Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification

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SUMMARY

The *Drosophila* oocyte develops within a cyst of 16 germline cells interconnected by ring canals. Polarized, microtubule-based transport of unknown determinants is required for oocyte formation, but whether polarity is established during or after cyst formation is not clear. We have analyzed how polarity develops in stem cells and dividing cysts by following the growth of the fusome, a vesiculated cytoplasmic organelle. Our studies show that the fusome grows by a regular, polarized process throughout the stem cell and cyst cell cycles. Each polarization cycle begins in mitosis, when the fusome segregates to a single daughter cell of each pair. Following mitosis, a 'plug' of fusomal material forms in each nascent ring canal and gradually fuses with the pre-existing fusome. In stem cells, the ring canal is transient and closes down after the fusome is partitioned through it. In dividing cysts, as the fusome plugs move toward the pre-existing fusome, their associated ring canals also move, changing the geometry of the cyst. At the end of each cycle of cyst growth, the fusome remains asymmetrically distributed within the cyst; one of the two cells with four ring canals retains a bigger piece of fusome than any other cell, including the other cell with four ring canals. Based on these observations, we argue that the oocyte is specified at the first cyst division.

Key words: Oogenesis, Germline, Stem cell, Ring canal, Fusome, Anillin, Polarity, *Drosophila*

INTRODUCTION

Gametes in many organisms develop within small clusters or cysts of germline-derived cells (reviewed in Telfer, 1975; Büning, 1994; de Cuevas et al., 1997). Germ cell cysts are formed by a process of mitosis with incomplete cytokinesis, which keeps the daughter cells linked by stable intercellular bridges called ring canals. In most male cysts, the cells remain synchronized and share a common fate throughout their development into spermatocytes. In female cysts, however, generally only one cell develops into an oocyte; the others become nurse cells, which synthesize materials that are transported through the ring canals into the oocyte, to support its growth. Organisms that use this strategy, such as higher insects, are able to produce relatively large eggs much more quickly than those without persistent nurse cells.

In the *Drosophila* ovary, cysts arise from germline stem cells located at the tip of each ovariole, in the germarium (Fig. 1; see Spradling, 1993, for a review of oogenesis). These stem cells divide asymmetrically to produce a new stem cell and a cystoblast, which then goes through four synchronized rounds of mitosis to form a cluster of 16 cells, or cystocytes. Because each division plane is oriented relative to the others during these mitoses, the 16 cystocytes are always interconnected in a stereotypical pattern, with two cells containing four ring canals each, and the others containing three, two or one. Following cyst formation, oocyte-specific transcripts, proteins and other cytoplasmic components accumulate in one cell; invariably, it is one of the two cells with four ring canals. This accumulation, which depends on polarized, microtubule-based transport, appears to be required for oocyte differentiation (Mahowald and Strassheim, 1970; Koch and Spitzer, 1983; Suter and Steward, 1991; Theurkauf et al., 1993). The transported oocyte determinants have not yet been identified, but they are likely to include factors that establish and maintain meiosis in the oocyte (Lilly and Spradling, 1996).

How the oocyte is specified is controversial. One model is that oocyte specification occurs after the cyst mitoses are complete: first, both of the cells with four ring canals are selected as 'pro-oocytes', but later one is chosen as the oocyte while the other reverts to the nurse cell fate (Carpenter, 1994; González-Reyes et al., 1997). This view is based on the observation that the two cells with four ring canals are initially difficult to distinguish; both enter meiosis and form synaptonemal complexes, and both accumulate factors transported from the nurse cells (Mahowald and Strassheim, 1970; Carpenter, 1975, 1979). How the asymmetry between the two pro-oocytes could arise is unclear. Another model is that the oocyte is specified at the first cyst division, by the establishment of some system of polarity that is maintained in

subsequent divisions (Lin et al., 1994; Theurkauf, 1994). Some support for this model comes from studies that show the fusome, a prominent cytoplasmic organelle, associates with only one pole of the spindle at every cyst mitosis (Storto and King, 1989; Lin and Spradling, 1995; McGrail and Hays, 1997). Thus, each cystocyte division is inherently asymmetric.

Fusomes are found in developing cysts in *Drosophila* and many other insects (reviewed in Telfer, 1975; Büning, 1994; de Cuevas et al., 1997; McKearin, 1997). Ovarian fusomes were first identified in stained *Dytiscus* (diving beetle) specimens (Giardina, 1901) and later in electron micrographs of *Drosophila* germaria, where the organelle appears as a distinct and highly vesiculated region of cytoplasm (Koch and King, 1966; Mahowald, 1971; Telfer, 1975). Cystoblasts contain a small spherical fusome, which grows during cyst formation into a large branched structure that extends through the ring canals into every cell. Once the cyst has stopped dividing, the fusome begins to break down, usually disappearing soon after the cyst leaves the germarium. In *Drosophila*, a spherical fusome, also called a spectrosome, is found in germline stem cells (Lin et al., 1994). Although the association of the fusome with one pole of the mitotic spindle has been well documented in both stem cells and cysts (Storto and King, 1989; Lin and Spradling, 1995, 1997; Deng and Lin, 1997; McGrail and Hays, 1997), its growth and behavior at other points in the cell cycle have not been analyzed.

Recent studies have identified several components of the fusome and have begun to shed light on its function. Fusomes in *Drosophila* females contain the membrane skeletal proteins α-spectrin, β-spectrin, ankyrin and the adducin-like hu-li tai shao (hts) protein (Lin et al., 1994; de Cuevas et al., 1996). Mutations in the genes encoding hts and α-spectrin essentially abolish the fusome and cause the formation of cysts with fewer than 16 cells, which usually lack an oocyte (Yue and Spradling, 1992; de Cuevas et al., 1996). The microtubule motor dynein associates with the fusome in a cell cycle-dependent manner and is also required for fusome integrity and cyst division (McGrail and Hays, 1997). Fusomes in *Dynein heavy chain (Dhc64C)* mutants are misshapen and fragmented, and *Dhc64C* cysts contain too few cells and no oocyte. Another fusome component, the protein expressed by the *bag-of-marbles (bam)* gene, is thought to be involved in forming the membranous network of the fusome; it is required for cystoblast differentiation and may regulate cyst divisions (McKearin and Ohlstein, 1995; McKearin, 1997). Finally, the cell cycle regulator cyclin A also associates with the fusome and is involved in regulating cyst formation (M. Lilly, M.C. and A.C.S., unpublished data). Taken together, these results show that the fusome is an integral part of the mechanisms that guide cyst construction. Moreover, they suggest that the fusome not only is a marker of cyst polarity, but

also participates directly in establishing or maintaining this polarity.

