The fusome organizes the microtubule network during oocyte differentiation in Drosophila

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SUMMARY

Differentiation of the Drosophila oocyte takes place in a cyst of 16 interconnected germ cells and is dependent on a network of microtubules that becomes polarized as differentiation progresses (polarization). We have investigated how the microtubule network polarizes using a GFP-tubulin construct that allows germ-cell microtubules to be visualized with greater sensitivity than in previous studies. Unexpectedly, microtubules are seen to associate with the fusome, an asymmetric germline-specific organelle, which elaborates as cysts form and undergoes complex changes during cyst polarization. This fusome-microtubule association occurs periodically during late interphases of cyst divisions and then continuously in 16-cell cysts that have entered meiotic prophase. As meiotic cysts move through the germarium, microtubule minus ends progressively focus towards the center of the fusome, as visualized using a NOD-lacZ marker. During this same period, discrete foci rich in γ tubulin that very probably correspond to migrating cystocyte centrosomes also associate with the fusome, first on the fusome arms and then in its center, subsequently moving into the differentiating oocyte. The fusome is required for this complex process, because microtubule network organization and polarization are disrupted in hts† mutant cysts, which lack fusomes. Our results suggest that the fusome, a specialized membrane-skeletal structure, which arises in early germ cells, plays a crucial role in polarizing 16-cell cysts, at least in part by interacting with microtubules and centrosomes.

Key words: Drosophila, Oogenesis, Microtubules, α- and γ-tubulin, GFP, Oocyte differentiation, Axis formation, Polarity, Fusome, NOD-lacZ

INTRODUCTION

The Drosophila oocyte develops within a polarized cyst of 16 interconnected germ cells (reviewed in King, 1970; Spradling, 1993). Cysts form by a process of incomplete cell division in region 1 of the germarium, a tripartite structure at the anterior of each ovariole (reviewed in de Cuevas et al., 1997; see Fig. 1A-C). Although they appear equivalent initially, one of the 16 cells (cystocytes) in each cyst differentiates as an oocyte and the other 15 become nurse cells, while the cyst moves in line with neighboring cysts through germarium regions 2a and 2b. The first definitive morphological sign of oocyte differentiation is the re-orientation of the pro-oocyte from its initial random position along the antero-posterior (AP) axis of the germarium to a posterior position near the end of region 2b (reviewed in van Eeden and St Johnston, 1999). However, oocyte differentiation depends on an intercellular (intra-cyst) polarity-asymmetry that begins to be elaborated much earlier (Suter and Steward, 1991). The molecular basis of cyst polarity remains a central issue in Drosophila oogenesis that is only beginning to be unraveled (reviewed in Cooley and Theurkauf, 1994; de Cuevas et al., 1997).

A polarized set of microtubules (MTs) plays an important role in intra-cyst asymmetry and arises as 16-cell cysts traverse the germarium (polarization) (Koch and Spitzer, 1983; Theurkauf et al., 1992, 1993). When stained with anti-tubulin (anti-tub) antisera, organized microtubules could first be detected within region 2a cysts, and these microtubules appeared to emanate from a microtubule-organizing center (MTOC) located in a central cyst cell. In region 2b, a large number of MTs clearly extend from the posteriorly positioned oocyte into the nurse cells, with their minus ends focused into the oocyte (Theurkauf et al., 1993; Clark et al., 1997). During this same developmental period, centrioles migrate from the pro-nurse cells into the oocyte (Mahowald and Strassheim, 1970) but they are thought to lack MT nucleation activity and to play no role in polarization (reviewed in Cooley and Theurkauf, 1994).

The microtubule cytoskeleton has been postulated to underlie many aspects of intra-cyst asymmetry. Specific RNAs and proteins accumulate in a polarized manner within cysts during this period. One or two cells in cysts midway through region 2a begin to display elevated levels of Bicaudal D (BicD), oskar (osk), orb, and hu-li-tai-shao (hts) RNAs (Suter et al., 1989; Ephrussi et al., 1991; Lantz et al., 1994; Yue and Spradling, 1992); by the end of region 2b high levels persist.
only in the definitive oocyte. A similar pattern of polarized accumulation is seen for BIC-D and ORB. Selective, microtubule-dependent transport of key gene products into a single cell within each cyst is thought to promote and maintain oocyte differentiation. In support of this model, both the polarized accumulation of gene products and oocyte differentiation are disrupted by microtubule depolymerizing drugs (Koch and Spitzer, 1983; Pokrywka and Stephenson, 1995) and by mutations in Dynemin heavy chain 64C (Dhc64C) encoding a microtubule motor subunit (Li et al., 1994; McGrail and Hays, 1997), and Lissencephaly-1 (Lis1), encoding a candidate dynactin subunit (Liu et al., 1999; Swan et al., 1999). Mutations in egalitarian (egl) and BicD, whose gene products are proposed to mediate transport along microtubules, also disrupt cyst polarization and oocyte formation (Carpenter, 1994; Mach and Lehmann, 1997; Ran et al., 1994; Schüpbach and Wieschaus, 1991; Suter et al., 1989; Wharton and Struhl, 1989). However, it remains unclear whether all aspects of cyst polarization are dependent on microtubules.

