cytoskeleton. In cysts entering region 2b, both pro-oocytes contain synaptonemal complexes (Carpenter, 1975). Microtubule asymmetry therefore develops before the oocyte can be unambiguously identified by nuclear morphology. If the MTOC remains

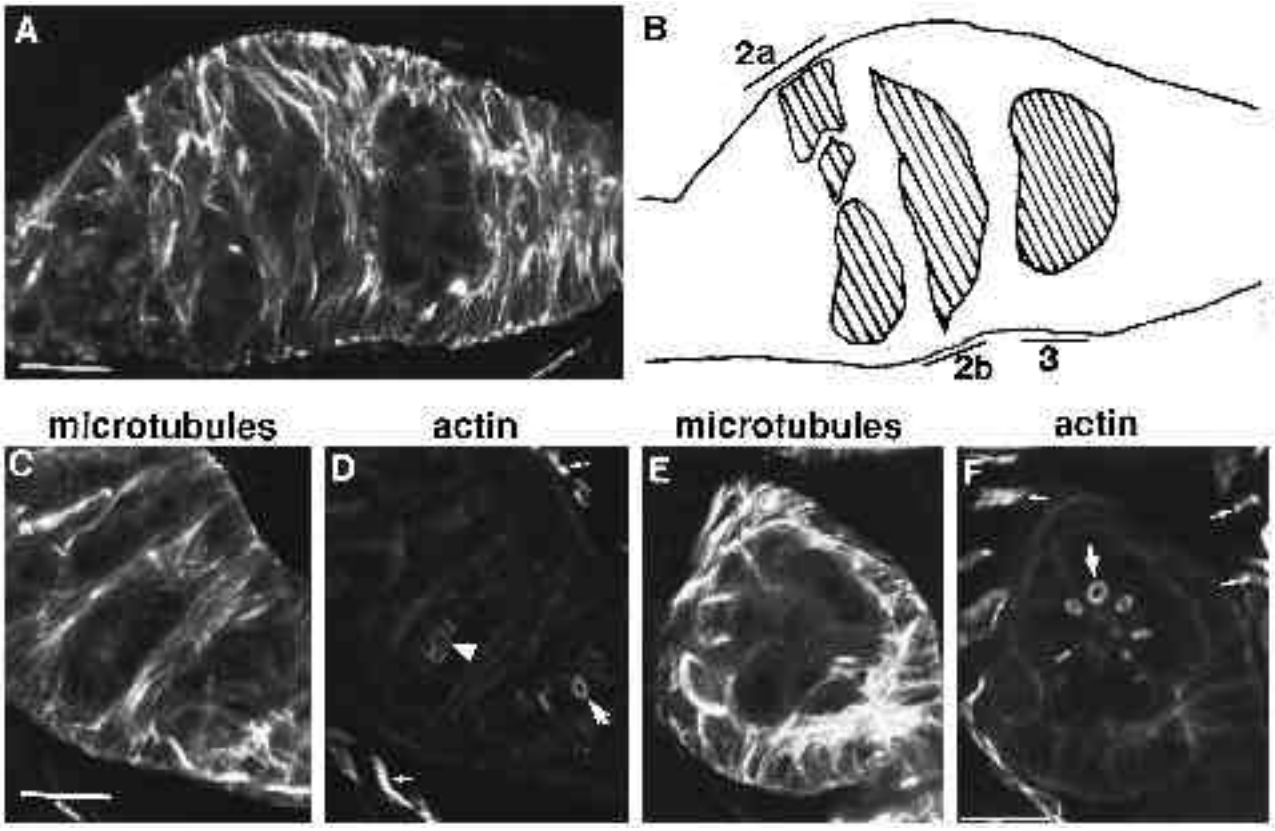

Fig. 7. Recessive *Bic-D* mutations block formation of the MTOC in germarial cysts. (A) Microtubule distribution in *Bic-D*^{PA66}*/Df(2)TW119* germarium. Microtubule foci are not apparent in any of the cysts. (B) Organization of the germarium shown in A. Germline cysts are shaded and somatic cells are unshaded. The positions of regions 2a, 2b, and 3 cysts within the germarium are indicated. (C,D) Microtubule (C) and actin filament (D) co-localization in a *Bic-D*^{PA66}*/Df(2)TW119* germarium. Microtubules are dispersed throughout the cysts. In contrast, actin preferentially accumulates at the ring canals associated with a single cell at the center of the cysts in region 2b (arrowhead) and 3 (large arrow). Microtubule (E) and actin (F) organization in a stage 3/stage 4 *Bic-D*^{R26}*/Df(2)TW119* mutant egg chamber. Microtubules are dispersed throughout the cyst (E). In contrast, ring canal size, revealed by actin accumulation, shows a wild type size gradient along the anterior-posterior axis (F). The large arrow in F indicates the largest ring canal in the cyst. The small arrows in D and F indicate actin filaments in the striated muscle sheath surrounding each ovariole. Bar, 10 μm.