To gain a greater understanding of how and when cysts become polarized, we have closely examined fusome morphogenesis throughout cyst formation, from stem cell to 16-cell cyst. Our analysis shows that the fusome grows in all cells by a regular, cyclic process of accumulation and fusion of fusome pieces. Furthermore, the fusome remains asymmetrically distributed within the cyst throughout this time, supporting the idea that the oocyte is specified at the first cystocyte division.

MATERIALS AND METHODS

Drosophila culture

All flies were maintained at 22-25°C on standard medium; they were transferred to bottles containing wet yeast for 1-2 days before dissections.

BrdU labeling

Ovaries were dissected in Grace's medium and incubated in Grace's medium plus 10 μM bromodeoxyuridine (BrdU) for 1 hour at room temperature. They were washed for 5 minutes in Grace's medium and 10 minutes in PBS (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 175 mM NaCl), fixed for 10-20 minutes in Buffer B (16.7 mM KH_2PO_4/K_2HPO_4, pH 6.8, 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$) plus 5% formaldehyde (Ted Pella Inc.), washed for 30 minutes in PBS plus 0.6% Triton X-100 and for 10 minutes in DNase I buffer (66 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM 2-
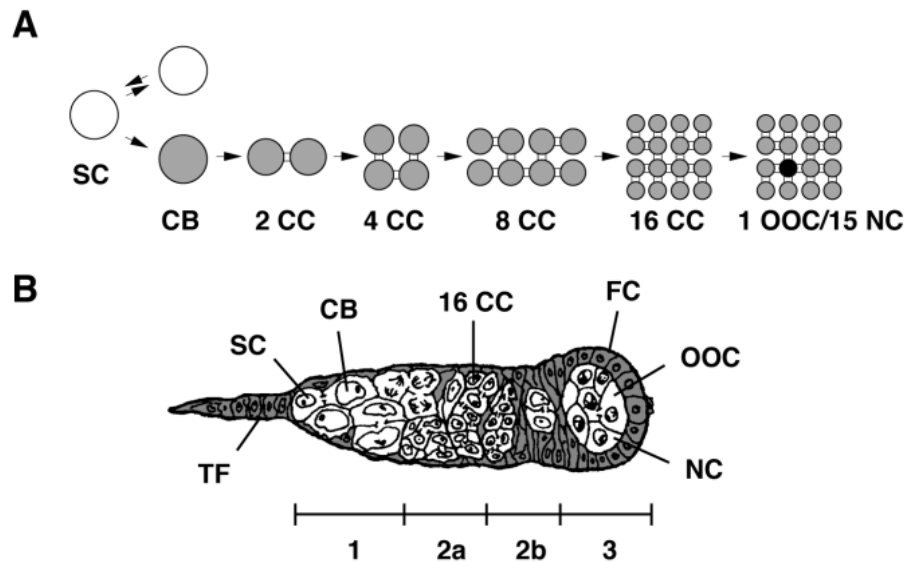

Fig. 1. Drawings of cyst formation and the germarium. (A) The pattern of germ cell divisions, from stem cell to differentiated 16-cell cyst. The oocyte is shown in black; it always forms from one of the two cystocytes with four ring canals. The remaining 15 cells become nurse cells. (B) The organization of cells in the germarium (drawing adapted from Koch and King, 1966). Anterior is to the left. Germline cells are shown in white, somatic cells in grey. The germarium is conventionally divided into four regions, 1, 2a, 2b and 3. Mitotically active germline cells are found in region 1; region 2 contains 16-cell cysts, which are enveloped by somatic follicle cells, forming an egg chamber, and become lens-shaped in region 2b; and in region 3, egg chambers round up again and bud off the germarium. The oocyte can be distinguished from its sister cystocytes by the end of region 2a, but how and when cyst polarity is established are not known. Abbreviations for germline cell types include: stem cell (SC), cystoblast (CB), cystocyte (CC), oocyte (OOC), nurse cell (NC); for somatic cell types: follicle cell (FC), terminal filament cell (TF).

mercaptoethanol), incubated in 0.5 ml DNase I buffer plus 25 u DNase I (Boehringer Mannheim Biochemicals) for 30 minutes at 37°C, and washed for 30 minutes in PBS plus 0.1% Triton X-100. Ovaries were then labeled with anti-BrdU and other antibodies (see below).

Antibodies

Affinity-purified rabbit anti-anillin antibodies (Field and Alberts, 1995), unlabeled or directly labeled with Oregon Green, were a gift from C. Field and were used at a concentration of 1-5 µg/ml. Bam protein was labeled using a mouse anti-BamF antiserum (McKearin and Ohlstein, 1995), which was a gift from D. McKearin, at a 1:100 dilution. Mouse anti-BrdU antibodies were purchased from Becton-Dickinson and used at a 1:20 dilution. Anti-α-spectrin, anti-hts and secondary antibodies are described in de Cuevas et al. (1996).

Immunostaining and fluorescence microscopy

Ovaries were dissected, fixed and stained as described previously (de Cuevas et al., 1996). They were mounted in PBS plus 50% glycerol and 1 mg/ml p-phenylene diamine (Sigma) for microscopy. Stained ovaries were examined by epifluorescence and confocal microscopy on a Leica TCS NT or on a Zeiss Laser Scan Microscope. Figs 2F, 2I, 3C-E and 7A-C are single optical sections; all other confocal images are stacks of multiple sections. DNA stained with 4,6-diamidino-2-phenylindole (DAPI) was photographed with a Princeton Instruments cooled CCD camera.

RESULTS

Fusomes in dividing stem cells

To identify dividing germline cells, we labeled ovaries with antibodies that recognize the actin-binding protein anillin. In *Drosophila* embryos and tissue culture cells, anillin is expressed in actively dividing cells and is localized in a cell-cycle-dependent manner (Field and Alberts, 1995). During interphase, it accumulates in the nucleus, but in mitosis, following nuclear envelope breakdown, it is released into the cytoplasm and moves out to the cortex. At telophase, anillin becomes highly enriched in the cleavage furrow, where it remains until the connection between the sister cells is severed. It is usually not detected in cells that have left the cell cycle. In germline stem cells and cystocytes, anillin follows the same cell-cycle-dependent pattern of localization; however, it persists in the ring canals that link the cystocytes long after the cells have stopped dividing, at least until the cyst has left the germarium (Fig. 2A; see also Fig. 8 in Field and Alberts, 1995).