There are other indications of developing intra-cyst asymmetry. Oocyte differentiation, as reflected by events of meiosis, also progresses in a polarized manner within region 2a and 2b cysts. Most, if not all, cells in a newly formed 16-cell cyst enter meiosis transiently in region 2a, but usually only two cyst cells remain in meiotic prophase I by the time cysts reach region 2b, and only the definitive oocyte persists in meiosis as cysts prepare to exit the germarium (Carpenter, 1975, 1979).

There is strong evidence that cystocytes are unequal even before 16-cell cysts are completed at the beginning of region 2a. Cysts form from cellular precursors called cystoblasts by four synchronous cycles of incomplete cell division. Following each division, daughter cystocytes remain connected by a cytoplasmic bridge called a ring canal. After four rounds of division, two cells contain four ring canals, two cells retain three, four cells have two, whereas the remaining eight cells have only one ring canal. The oocyte invariably develops from one of the two cells with four ring canals (reviewed in de Cuevas et al., 1997).

At least one other aspect of cystocyte structure is asymmetric during the process of 16-cell cyst formation. Cystocytes contain an unusual germline-specific membranous organelle known as the fusome, which extends unequally throughout the cyst and its ring canals (reviewed in Telfer, 1975). The fusome contains high levels of membrane skeletal proteins such as α and β-spectrin (alpha-Spec and beta-Spec) as well as the adducin-like HTS protein (Lin et al., 1994; de Cuevas et al., 1996). The fusome grows and branches during each cycle of cyst growth, but remains asymmetrically distributed. At the time a 16-cell cyst is completed, the ‘oldest’ cell in the cyst (one of two with four ring canals) retains the largest amount of fusomal material, while younger cells contain proportionately less fusome (de Cuevas and Spradling, 1998). As cysts become polarized in region 2, the fusome undergoes complex changes and eventually breaks down after the cyst has acquired follicle cells and budded off the germarium as a new follicle. Morphological observations during this time do not reveal how long fusomal polarity is retained, or whether this polarity presages which cell with four ring canals will become the oocyte.

Because mutations in hts or alpha-Spec disrupt both fusome structure and oocyte differentiation (Yue and Spradling, 1992; de Cuevas et al., 1996), the fusome has been postulated to play an important role in generating cyst polarity (Lin et al., 1994; Knowles and Cooley, 1994; Deng and Lin, 1997). However, the mechanism by which the fusome affects oocyte differentiation has been elusive. Several potential roles of the fusome have been proposed to account for this requirement. The larger amount or character of the fusome retained in the initial cystocyte was postulated to organize an MTOC within young 16-cell cysts (Lin and Spradling, 1995; de Cuevas and Spradling, 1998). Alternatively, asymmetric molecular structures within the fusome might template microtubule asymmetry during cyst polarization (Lin and Spradling, 1995).

Here, we demonstrate a close physical and functional relationship between the fusome and the MT network. The fusome interacts with and organizes the microtubule network during late interphase in both mitotic and meiotic cysts. At a specific time in region 2a cysts, microtubule minus ends focus towards the center of the fusome. Nurse-cell centrioles migrate along the fusome toward the oocyte, and appear to participate more actively in intra-cyst polarization than previously supposed. Our studies reveal that the fusome plays a central role in a complex and dynamic process that leads to the production of a polarized germline cyst containing a single oocyte.