within the cell in which it originally forms, the single MTOC in region 2a cysts is within the cell that will form the oocyte, and formation of the MTOC precedes and predicts oocyte specification.

Several observations lead us to conclude that polarization of the microtubule cytoskeleton is required for oocyte differentiation. As discussed above, germline microtubule reorganization correlates temporally with oocyte specification. Furthermore, microtubule assembly inhibitors block oocyte differentiation (Fig. 4). In addition, sensitivity to microtubule depolymerization appears to be restricted to cysts within regions 2a and b, which correlates temporally with microtubule reorganization (Figs 2, 3). Finally, the *Bic-D* and *egl* mutations studied here block oocyte differentiation and disrupt establishment and maintenance of the polarized microtubule cytoskeleton (Figs 5, 6). Taken together, these observations indicate that reorganization of the microtubule cytoskeleton is an essential step in oocyte differentiation.

A multi-step model for oocyte differentiation

The analysis of cytoskeletal elements in wild type and mutant ovaries described here, along with previous studies on mRNA and protein localization in *Bic-D* mutant females, indicate that oocyte differentiation involves at least three distinct steps: (1) specification of the cytoplasmic compartment that will form the oocyte; (2) reorganization of microtubules within the 16 cell cyst in response to this asymmetry; (3) maintenance of the resulting polarized cytoskeleton.

Microtubule reorganization appears to be downstream of the initial asymmetry that specifies the oocyte. In wild-type females, the Bic-D protein accumulates in a single cell in region 2a and 2b germarial cysts, and is found specifically in the oocyte in later egg chambers (Suter and Steward, 1991). Accumulation of Bic-D protein therefore appears to mark the cell that will become the oocyte. In *Bic-D*^{R26} hemizygous germaria, localization of Bic-D protein is normal. Because *Bic-D*^{R26} blocks initial reorganization of the microtubule cytoskeleton (Fig. 7), microtubule polarization is not required to generate asymmetric Bic-D protein localization, and is therefore downstream of the asymmetry that is revealed by this localization. Based on these observations, we propose that oocyte differentiation begins by establishing an initial asymmetry within the cysts, and is followed by microtubule polarization in response to this asymmetry.