We have found that anillin also localizes to a transient ring canal that arises when a stem cell divides to produce a daughter stem cell and a cystoblast. When it is first formed, this ring canal is similar in size and shape to the ring canals that link dividing cystocytes (Fig. 2A,B). It is not a permanent structure, however; before either cell divides again, the ring canal shrinks in diameter and the hole in its center disappears, severing the connection between the two cells (Fig. 2C). Usually no detectable anillin remains at the site of the severed connection, though occasionally a small spot can be found on the membrane of a dividing cystoblast (not shown). This stem cell ring canal was identified previously in electron micrographs of
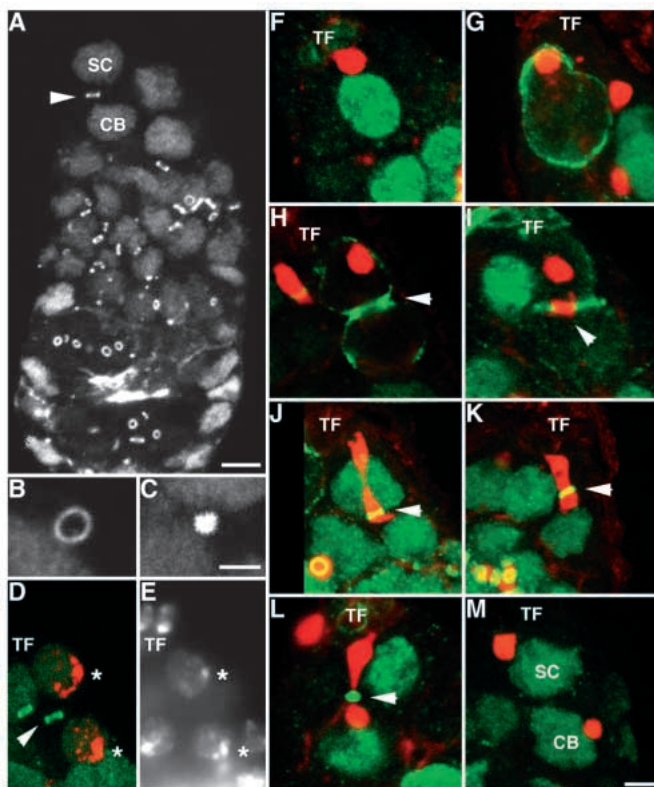

Fig. 2. Stem cell division. Stem cells were identified by their location at the anterior tip of the germarium, adjacent to the terminal filament (TF). Anterior is at the top in all panels. (A-C) Stem cells and cystoblasts are connected by a transient ring canal. (A) A wild-type germarium stained with anti-anillin antibodies. Anillin is present in the nuclei of mitotically active germline and somatic cells, in the ring canals between cystocytes, and in the ring canal (arrowhead) between a stem cell (SC) and cystoblast (CB). Some of the ring canals, including the one between SC and CB, are seen from the side and therefore look like bars instead of rings. (B,C) Higher magnification images of SC-CB ring canals stained with anti-anillin antibodies. A newly formed ring canal is open (B) but later in interphase it closes, severing the connection between the two cells (C). (D,E) SC and CB are connected and synchronized through S-phase. Ovaries were incubated with BrdU to label S-phase cells, then fixed and triple-labeled with anti-anillin antibodies (green), anti-BrdU antibodies (red) and DAPI (E). SC and CB, connected by an open ring canal (arrowhead), are both labeled with BrdU mostly over the heterochromatic DAPI-bright spot (asterisks) and are therefore in late S-phase. (F-M) The fusome is partitioned asymmetrically between SC and CB. Ovaries were triple-stained with anti-anillin antibodies (green), anti-hts antibodies (red), which label the fusome, and DAPI (not shown). (F) In late interphase, the SC nucleus contains a high level of anillin; the fusome is spherical and abuts the base of the TF. (G) At metaphase, anillin is concentrated at the cell cortex; the fusome does not change. (H) At cytokinesis, anillin becomes enriched in the cleavage furrow; the fusome begins to move away from the anterior tip of the cell. (I) In early interphase, before any anillin is visible in the nuclei, a 'plug' of fusomal material forms in the nascent ring canal; the original fusome moves towards it. (J,K) As interphase progresses, anillin accumulates in the nuclei of both cells; the fusome and plug gradually fuse, forming a bar-shaped structure that extends through the ring canal. (L) In G2 (see text), the ring canal closes and appears to pinch the fusome into two unequal pieces; about one-third ends up in the CB, the rest in the SC. (M) When the SC and CB are no longer connected, the fusome reforms a sphere in each cell. In H-L, cleavage furrows and ring canals are indicated by arrowheads. In G,H,L fusomes in adjacent stem cells are also visible. The scale bar in A is 5 µm; in C, for B,C, 2 µm; and in M, for D-M, 3 µm.
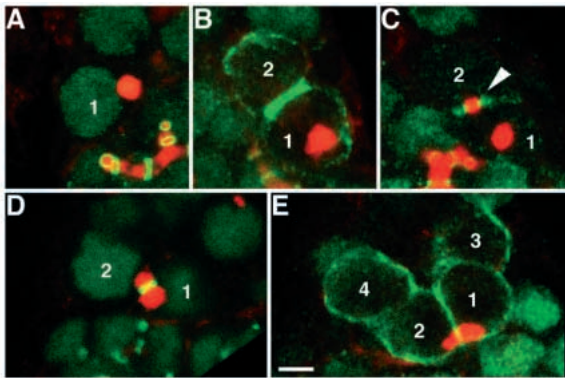
Fig. 3. Growth of the fusome from cystoblast to 2-cell cyst. Ovaries were triple-stained with anti-anillin antibodies (green), anti-hts antibodies (red) and DAPI (not shown). Cystoblasts were identified by their location in the germarium: any single cell separated from the base of the terminal filament by at least one other cell was considered a cystoblast. Anterior is at the top in each panel. (A) In late interphase, after separating from a stem cell, the cystoblast (1) has a spherical fusome, usually located in the anterior half of the cell. (B) At cytokinesis, anillin is enriched in the cleavage furrow; the fusome is often located in the more posterior of the two cells. (C) In early interphase, before anillin is visible in the nuclei of the two cells, a fusome plug forms in the nascent ring canal (arrowhead); the original fusome begins to move towards it. (D) As interphase progresses and anillin accumulates in the nuclei, the two fusome pieces fuse; after fusion, the cell that retained the original fusome at cytokinesis (1) always contains a bigger piece of fusome than its sister cell (2). (E) By the following mitosis, which will form four cells from two, cell 1 still contains a bigger piece of fusome than cell 2. The scale bar in E, for all panels, is 3 μm.