MATERIALS AND METHODS

Construction of transgenic Drosophila

p[w+;UASp-GFPS65C-α-tub-A488B]: A fragment encoding GFP65C was fused by PCR in frame to the N-terminal region of α-tubulin, encompassing amino acid 2 of the putative α-tubulin protein up to the internal BamHI site, using clone 99.41 (a generous gift from Andy Fire) containing GFP65C (Heim et al., 1995) and the α-tubulin 84B (α1) genomic clone pTr1.5′-3′ (Kalfayan and Wensink, 1981; Theurkauf et al., 1986). Primers, RIAspNFGP cggaatctggtggtctttgtgttcatccatcga, GPalphagatgacttacagttgaggaagatggtggttcatccatcga, were used to introduce an EcoRI and an Asp718 restriction site at the 5′ end of this fusion product. The GFP-N-terminal α-tubulin fragment was cloned into pGEM-T (Promega) and sequenced. The fragment was recovered by EcoRI, BamHI restriction and cloned into the EcoRI, ClaI site of pHSS6 together with a BamHI, ClaI fragment of genomic clone pTr1.5′-3′ encoding the C-terminal part of α-tubulin, as well as its 3′UTR. A NorI fragment encoding the GFP65C-α-tubulin fusion was cloned into the NorI site of pUASp (Rorth, 1998).

p[w+;UASp-nod-lacZ]: An XbaI fragment encoding NOD-lacZ was recovered from pUAST-nod-lacZ (Clark et al., 1997) and subsequently cloned into the XbaI site of pUASp (Rorth, 1998).

These transformation plasmids were used for injection into y w flies (see strains) as described (Spradling and Rubin, 1982), with modifications (available on request). Transformants on the second, third or X chromosome were recovered. Transformants varied in expression level but not in the pattern they revealed.

Drosophila strains

y ac w118 (Bello et al., 1998) or y w67c23(2) (Tulin et al., 1998) were used for injections to obtain P-element-mediated transformants. The following strains were used for GAL4-induced expression: w pCOG-Gal4-VP16 (Rorth, 1998), NGT40 (Tracey et al., 2000), w nos-Gal4-VP16/A488BIII (Van Doren et al., 1998). These driver lines express GAL4 in the germline of the ovary, but not in the surrounding somatic
cells (Rørth, 1998), inducing expression of UASp transgenes. For analysis of the MT network in the germline using UASp-GFP-α-tub, the following strains were used: flies of the genotype w; NGT40/SM6b; p[w]+, UASp-GFP-α-tub/MKRS were crossed to males of the genotype w; NGT40/SM6a; nos-Gal4-Vp16/TM3 or to males of the genotype w; pCOG-Gal4-Vp16/Y; NGT40/SM6b; nos-Gal4-Vp16/TM2. Ovaries from females containing two copies of NGT40 and one copy of each nos-GAL4-VP16 and UASp-GFP-α-tubulin were analyzed for their MT organization; rarely pCOG-GAL4-VP16 was used. For analysis of GFP-α-tubulin organization in hts1 mutants (Yue and Spradling, 1992), the stocks w; hts1/SM6b; nos-GAL4-VP16/ MKRS and w; hts1/SM6b; p[w]+, UASp-GFP-α-tub/MKRS were crossed. Ovaries of homzygous and heterozygous hts1 mutants were fixed and stained in the same tube to avoid fixation differences among samples.

To achieve NOD-lacZ expression in the germlarium, flies of genotype nos-GAL4-VP16 or p[w]+, UASp-α-tub-J/SM6a; nos-GALA-VP4/MKRS were crossed to y w p[w]+, nod-lacZ/F or constructs on other chromosomes.

Survival measurements

100 embryos were aligned on agar plates and allowed to develop at 22-23°C. Hatching larvae were collected and counted. After several days, dead embryos were counted. Survival rates were calculated as the number of aligned embryos minus the number of dead embryos ± 100 and are displayed in Fig. 2D.

Immunohistochemistry and fluorescence microscopy

Ovaries were dissected in 1x Grace’s medium (Gibco BRL), which was equilibrated at room temperature. The dissected ovarioles were fixed for 10 minutes at room temperature, using typically a mixture of 350 µl Grace’s medium and 200 µl 16% electronmicroscopy-grade formaldehyde (Ted Pella, Redding, CA). After several washes in 1x PBS, 0.1% Triton X-100 (PBT), the ovarioles were blocked using PBT containing 5% normal goat serum (Jackson Labs, West Grove, PA) (PBT-NGS) for at least 30 minutes. Incubation with primary antibodies was carried out either for 4 hours at room temperature or overnight at 4°C in PBT-NGS, followed by several washes with PBT. After blocking with PBT-NGS for at least 30 minutes, incubation with fluorescent secondary antibodies was carried out for 4 hours at room temperature. Subsequently the ovarioles were stained with DAPI, washed extensively with PBT and equilibrated with 1x PBS before mounting in Vectashield (Vector Laboratories, Burlingame, CA). Samples were examined by confocal microscopy using a Leica TCS-NT microscope.