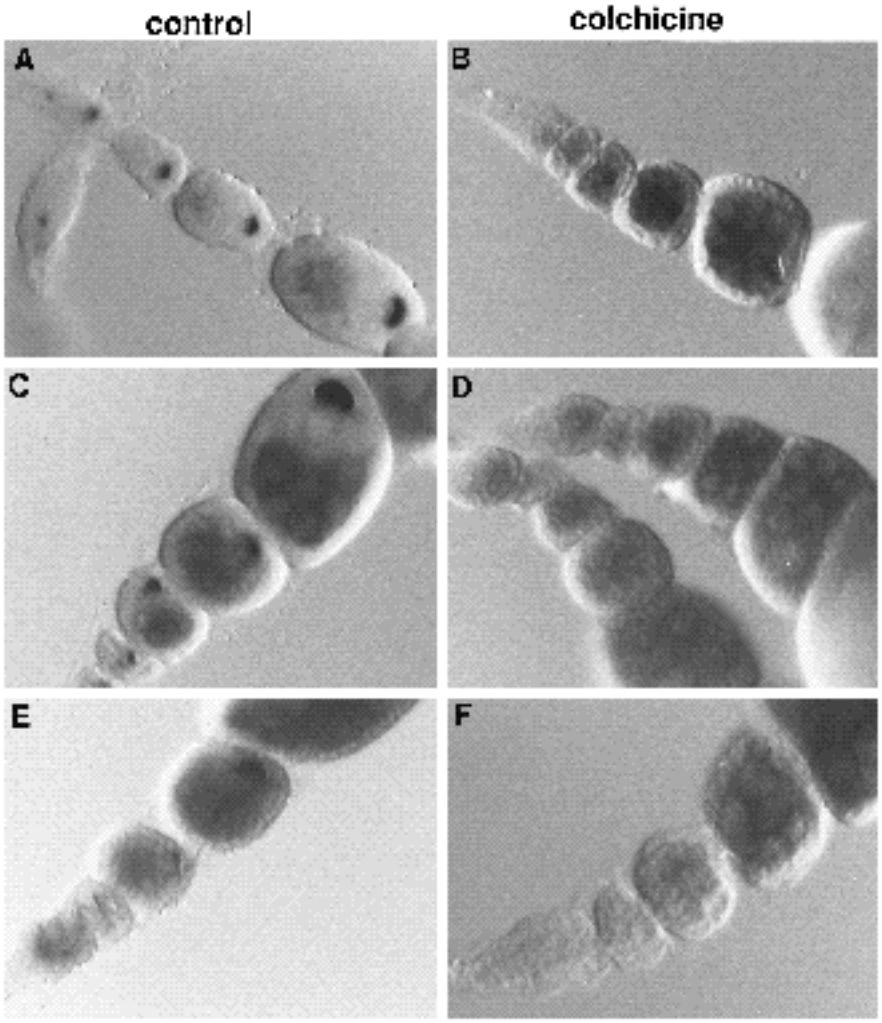

Fig. 8. mRNA localization in colchicine-treated ovarioles. Ovarioles from control and colchicine fed females were probed for oskar (A,B), cyclin B (C,D), and 65F (E,F) mRNAs. All three mRNAs are enriched in the oocyte in control egg chambers (A,C,E), but uniformly distributed in egg chambers isolated from flies fed 50 μg/ml colchicine for 12 hours (B,D,F). Duplicate samples were immunolabeled to reveal microtubule distributions, confirming that germline microtubules were disrupted in each experiment (not shown).

Furthermore, these observations indicate that the Bic-D protein is required for microtubule polarization.

A third step in oocyte differentiation is revealed by microtubule organization in *egl* ovaries. In these ovaries, the MTOC begins to form normally, but the resulting polarized array breaks down (Fig. 6). Establishing the MTOC is therefore genetically distinct from maintaining the resulting polarized array, and these processes involve the sequential action of the *Bic-D* and *egl* genes.

What is the function of the polarized microtubule cytoskeleton within the syncytial cysts? Two observations indicate that a primary function of germline microtubules is to mediate the asymmetric localization of mRNAs within the syncytial cysts. First, the oocyte-specific localization of several mRNAs is disrupted by the *Bic-D* and *egl* mutations, which disrupt germline microtubule organization (Suter and Steward, 1991). Second, inhibitor-induced microtubule depolymerization reverses oocyte-specific mRNA localization (Fig. 8).

Oocyte-specific mRNAs show differential sensitivity to both forced microtubule depolymerization and to the *Bic-D* mutations that disrupt microtubule reorganization. For example, *osk* mRNA is initially localized in *Bic-D* mutant germaria, while the *Bic-D* and *K10* mRNAs are not (Suter and Steward, 1991). We have found that osk mRNA localization is also somewhat resistant to microtubule depolymerization, while oocyte-specific cyclin B and 65F mRNA localization is readily disrupted by colchicine treatment (see Results). These observations suggest that both microtubule-dependent and microtubule-independent mechanisms may lead to oocyte-specific mRNA accumulation.

We speculate that the key function of the microtubule cytoskeleton in oocyte differentiation is to mediate the transport of an oocyte differentiation factor to the future oocyte, and that this factor must accumulate to a critical concentration to induce oocyte formation. Disrupting microtubule organization, either genetically or with microtubule assembly inhibitors, would therefore prevent accumulation of this factor to the required level and therefore lead to the formation of 16 nurse cell cysts. We predict that mutations disrupting such a factor would lead to production of 16 nurse cell cysts in which the microtubule cytoskeleton reorganizes normally. The best candidate for an oocyte differentiation factor is the product of the *egl* locus. The MTOC forms within early *egl* germarial cysts, and this mutation leads to production of egg chambers with 16 nurse cells. However, the MTOC is not maintained in *egl* mutant egg chambers. Maintenance of the MTOC during oogenesis