Drosophila germaria (Carpenter, 1981), but to our knowledge anillin is its first molecular component to be identified.

Having a marker for the stem cell ring canal has allowed us to identify stem cells that have recently completed mitosis, whose fusomes are likely to be in the process of segregating between the two daughter cells. To analyze the behavior of the fusome in these cells, we fixed ovaries and triple-stained them with anti-anillin antibodies, anti-hts antibodies (which label the fusome) and the DNA dye DAPI; we then examined them by immunofluorescence and confocal microscopy. Stem cells were identified by their location in the germarium, directly abutting the base of the terminal filament. We determined the cell cycle phase of a stem cell from its nuclear morphology, from the location of anillin within the cell, and from the presence or absence of a ring canal. The results of this analysis are shown in Fig. 2F-M (DAPI not shown). In late interphase, when the stem cell has a high level of anillin in its nucleus and there is no ring canal attaching it to any other cell, the fusome is spherical in shape and is located at the anterior tip of the stem cell, adjacent to the base of the terminal filament (Fig. 2F). Throughout mitosis, when anillin is enriched at the cell cortex, the fusome stays in this location and usually remains spherical (Fig. 2G), although occasionally it flattens out along the membrane of the stem cell, forming a wedge-shaped structure (not shown). Regardless of its shape, however, in most stem cells the fusome appears to stay the same size throughout mitosis; we did not see an eight-fold reduction in its volume, as reported by Deng and Lin (1997). Previous studies have shown that, at metaphase and anaphase, the fusome is also

adjacent to the anterior pole of the mitotic spindle (Deng and Lin, 1997; Lin and Spradling, 1997).

After mitosis, when the nuclear membrane has reformed and the cleavage furrow is well advanced, the fusome begins to migrate posteriorly towards the cleavage furrow (Fig. 2H,I). At the same time, a small amount of fusomal material accumulates in the nascent ring canal, forming a 'plug' in the ring. Although we could not determine its origin(s) from this experiment, we suggest that the plug is formed at least in part from newly synthesized material, since the original spherical fusome does not become noticeably smaller as it forms. As interphase progresses, the plug enlarges and the original fusome elongates along the anterior-posterior axis of the stem cell, eventually contacting the growing plug (Fig. 2J). The resulting single fusome then reorganizes and/or accumulates new material until it forms a thick bar-shaped structure that extends through the ring canal (Fig. 2K). Later, as the ring canal closes, it appears to squeeze the bar-shaped fusome into two pieces (Fig. 2L). Because of the appearance of the fusome, we call this the 'exclamation point' stage. At this stage, the fusome is usually distributed unequally between the two cells; thus about one-third of it ends up in the cystoblast, and the remainder in the stem cell. The bar-shaped fusome and its unequal distribution between the two cells were previously reported by Deng and Lin (1997).

When there is no anillin at the membrane (and presumably no connection) between the stem cell and cystoblast, the stem cell fusome returns to the anterior tip of the cell and regains its spherical shape (Fig. 2M). In stem cells that appear to have separated only recently from a cystoblast, the fusome looks smaller than in other stem cells (compare Fig. 2F and M); hence it might accumulate more material before the stem cell divides again. In some stem cells, the fusome appears to remain connected to the anterior tip of the cell throughout interphase. After the bulk of the fusome has migrated posteriorly, a thin 'thread' of material is visible between it and the anterior membrane. The fusome can also separate into two pieces; one piece migrates posteriorly and fuses with the new fusome plug, while the other piece remains at the anterior membrane of the stem cell. The behavior of the fusome in these cases suggests that there is a specific cortical attachment site for the fusome, from which it does not always detach completely.

Because most germaria have at least one stem cell, and sometimes several, with a ring canal and an elongated fusome, we reasoned that these stages of the fusome cycle must occupy a fairly large portion of the cell cycle following mitosis. To determine how long the transient ring canal persists, we incubated ovaries in the nucleotide analog BrdU, which incorporates into S-phase nuclei; we then fixed and triple-labeled them with anti-BrdU and anti-anillin antibodies, and with DAPI (Fig. 2D,E). In about 8-10 ovaries, we found 43 stem cells that were labeled with BrdU; each one was connected to a cystoblast by a ring canal that was fully open. Furthermore, in every case, the connected cystoblast was also labeled with BrdU. No single, unconnected stem cells or cystoblasts, and no stem cell-cystoblast pairs connected by a closed ring canal, were labeled with BrdU. These results show that the ring canal between stem cell and cystoblast stays open until after S-phase of the following cell cycle is complete in both cells. Thus, we conclude that the exclamation point stage of the fusome cycle occurs in G2. We have confirmed this result by examining ovaries double-labeled with anti-BrdU and anti-hts antibodies (not shown).

The results of these experiments also suggest that the stem cell and cystoblast enter and exit S-phase together. By examining the BrdU-labeled stem cell and cystoblast nuclei more closely, we determined that the BrdU was distributed over the same portion of the nucleus – over euchromatin, over heterochromatin, or over both – in every labeled pair of cells. Thus, we conclude that the cell cycles of the stem cell and cystoblast are synchronized through S-phase. Our findings are supported by those of Carpenter (1981), who labeled ovaries with [³H]thymidine, which incorporates into S-phase nuclei, and examined them by EM autoradiography. She identified 20 stem cell-cystoblast pairs with a connecting ring canal; in 4 of these pairs, both stem cell and cystoblast were labeled, but neither cell was labeled in the other pairs. She also found 34 single stem cells and 29 single cystoblasts, none of which was labeled. From these observations, she concluded that the stem cell and cystoblast remain connected and synchronized at least through S-phase. We suggest that they become asynchronous soon after the cells are separated, however, since we almost never saw a stem cell and a cystoblast in mitosis at the same time. We also found several germaria each with two labeled stem cell-cystoblast pairs; thus, two stem cells in one germarium are not prohibited from entering S-phase at the same time.

Fusomes in dividing cystoblasts

We next examined the growth and behavior of the fusome in dividing cystoblasts, in ovaries that were triple-labeled with anti-anillin antibodies, anti-hts antibodies and DAPI (Fig. 3). Cystoblasts were identified by their position in the germarium: any single cell without a ring canal, which was separated from the base of the terminal filament by at least one other cell, was considered a cystoblast. Its cell cycle phase was determined by the location of anillin and by its nuclear morphology. A new cystoblast, which has separated from the stem cell but not yet entered mitosis, contains a spherical fusome slightly smaller than a stem cell fusome; it is usually located in the anterior half of the cell, near the plasma membrane (Figs 2M, 3A). In mitotic cystoblasts, the fusome keeps its spherical shape and size but is often located in the posterior half of the cell (not shown). Thus, at cytokinesis, the fusome is often retained by the more posterior of the two daughter cells (Fig. 3B). This finding contradicts that of Lin and Spradling (1995), who stated that the fusome remains in the anterior-most cell. We did not determine whether the fusome changes its location by migrating through the cystoblast before mitosis begins, or whether the entire cell rotates; we suggest, however, that the fusome migrates towards the centrosome, which is likely to be located posteriorly.