The primary antibodies used were as follows: cell culture supernatant of mouse monoclonal antibody (mAb) 1B1 (1:4-5), (which was produced from a cell culture stock provided by H. D. Lipshitz using standard cell culture procedures) (Zaccai and Lipshitz, 1996); rabbit anti-α-spectrin 354 (1:100); rabbit anti-β-spectrin 337 (1:200) sources are described in de Cuevas et al. (1996); anti-phosphotyrosine (1:1000) PY20 and PY69 (ICN Biomedicals, Costa Mesa, CA); mouse mAb anti-γ-tubulin GTU88 (1:350) (Sigma); mouse mAb anti-β-tubulin 4A2 (1:10) (Piperno and Fuller, 1985); mouse mAb anti-β-gal (1:400) (Promega) or rabbit anti-β-gal (1:400) (Cappel). As secondary antibodies goat anti-rabbit or mouse Alexa-568 (Molecular Probes) and Cy5 (Jackson Labs) were used (1:400).

Signals for γ tubulin were quantified using IP Labs software. These were typically five- to tenfold above background.

RESULTS

GFP-α-tubulin, a reporter for the MT network

To analyze the MT network in detail within early germ cells, we made a GAL4-inducible GFP-α-tubulin construct that contained an in-frame fusion, similar to one previously described in yeast (Carmini and Sterns, 1997), between GFP565C (Heim et al., 1995) and the N-terminus of α-tubulin 84B (Theurkauf et al., 1986; Materials and Methods). To promote expression in Drosophila germ cells the construct used the pUASP vector (Rørth, 1998). When combined with two or three different GAL4 driver lines, GFP-α-tubulin was visible throughout the gerarium (Fig. 1). The MT network in budding and young egg chambers could also be seen, although the strength of the labeling varied. In response to these GAL4 lines, GFP-α-tubulin was also deposited into the egg (Fig. 2).

To find out whether the modified tubulin was functional and compatible with Drosophila development, we undertook a few tests. Western-blot analysis confirmed that a GFP-tagged protein of roughly 80 kDa was present in GAL4-induced ovarian extracts, which corresponds to the expected 77 kDa for the fusion-protein (data not shown). Inspection of living embryos laid by mothers expressing GFP-α-tubulin, revealed that the modified tubulin was incorporated into the MTs of syncytial blastoderm embryos (Fig. 2A-C). The observed MT organization resembled that previously reported for wild-type (wt) embryos (Kellogg et al., 1988), although the amount of nonincorporated GFP-α-tubulin was high. Most of the embryos laid by these mothers gave rise to living larvae (Fig. 2D) and also developed to adulthood (data not shown). Only occasionally were defects detected during oogenesis. These tests suggest that the GFP-α-tubulin construct encodes a functional tubulin that is compatible with development and suitable for the analysis of the MT network in the ovary. In addition, our observations of MT behavior were confirmed by staining with anti-tubulin antibodies (data not shown).

MT organization in the germline of meiotic cysts and young egg chambers

A Drosophila ovary contains about 16 ovarioles. Each ovariole can be considered a linear assembly line for the formation of eggs, and contains progressively older egg chambers towards the posterior. The oocyte develops in an egg chamber, containing a cyst of 16 interconnected germ cells surrounded by a layer of follicle cells (Fig. 1A,B). The gerarium, which resides at the anterior tip of the ovariole, is the site of egg-chamber formation and typically contains 7-12 developing 16-cell cysts (Fig. 1C). Cyst location within the gerarium correlates with developmental age and stage. The organization of the MT network described below and in Fig. 3 is usually observed at the indicated position within the gerarium.

To describe more accurately the process of intra-cyst polarization, we found it useful to categorize nine specific stages of cyst development within the gerarium based on consistently observed shape changes (Fig. 1C), in addition to the classically defined regions 1-3 (King, 1970). Region 1 contains stem cells, cystoblasts and dividing cysts. Region 2a begins when 16-cell cysts have been formed. As young 16-cell cysts move through region 2a, they remain randomly oriented with respect to the anterior-posterior (AP) axis (oblique, type 1). As cysts approach the posterior part of region 2a, they start to interact with ingrowing follicle cells and become oriented (aligning, type 2). Subsequently, they stretch out (stretching, type 3 and 4), straighten (straightening, type 5), and finally span the entire width of the gerarium and appear flat (straight or lens-shaped, type 6). Cysts then bulge slightly with the
differentiating oocyte facing posteriorly (bulging, type 7). Finally, late in region 2b, cysts begin to round up (budding, type 8) and complete the process of budding as new egg chambers (region 3/stage 1, type 9).