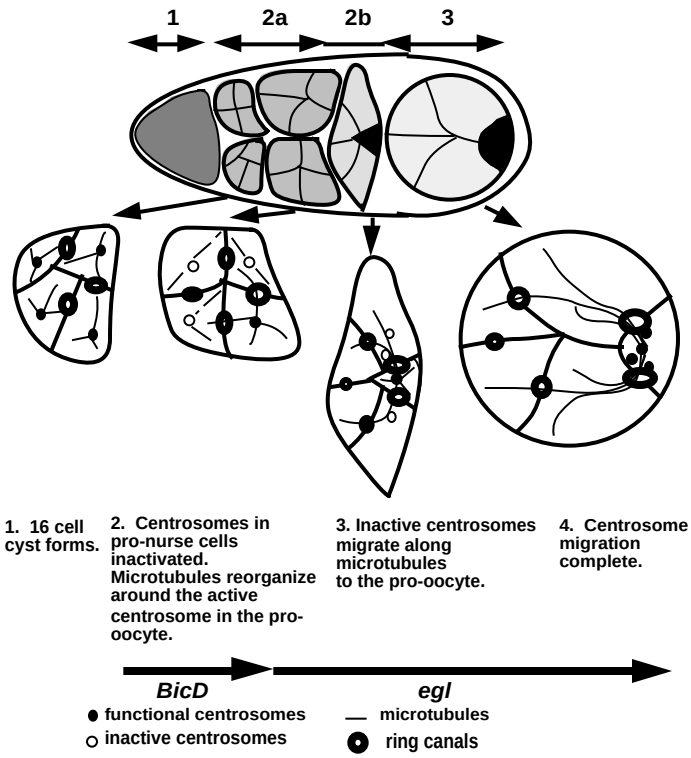

Fig. 9. Model for microtubule reorganization in the germarium. *Bic-D* function is required during the initial stages of microtubule reorganization (step 2), while *egl* function is required later (steps 3 and 4) to stabilize the microtubule organization established earlier. The positions of follicle cells, germline cells, and the oocytes are indicated as in Fig. 1.

may depend on oocyte differentiation. If this is the case, a failure in oocyte differentiation would produce the *egl* phenotype.

A model for germline microtubule reorganization

How is a single microtubule cytoskeleton formed within the 16 cell germarial cysts? We speculate that, shortly after the 16 cell cysts have formed, the centrosomes in the pro-nurse cells are inactivated and no longer nucleate microtubules, and that only the centrosome in the pro-oocyte remains active. Microtubules in the pro-nurse cells therefore depolymerize, and the resulting tubulin subunits are available for assembly onto microtubules nucleated within the pro-oocyte. Because all of the cells in the cysts are interconnected by cytoplasmic bridges, and microtubule polymer is dynamic (Mitchison and Kirschner, 1984), microtubules nucleated at the MTOC eventually extend from the pro-oocyte, through the ring canals, and into the adjacent pro-nurse cells, and a single microtubule cytoskeleton is formed. This model is summarized in Fig. 9.

Centrioles migrate through the ring canals and accumulate in the oocyte as cysts pass through the germarium (Mahowald and Strassheim, 1970). We speculate that the migrating centrioles are the core structures of the inactivated pro-nurse cell centrosomes, and that these inactive centrosomes move to the oocyte along microtubule tracks. Centriole migration occurs in late region 2a and region 2b cysts, and the polarized microtubule cytoskeleton appears to be established in region 2a. The polarized microtubule cytoskeleton is therefore present at the time of centriole migration (Figs 2, 3). This model predicts that centriole migration will be blocked in *Bic-D* mutants and colchicine-treated germaria. Ultrastructural analysis of mutant and inhibitor-treated cysts will therefore provide an important test of this model.

We propose that selective stabilization of the centrosome in the future oocyte is the key process leading to microtubule reorganization and oocyte differentiation. The cytoskeletal phenotype of recessive *Bic-D* mutations suggests that Bic-D protein could be directly involved in a very early step in this process. The Bic-D protein is predicted to be largely α-helical, and to form a coiled-coil structure similar to the tail domain of myosin (Suter et al., 1989). Pericentrin, a component of the centrosome in vertebrate cells, is also predicted to be an α-helical coiled-coil (S. Doxsey, personal communication). It is tempting to speculate that Bic-D protein, like pericentrin, is a centrosomal component, and that this protein binds to, and stabilizes, the centrosome in the pro-oocyte.

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