Following mitosis, as in the stem cell, a small plug of fusomal material accumulates in the arrested cleavage furrow (Fig. 3C). As interphase progresses, the original fusome and plug move closer together, additional fusomal material accumulates between them, and the two pieces of fusome eventually fuse, forming a snowman-shaped structure with its 'neck' in the ring canal (Fig. 3D). In the cystoblast, however, the original fusome does not elongate towards the plug as they fuse. Thus, we could not determine if the original fusome moves towards the plug, or if the plug and ring canal move towards the original fusome. Because it is formed from two smaller fusomes, the fused fusome has also
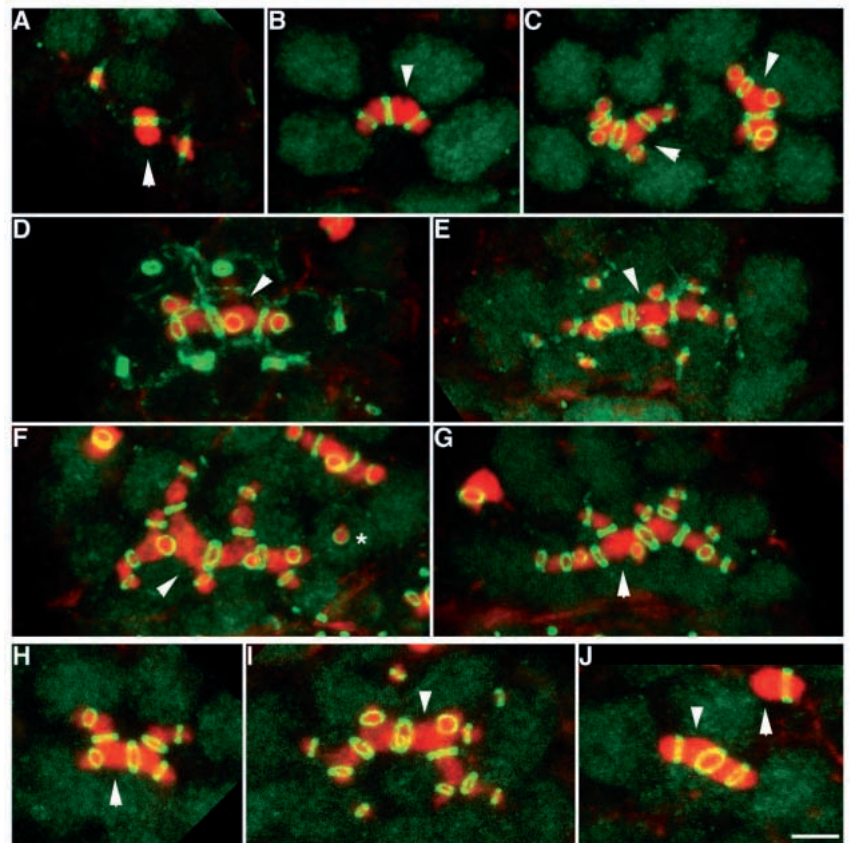

Fig. 4. Growth of the fusome in wild-type and mutant cysts. Ovaries were stained with anti-anillin (green) and anti-hts (red) antibodies. Anterior is at the top in each panel. (A-G) Fusomes in wild-type cysts. (A) A 4-cell cyst in early-mid interphase; anillin is just beginning to accumulate in the nuclei. Two fusome plugs have formed, one in each new ring canal. (B) A 4-cell cyst in late interphase; anillin has accumulated to high levels in the nuclei. The plugs have fused with the original fusome, forming one continuous structure that spans the three ring canals. (C) A pair of 8-cell cysts in late interphase. In each cyst, the four new plugs, in the four smallest ring canals, have already fused. (D) A 16-cell cyst in early interphase. Fusomal material is beginning to accumulate in the new ring canals but has not yet formed distinct plugs. Only seven of the eight new ring canals are visible here. (E,F) 16-cell cysts in mid interphase. The plugs have enlarged and are beginning to fuse, though not all are fusing at the same rate. In E, only 14 ring canals are visible; the other is out of focus. In F, only one of the plugs (asterisk) has not yet fused with the rest of the fusome. (G) A 16-cell cyst in late interphase. All fusome plugs have fused; the fusome spans all 15 ring canals. (H,I) Fusomes in *Bic-D^{PA66/R26}* cysts. (H) An 8-cell cyst in late interphase. (I) A 16-cell cyst in mid interphase; the plugs are beginning to fuse. (J) Fusomes in *egl^{WU50/RC12}* cysts. A 2-cell cyst and a 4-cell cyst, both in late interphase. Fusomes in *Bic-D* and *egl* cysts appear to form normally. In both wild-type and mutant cysts, one of the two cells with the most ring canals always contains more fusomal material than its sisters (arrowheads). Thus, throughout cyst formation, the fusome is asymmetrically distributed within the cyst. The scale bar in J, for all panels, is 3 μm.

been called a 'polyfusome' (King et al., 1982). After fusion, the cell that lacked a fusome at cytokinesis contains less fusomal material than its sister cell and, apparently, it does not accumulate more during the rest of interphase. By the next mitosis, which forms four cells from two, the fusome is still asymmetrically distributed within the cyst (Fig. 3E).

Fusomes in dividing cysts

In 4-, 8- and 16-cell cysts, the fusome grows in a similar manner as in 2-cell cysts: after each round of mitosis, plugs form in each nascent ring canal and gradually fuse with the original fusome (Fig. 4A-G). Fusion takes place as the plugs move towards the central fusome and as fusomal material accumulates in the gaps between them. All plugs fuse before the next round of mitosis begins, but they do not always fuse at the same rate; we saw many cysts in which some plugs had already fused while others were still separated from the central fusome by a large distance (eg. Fig. 4E,F). In 16-cell cysts, fusion is always complete before the cyst becomes lens-shaped and the fusome begins to disaggregate. Thus, 16-cell cysts whose fusomes are in the process of fusing are located in region 2a of the germarium. It is likely that some fusomal material is newly synthesized or assembled as the cyst grows, since fusomes in 16-cell cysts are much bigger than cystoblast fusomes.