We used GFP-α-tubulin to survey MT behavior in the gerarium and in young egg chambers (stages 1-7) and to compare it with previous observations made with immunohistochemistry (Theurkauf et al., 1992, 1993). We saw many of the general structures reported previously but at higher resolution and in greater detail. MTs are enriched in the oocyte of young egg chambers (Fig. 1D; Theurkauf et al., 1992). The oocyte-resident MT network appeared complex. MTs clearly extend from the posterior side of the oocyte into the neighboring nurse cells (Fig. 1E,F; Theurkauf et al., 1992). These MTs radiated from all directions within the oocyte and left the oocyte through ring canals, extending into the neighboring nurse cells (Fig. 1D-G). As shown for very young egg chambers, many MTs ran along the periphery of the oocyte, forming a tent-like structure over the oocyte nucleus (Fig. 1G). Some MTs focus close to the oocyte nucleus (Fig. 1H). Typically, at least one major focus is evident; it is unclear whether the peripheral MTs converge onto this focus.

To investigate how the oocyte-specific MT network...
develops, we analyzed MT organization in progressively younger 16-cell cysts within the germarium (Fig. 1I-M). As cysts bud (Fig. 1I,J, type 8), the MTs already focus in the oocyte and run into the nurse-cell cluster through ring canals. In contrast to young egg chambers, many MTs at this stage are found at the anterior rather than the posterior of the oocyte nucleus (also see Theurkauf et al., 1993; Clark et al., 1997). Even in bulging cysts, more MTs focus into one cell, which is at the leading edge of the cluster, presumably the oocyte (Fig. 1K, type 7). In younger region 2b cysts, we observed a somewhat different pattern of MT organization than previously reported (Theurkauf et al., 1993). Abundant MTs at the anterior side of straight 2b cysts run along the length of the cyst (Fig. 1K, type 6). In oblique cysts (Fig. 1L,M, type 1),

![Fig. 2](image)

**Fig. 2.** GFP-α-tubulin incorporates into the MT network.

(A) Blastoderm embryo in metaphase, (B) in anaphase and (C) in prophase. (D) hatching rate: 1, y w; 2, GFP-tubulin expressing (NGT40/NGT40; nos-GAL4-VP16/UAS-GFP-tub); 3, nos-GAL4-VP16; 4, NGT40; 5, UAS-GFP-tub. Grey and black bars: replicate experiments. Scale bars: 5 μm in A,B; 2.5 μm in C.

![Fig. 3](image)

**Fig. 3.** MTs are embedded in the fusome starting in late interphase. The fusome is stained in red, GFP-α-tubulin is green. Confocal images display only optical slices of a cyst; anterior is to the left and posterior to the right. (A) Fourth division (metaphase). One pole of each spindle is associated with fusome branches. (B) Early meiotic interphase, oblique, region 2a 16-cell cyst. ‘Spindle remnants’ are symmetric structures extending on either side of the ring canal (marked by the fusome plug); only a few MTs are associated with the ‘mother’ fusome. (C) 16-cell cyst in late interphase. Most MTs clearly associate with the fusome. (D) Aligning cyst in region 2a (type 2). (E) Stretching cyst (type 3). MTs appear weak. (F) Lens-shaped cyst. MTs appear stronger in the center of the cyst, MTs associate with the regressing fusome. (G) Budding cyst (type 8). Some MTs are still associated with the fusome; MTs on the anterior side of the oocyte appear slightly displaced from the fusome. Green channel: GFP-α-tubulin (A’-G’). Red channel: fusome (A’”-G’’); α-spectrin (A’”-C’”;E’’); 1B1 (B’;D’); β-spectrin (F’;G’). Scale bar: 5 μm.
MTs cluster towards the middle of the rosette-shaped cyst. A large MT cluster, which has been described previously as an MTOC (Theurkauf et al., 1993), was not confined to a single cell as previously reported, but extended throughout all the cells of the cyst. The MTs appeared to outline a large central structure within the 16 cystocytes, and also extended away from it. The shape of the MT organization in 2a and young 2b cysts was strongly reminiscent of the fusome.

Microtubules associate with the fusome in late interphase

To investigate whether microtubules associate with the fusome, GFP-α-tubulin-expressing ovaries were stained with antibodies that recognize the fusome components HTS, α- or β-spectrin. Fusome structure is dynamic and related to the cell-cycle stage (de Cuevas and Spradling, 1998; Lilly et al., 2000; M. de Cuevas and A. S., unpublished) which allowed us to infer how MT-fusome interactions changed during the meiotic cell cycle. As 16-cell cysts form in the fourth cyst division (M-phase), we observed one of the spindle poles associated with the fusome, as expected (Storto and King, 1989; Fig. 3A). In early interphase, these spindle MTs did not break down, but instead were modified into large structures extending equally on either side of the ring canals (Fig. 3B). These modified spindles correspond to the ‘spindle remnants’ postulated by early researchers (see Telfer, 1975), and will be described in detail elsewhere (M. de C., N. G. and A. S., unpublished).

Whereas only a few MTs associated with the fusome during

Fig. 4. MT minus ends focus on the fusome during the polarization process in 16-cell cysts. In all images, anterior is to the left and posterior to the right. Fusome is in red (α-spectrin) and NOD-lacZ in green (β-Gal). (A) Two fully formed oblique sixteen-cell cysts. (B) Anterior cysts are oblique and posterior cysts are aligning. NOD-lacZ is found first on an extended area of the fusome (open arrowhead), then close to the central part of the fusome (white arrowhead). (C) Lens-shaped cysts, NOD-lacZ is still localized to the central part of the fusome (arrowhead). (D) Budding cyst. NOD-lacZ is highly enriched in the oocyte and is displaced from the fusome, both at the anterior (open arrowhead) and the posterior side of the oocyte nucleus (white arrowhead). Scale bar: 5 μm.

Fig. 5. Centrioles migrate along the fusome. In all pictures, anterior is to the left and posterior to the right. (A-G) γ-tubulin is in red, (A,B,C,G) GFP-α-TUB is in green and the fusome in either blue (B,C,G) or green (B’,D,F). (A) Disk-like structure at the posterior of the stage 3 oocyte (arrow); arrowhead points to MTs, bundled by ring canals. (A’) single channel (γ-tubulin). (B) 16-cell cyst in 2a. (B’) section of (B) but only fusome and γ-tubulin channels shown. Arrows point to a γ-tubulin spot on the fusome of an oblique 16-cell cyst, arrowheads point to the γ-tubulin spots on the fusome of a nearby cyst in mitosis. (C) Section of another oblique 16-cell cyst. The arrow points to a γ-tubulin spot located close to but not on the fusome, the arrowhead points to a γ-tubulin spot located on fusome. (D,E) Two examples of aligning cysts. γ-tubulin spots are localized to fusome arms. (F) Lens-shaped cyst. Spots of γ-tubulin are predominately found in the center of the cyst (open arrowhead) and are stronger than the fewer peripheral γ-tubulin spots (white arrowhead). (G) Budding cyst. The γ-tubulin spots in the oocyte are displaced from the fusome (open arrowhead). The arrow points to a posteriorly placed γ-tubulin spot. Nurse-cell γ-tubulin spot is on fusome (closed arrowhead). The following numbers of foci were revealed by computer analysis: (B) 14 spots, (D) 13 spots, (E) 16 spots, (F) 6 spots and (G) 6 spots. Scale bar: 5 μm.
M and early interphase, the fusome was embedded in a complex network of MTs in late interphase (Fig. 3C). MTs extended away from the fusome but also ran along the fusome. However, a small number of MTs seemed to be part of the fusome and penetrate within it at all stages of the cell cycle. The interaction between the fusome and the MTs persisted throughout region 2a and 2b as the fusome changed and regressed (Fig. 3D-G). When cysts start to stretch out and interact with follicle cells, the MT network appeared strong and remained associated with the stretching fusome (Fig. 3D). Subsequently (type 3), the MT network weakened but the fusome remained at its center (Fig. 3E). This may reflect changes in MT amount or stability. In progressively older cysts (type 4), the MT network again strengthened. Although the fusome is breaking down, many MTs remained in close proximity as cysts became lens-shaped (Fig. 3F). Even in budding cysts, some MTs associated with fusome remnants in nurse cells (Fig. 3G). On the anterior side of the oocyte, many MTs became slightly displaced from the fusome; others moved towards the posterior of the oocyte (as described in Fig. 1I,H).

Many MT minus ends focus first on the fusome and then into the differentiating oocyte