As in 2-cell cysts, after every round of fusome fusion, one cell appears to contain more fusomal material than its sister cystocytes; this cell is always one of the two with the greatest number of ring canals (Fig. 4A-G). Based on this observation, the cell with the most fusomal material in a 16-cell cyst is probably the same cell that retained the fusome at cytokinesis in the cystoblast. Thus, the initial asymmetry of the fusome is maintained throughout cyst formation. In 4-, 8- and newly formed 16-cell cysts (region 2a), the cell with the most fusomal material is positioned at random in the cyst with respect to the axes of the germarium; we could not accurately determine its location in older 16-cell cysts (region 2b), however, because the fusome begins to break down before this point.

We also examined fusome morphogenesis in two mutants in which cyst polarity appears to be disrupted. Mutations in *Bicaudal-D* (*Bic-D$^{PA66/R26}$*) and *egalitarian* (*egl$^{WU50/RC12}$*) cause the formation of cysts that fail to accumulate oocyte-specific factors in a single cell and thus differentiate into 16 nurse cells (Suter et al., 1989; Suter and Steward, 1991; Schüpbach and Weischaus, 1991; Mach and Lehmann, 1997). Fusomes appear to form normally and are still asymmetrically distributed in these cysts, however, indicating that the polarizing mechanisms that underlie fusome formation are not disrupted by these mutations (Fig. 4H-J). Thus, the fact that the oocyte fails to differentiate in these mutants is apparently not caused by a lack of polarity in dividing cysts. Consistent with this result, one of the two cells with four ring canals still localizes to the posterior end of these mutant cysts in region 2b, indicating that some polarity is retained in older cysts as well (Suter and Steward, 1991; Ran et al., 1994; Carpenter, 1994).

Changes in cyst geometry

After each round of mitosis, as the fusome plugs move towards the central fusome, their associated ring canals move with them. To quantify this movement, we measured the distances between new and old ring canals both before movement (in cysts with newly arrested cleavage furrows, which did not yet

contain a distinct plug) and after movement (in cysts whose plugs had all fused with the central fusome). After plug fusion, the newest ring canal in each cell can still be distinguished by its smaller size. In cells with more than one old ring canal, we recorded only the distance from the new ring canal to the closest old ring canal. The results of this analysis show that new ring canals move about 2-3 μm closer to old ring canals after each round of mitosis (Table 1). A similar conclusion was reached by Storto and King (1989), based on measurements made from electron micrographs of a single dividing cyst.

We could not determine from this experiment if new ring canals actually flow through the plasma membrane, or if they are brought closer to old ring canals by removal of plasma membrane from one side of the cell and its replacement elsewhere. Regardless of how it happens, the centripetal ring canal 'movement' results in a change in the cyst's geometry. This change occurs after every round of mitosis but is best illustrated in 4-cell cysts, as shown in Fig. 5. In newly formed 4-cell cysts, just after cytokinesis, the cells are arranged in a linear or V-shaped structure (Fig. 5A; see also 3E). In older 4-cell cysts, however, the cells radiate out like flower petals from the central clump of ring canals. This arrangement, called a 'rosette' (Telfer, 1975), can be seen most clearly in cysts that are just entering mitosis, when anillin outlines the cortex of the cells (Fig. 5B). Rosettes have been found in many other insect species and their formation appears to be a general characteristic of cyst morphogenesis (Telfer, 1975; Büning, 1994). The effect of rosette formation is to shorten the distances between cells that are not directly connected; thus, it is tempting to speculate that this change in cyst geometry facilitates synchronization or communication between cystocytes. We also suggest that ring canal movement and rosette formation might be facilitated by the reduced amounts of membrane skeletal proteins found at the plasma membrane of dividing cystocytes (Lin et al., 1994; de Cuevas et al., 1996).

Formation of fusome plugs

To characterize fusome formation in more detail, we labeled ovaries with antibodies against two other fusome components, α-spectrin and bag-of-marbles (bam) protein. Both of these proteins, like hts protein, localize to fusome plugs in nascent ring canals and are distributed asymmetrically within the cyst
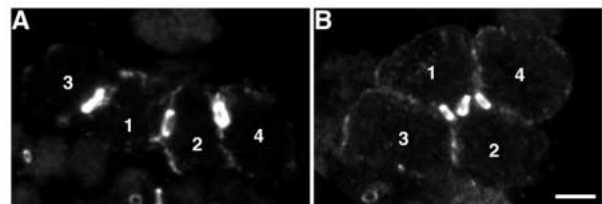

Fig. 5. Rosette formation. Ovaries were stained with anti-anillin antibodies. (A) A 4-cell cyst in early interphase. Fusomal material is just beginning to accumulate in the new ring canals at this stage (not shown). The new ring canals (cells 3-1 and 2-4) are each located a cell diameter away from the old ring canal (cells 1-2) and the cyst is approximately linear. (B) A 4-cell cyst in early mitosis; anillin is beginning to accumulate at the cortex. The fusome has completely fused by this stage (not shown). The ring canals are clumped in the center of the cyst and the cells radiate out from them like petals, forming a rosette-shaped structure. Note that cells 3 and 4 are closer to each other in B than in A. The scale bar in B, for both panels, is 3 μm.

Table 1. Distances between new and old ring canals in 4-, 8- and 16-cell cysts

Cyst size	Early interphase	Late interphase
4 cells	5.2±1.2*	1.6±0.3
8 cells	4.9±1.3	1.3±0.3
16 cells	4.3±0.8	1.5±0.4

In cells with more than one old ring canal, only the distance between the new ring canal and its closest old ring canal was recorded. Early and late interphase were distinguished by the morphology of the ring canal and fusome (see text).

*Measurements are in microns. n=14-83 for all samples.

throughout its formation (Fig. 6A-D). We have not yet found any difference in composition between the plugs and central fusome, but it is possible that other components might associate with only one part of the fusome.