The fusome is located at the center of a complex and dynamic MT network during the polarization process of 16-cell cysts (Fig. 3). Microtubules are intrinsically polar structures, made up of α- and β-tubulin subunits; they have a slowly growing minus end and a faster growing plus end (reviewed in Desai and Mitchison, 1997; Wiese and Zheng, 1999). The NOD-lacZ fusion protein localizes to MT minus ends in vivo (Clark et al., 1997) and can mark the polarity of the MT network. Since available NOD-lacZ constructs express poorly in the germarium, we re-cloned the NOD-lacZ gene into the UASp germline expression vector (Materials and Methods). To see how MT polarity was related to the fusome, we stained ovaries expressing NOD-lacZ protein, with antibodies that recognize both β-gal and the fusome (1B1 or α-spectrin). As reported previously (Clark et al., 1997), the NOD-lacZ motor protein localized both on the anterior as well as the posterior side of the oocyte nucleus in budding cysts (Fig. 4D). We followed how the minus-ends of MTs become concentrated into the oocyte during the budding and polarizing process. Initially, only a small amount of NOD-lacZ localized to any specific location in young region 2a cysts (white arrowhead) and 2b cysts (open arrowhead), as well as in region 3 (arrow). (B) hts1/hts1 mutant germarium shows no such central MT clusters either in regions 1-2a (closed arrowhead) or region 3 (arrow). MTs appear to run along the periphery of individual cyst cells (B,E). GFP-α-tubulin expression in hts1 mutants (C-E), and in control ovaries (F). Normal-looking MT organization is observed in M phase (C) and early interphase (D), but the typical central late interphase MT structures are absent. Linear hts1 mutant cyst in (E) probably corresponds to a late interphase cyst. For comparison, a wild-type eight-cell cyst in late interphase is shown in (F). Scale bar: 5 μm.
lacZ staining concentrated towards the central part of the fusome (Fig. 4B,C, closed arrowhead), the location of the two four ring canal cells. Interestingly, as cysts underwent budding some NOD-lacZ staining remained at the anterior side of the oocyte (Fig. 4D); however, it was now displaced from the fusome (Fig. 4D; open arrowhead). These findings reveal that a large pool of MTs first focuses on the fusome before becoming restricted to the oocyte. The fusome may therefore be involved in organizing and giving directionality to the MTs, at least in part by interacting with their minus ends.

Potentially active centrosomes migrate into the oocyte along the fusome

Changes in the structure and polarity of the MT network might be mediated by the nurse-cell centrosomes. Centrosomes typically contain a pair of centrioles, which are surrounded by an amorphous peri-centriolar material with microtubule nucleating activity. An universal and essential component of the peri-centriolar material is γ-tubulin (reviewed in Wiese and Zheng, 1999). It is required for nucleation at minus ends, and the amount of γ-tubulin correlates with the number of MTs that are nucleated from a given centrosome (Khodjakov and Rieder, 1999). Using EM serial section re-constructions, Mahowald and Strassheim showed that the pro-nurse cell centrioles migrate through the ring canals and into the oocyte as cysts pass through region 2. After arriving in the oocyte, many accumulate in a disc-like cluster at the posterior of the oocyte beginning in stage 1 (Mahowald and Strassheim, 1970). However, migrating centrioles have been thought to lack MT-nucleating activity, and previous attempts to detect them using immunofluorescent markers yielded negative results (Wilson et al., 1997).

To visualize the behavior of germline centrosomes during oogenesis, we used a monoclonal antibody against γ-tubulin (Materials and Methods). Under the conditions of our experiments, many meiotic cysts contained discrete γ-tubulin spots. These signals were considerably weaker than those associated with M phase centrosomes (see Fig. 5B’); discrete staining could not always be seen above background, and only a limited number of 16-cell cysts were reliably analyzed. Nevertheless, the γ-tubulin staining corresponded to bone fide centrosomes, because we observed the characteristic disk-like cluster in stage 3 oocytes (Fig. 5A,A’). Moreover, this staining was localized to the posterior portion of the oocyte close to the MT network (Fig. 5A).

In oblique 16-cell cysts, 13-20 γ-tubulin spots were observed, and many but not all were present on the tips of the fusome (Fig. 5B,C). Thus, the location of γ-tubulin spots correlated with the pattern of NOD-lacZ staining, which indicated that some, but not all, minus ends were fusome associated at this time. As cysts started to align with the A-P axis later in region 2a, we observed discrete γ- tubulin spots predominantly localized to the tip of the fusome arms (Fig. 5D,E). In lens-shaped cysts, about three strong γ- tubulin spots concentrated around the central portion of the fusome (Fig. 5F), a pattern that again resembled the central concentration of NOD-lacZ in cysts of this age (Fig. 4C). In addition, one to three weaker spots were found in a peripheral location (Fig. 5F). In budding cysts a few strong γ-tubulin spots were detected in the oocyte, displaced from the fusome (Fig. 5G). Some of these were located anterior to the oocyte nucleus and some were located towards the posterior. A few weak spots were still detected in nurse cells and remained localized to the fusome. Our results suggest that the γ-tubulin spots correspond to the nurse cell centrosomes, as their position, number and behavior correlates with that of the migrating centrioles (Mahowald and Strassheim, 1970). Thus, centrioles migrate to the oocyte along the fusome, and may retain microtubule nucleating activity that contributes to the dramatic reorganization of the MT cytoskeleton and MT minus ends.