We also examined plug formation more closely with these antibodies. By examining images at higher magnification, we found that the plugs are formed initially by material that appears to approach the ring canal from both sides. Moreover, especially when labeled with anti-hts antibodies, this material appears to approach the ring canal in lines, as if it is travelling along fibers (Fig. 6E). Thus, we suggest that the fusome plugs are assembled from material that is transported to the site of the spindle midbody, around which the ring canal forms, perhaps along microtubules that are remnants of the mitotic spindle.
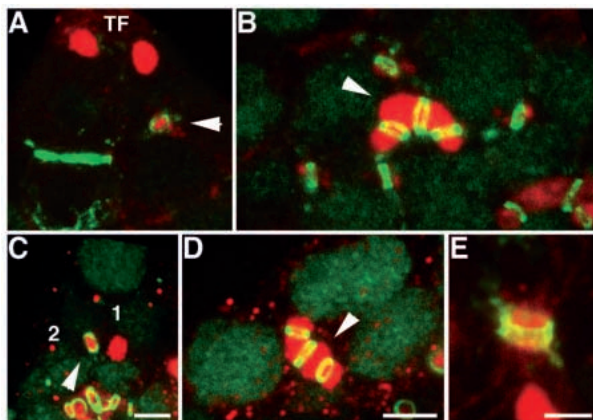
Ring canals between follicle cells

In the course of these experiments, we found that anillin is also present in the somatic ring canals that link ovarian follicle cells (Fig. 7A-D; see Robinson and Cooley, 1996, for a review of ring canals in follicle cells). These ring canals, which are first visible in region 2 of the germarium, increase in number as the follicle cells divide; thus by late oogenesis many follicle cells have multiple ring canals associated with them. Unlike germline ring canals, somatic ring canals are very small – about 0.5 μm in diameter – and do not enlarge as oogenesis progresses. They also continue to stain with anti-anillin antibodies throughout oogenesis. We did not see ring canals between the cells that comprise the interfollicular stalks (except when the stalks are forming, in stages 1-2; not shown) but they are present in the follicle cells that remain over the nurse cells after the others migrate posteriorly in stage 10 (Fig. 7D). Somatic ring canals in later egg chambers also contain actin (H. Frydman and A.C.S., unpublished data). In dividing follicle cells that were double-labeled with anti-anillin and anti-hts antibodies, we did not see any fusome-like structure spanning the ring canals.

Fig. 6. Formation of fusome plugs. Ovaries were stained with anti-anillin antibodies (green) and with anti-α-spectrin (A,B), anti-bam protein (C,D), or anti-hts (E) antibodies (red). Anterior is at the top in all panels. (A-D) α-spectrin and bam protein are also found in fusome plugs and are asymmetrically distributed in dividing cysts. (A) Two stem cells are shown. The one on the left is beginning cytokinesis and anillin is enriched in the cleavage furrow. The one on the right is in early interphase and connected to a cystoblast; α-spectrin is beginning to accumulate in the ring canal (arrowhead). (B) An 8-cell cyst in early interphase. α-spectrin is found in the four new ring canals. (C) A 2-cell cyst in early interphase. Bam protein is accumulating in the ring canal (arrowhead). (D) A 4-cell cyst in late interphase; the fusome has finished fusing. (B,D) The cell that appears to contain the most fusomal material is indicated (arrowheads). (E) A newly formed ring canal from a 2-cell cyst, shown at high magnification. Hts protein appears to be approaching the ring canal along fibers (see text). The scale bars in C, for A and C, and in D, for B and D, are 3 μm; the bar in E is 2 μm.
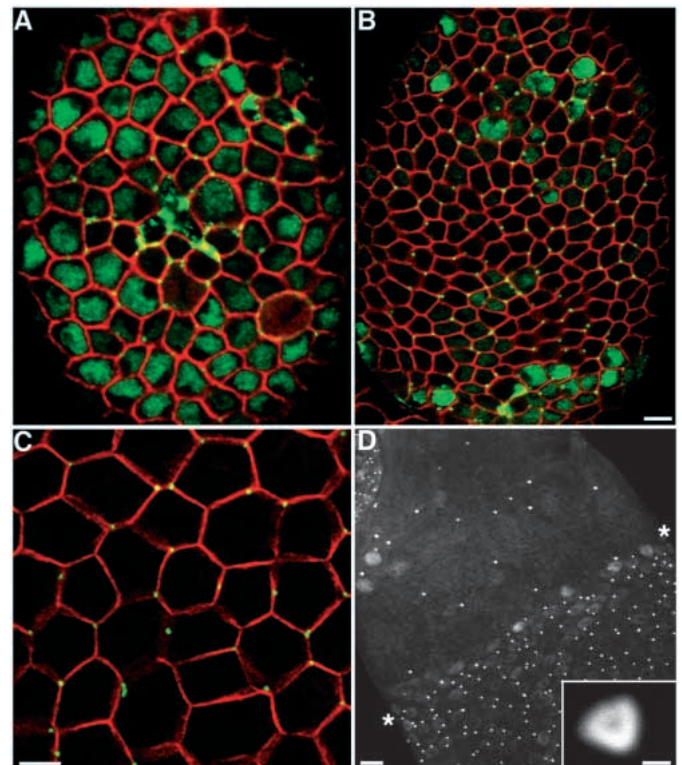

Fig. 7. Ring canals in follicle cells. Ovaries were stained with anti-anillin antibodies (green or white); those in A-C were also stained with anti-hts antibodies (red), which labels the plasma membrane of follicle cells. (A-C) Anillin is present in tiny ring canals between follicle cells at all stages of oogenesis and also in the nuclei, cytoplasm or cleavage furrows of cells that are dividing mitotically. The ring canals are often located at the vertices where three cells come together. (D) In stage 10, ring canals are visible in most follicle cells, including those that remain over the nurse cells after the others have migrated over the oocyte. The nurse cell-oocyte boundary is indicated (asterisks); the follicle cells covering the nurse cells are above the boundary. The inset shows a follicle cell ring canal at high magnification. The scale bars in B-D are 5 μm; A and C are at the same magnification. The bar in the inset is 0.5 μm.

Fig. 8. Cyst polarity and oocyte specification.
(A) Drawing of a dividing cystoblast from a *Dytiscus*
ovary (originally from Giardina, 1901; this image was
redrawn by Jörgensen, 1913). The fusome (F) and an
oocyte-specific ring of amplified rDNA (R) segregate to
the same cell at cytokinesis. (B) A model of the different
polarized events that contribute to the growth of the
fusome and cyst. (1) Spindles orient with one pole in the
fusome (red). (2) Fusome components move to the ring
canals. (3) Ring canals move to the center of the cyst; the
fusome (red) remains asymmetrically distributed within
the cyst after each division cycle.
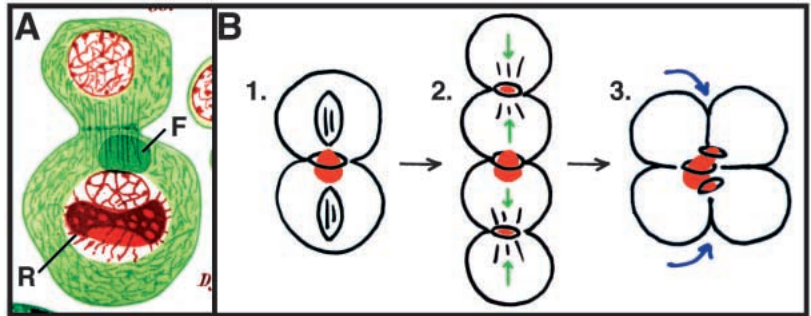
DISCUSSION

Cyst polarization and oocyte specification

The work presented in this paper strongly suggests that ovarian
cysts are polarized at the first division, that this polarity is
maintained during the subsequent rounds of division, and that
the fusome is a sensitive indicator of polarity in dividing cysts.
Thus, our observations support the idea that the oocyte is
specified early, during the cyst divisions, rather than later, after
the completion of the cyst mitoses. Previous studies had shown
that the fusome associates with only one pole of the spindle in
dividing cystoblasts (Lin and Spradling, 1995). Since the two
cells that are produced by this division give rise to the two cells
with four ring canals, one of which becomes the oocyte, this
result suggested that the fusome might mark the future oocyte.
It was not clear, however, how or even if the original
asymmetry between these two cells was maintained as the cyst
continued to divide. Our results provide the first evidence
suggesting that the initial polarity of the cyst is maintained.

One important question that remains is whether the cell that
originally inherits the fusome does in fact become the oocyte.
One of the earliest markers of the oocyte is its accumulation
of specific proteins including Bic-D and Cup (Suter and
Steward, 1991; Keyes and Spradling, 1997). Unfortunately, by
the time these proteins are localized to a single cystocyte, the
fusome has begun to break down, the ring canals have migrated
away from the center of the cyst, and it is not possible to
determine which cell originally contained the most fusomal
material (not shown). This question has been answered in
another insect, however. In the diving water beetle *Dytiscus*,
cyst formation is similar to that in *Drosophila*: cystoblasts go
through four rounds of synchronous mitosis to form a cyst of
16 cells, one of which differentiates into an oocyte while the
others become nurse cells. Unlike *Drosophila*, however, the
Dytiscus oocyte can be distinguished from its siblings as early
as the 2-cell stage because it contains a large ring of highly
amplified rDNA (Giardina, 1901; see also Gall, 1996).
Moreover, the cell that inherits the amplified rDNA ring at the
first division of the cystoblast is the same cell that inherits the
fusome (Fig. 8A). Thus, by analogy to *Dytiscus*, we propose
that the cell that retains the most fusomal material in
Drosophila is the one that becomes the oocyte.

What then is the mechanism of oocyte specification? One
possibility is that the asymmetric distribution of the fusome
reflects an underlying asymmetry in the cyst's cytoskeleton.
Although no obvious polarity has yet been found in the
distribution of microtubules in dividing cysts (Theurkauf et al.,
1993), other differences in the microtubule or actin-based

cytoskeleton might be present and might contribute to the
formation of the polarized microtubule array that directs
transport into the oocyte. Consistent with the view that the
asymmetric fusome is indicative of a polarized cytoskeleton,
fusomes in dividing male cysts, which are not likely to be
polarized, appear to be more evenly distributed (not shown).
Another possibility is that an oocyte-specifying factor that co-
segregates with the fusome might be inherited by the future
oocyte at each round of cyst division. For example, the
centrosome inherited by the future oocyte at the each division
could be distinct in some way (Theurkauf, 1994). The fusome,
which associates with one pole of the spindle at metaphase,
would thus be segregating with this marked centrosome at the
first division of the cystoblast.

To explain why both of the cells with four ring canals (the
two 'pro-oocytes') initially enter meiosis and accumulate
transported factors, we suggest that transport between these two
cells is delayed. As transport within the rest of the cyst begins,
if the ring canal connecting these two cells is somehow blocked,
then oocyte determinants would pile up in both cells and both
would initially appear to differentiate as oocytes. The fusome
itself might be involved in blocking this ring canal; the larger
piece of fusome associated with the oocyte might simply take
longer to break down than the fusome in the rest of the cyst.

Mechanisms of fusome and cyst growth

Our work, together with previous studies, shows that cyst and
fusome growth are highly regular, polarized processes (Fig. 8B).
In each division cycle, spindles orient with one pole in the fusome
(Storto and King, 1989; Lin and Spradling, 1995, 1997; Deng
and Lin, 1997; McGrail and Hays, 1997), fusome plugs
accumulate in the new ring canals, at the site of the spindle
midbody, and ring canals and fusome plugs move centripetally,
changing the cyst from a linear into a rosette-shaped structure. In
addition, our observations suggest several mechanisms that might
mediate these events. We have shown that fusome plugs are
formed from material that appears to approach from both sides
of the ring canals. If the spindle midbody contains the plus ends
of microtubules, then a plus-end directed, microtubule-based
motor might be involved in transporting fusome vesicles to the
ring canals. A different motor might be involved in moving the
fusome plugs, together with their associated ring canals, towards
the central fusome. Since this movement is directed towards the
centrosome, which remains associated with the original fusome
in each cell, it is likely to be mediated by a minus-end directed
motor. One such motor, dynein, localizes to the fusome and is
required for fusome assembly; mutations in the *Dynein heavy
chain (Dhc64C)* gene cause the formation of fragmented fusomes

(McGrail and Hays, 1997). Dynein is unlikely to play a direct role in transporting fusome plugs, however, because it apparently does not localize to the fusome in interphase, when this movement occurs. Instead, it appears to be required for anchoring spindle poles in the fusome during mitosis (McGrail and Hays, 1997). Perhaps, in the absence of this mitotic orientation, interphase microtubules are also misoriented and the fusome plugs are unable to move to the center of the cyst.

Similarities to other insects

Our results are supported by numerous studies of fusomes and cysts in other insects (reviewed in Telfer, 1975; Büning, 1994). These studies, based on light and electron microscopic observations of dividing cysts, show that cystocytes divide in synchrony, spindles orient with one pole adjacent to the fusome, vesicles and fibrils characteristic of the fusome accumulate within new ring canals, at the site of the spindle midbody, and many cysts form rosettes. Because these features of fusome and cyst formation are common in other insects, we suggest that the asymmetric distribution of the fusome, which we have now documented in *Drosophila*, may also be a general feature of ovarian cyst formation. Thus, it is tempting to speculate that oocytes are specified early in all organisms that form cysts, by the establishment of cyst polarity at the first division and its maintenance in subsequent divisions.

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