Fusome-MT interactions follow cell cycle changes

To analyze how the MT network relates to the fusome prior to meiosis, we studied developing cysts in region 1, where the fusome is still growing. Our results indicated that the MT network changed in a cyclic fashion throughout these divisions. Results for the eight-cell cyst division cycle are shown (Fig. 6). In each successive M phase one of the spindle poles of each dividing cell pair was attached to the fusome (Fig. 6A; Storto and King, 1989). As cells enter early interphase, the MTs elongated symmetrically relative to the ring canals, which contain growing fusome plugs (de Cuevas and Spradling, 1998). Subsequently, in both dividing and meiotic cysts (Figs 6B, 3B) microtubules became organized in association with the parent fusome as the plugs fuse (Fig. 6C). Finally, in late interphase, the fusome continued to reside in the middle of the MT network (Fig. 6D). The regular prominent shift of the MT network into association with the fusome and away from it within distinct cell cycle phases suggests that the fusome-MT interaction is cell-cycle regulated. A few differences were noted (compare Fig. 6C,D and Fig. 3C) that need to be resolved as more late-interphase markers become available. In particular, the MTs radiate away from the fusome more prominently during late interphase of mitotic cysts and appear to embed the fusome less than in meiotic cysts.

Our results suggest that the fusome and the MTs undergo a series of coordinated, cyclic changes during cyst divisions and in meiotic 16-cell cysts. In contrast to previous reports (Theurkauf et al., 1993), an organized MT network is present continuously during this period, and does not arise de novo in region 2a cysts.

The fusome is required to organize the MT network

If the fusome actively participates in organizing the MT network in late interphase, then mutations that disrupt the fusome should disturb the MT network at this time. To test this idea, we analyzed the MT organization in hts1 mutant females whose developing cysts lack a fusome (Lin et al., 1994). hts1 mutants produce defective cysts with a reduced number of cells that almost never form an oocyte (Yue and Spradling, 1992). The GFP signals observed were lower than in wild type because only one GAL4 driver line was present (Materials and Methods), hence to reveal the MT organization in hts1 mutants we also stained the mutant ovaries with an antibody against β-tubulin.

GFP-α-tubulin expression and β-tubulin antibody staining revealed the same results (Fig. 7). Control ovaries readily showed MTs organized in the center of the germ-cell clusters, as previously described (Fig. 7A). In contrast, a central clustering of MTs was not observed in hts1 mutant cysts in any position within the gerarium (Fig. 6B); instead the MTs simply outlined the individual cells (Fig. 7B,E). Even though
the organization of the MT network was disrupted, MTs were stable in the absence of a fusome. Interestingly, \textit{hts}\textsuperscript{1} mutant cysts typically appeared linear and not rosette shaped, suggesting that the altered MT cytoskeleton blocks programmed changes in cyst shape. Normal-looking spindles were readily observed in \textit{hts}\textsuperscript{1} mutants (Fig. 7C). Also the characteristic 'spindle remnants' were identified (Fig. 7D). Thus, the MT network in M phase and early interphase appeared normal in \textit{hts}\textsuperscript{1} mutants. We conclude that the fusome is needed to organize the MTs that associate with it in late interphase, but not to form mitotic spindles. These observations differ from a previous report that MTs are completely absent in \textit{hts}\textsuperscript{1} mutant germaria (Deng and Lin, 1997); our results show that microtubule organization is disrupted in a cell-cycle-dependent manner, with no clear change in overall MT levels.

DISCUSSION

The fusome plays a key role in cyst polarization

Polarity within germline cysts arises at two distinct times. First, polarizing forces build the asymmetric structure of the cyst in region I and are dependent on the fusome. Later, polarizing forces operate in fully formed 16-cell cysts to re-organize and polarize the MT network towards the differentiating oocyte. Since the oocyte is invariably one of the four-ring canal cells, early asymmetries must be preserved and re-used within polarizing 16-cell cysts; however, the mechanisms responsible for preserving and interpreting early polarity information have remained unclear. Our results show that the fusome is required to organize a complex and dynamic MT network both in dividing cysts of region I and also in polarizing 16-cell cysts. The experiments reported here strongly suggest that a persistent asymmetry within the fusome stores early polarity information for use in fully formed cysts. Moreover, this polarity is likely read out through an action of the fusome on microtubule organization. In the absence of a fusome, microtubule polarity in meiotic cysts is abolished and oocytes do not form.

The fusome organizes the MT network

The requirement of a fusome to organize MTs and polarize 16-cell cysts provides the strongest argument that the associations we observed between MTs and the fusome are functionally important. However, it could be argued that the MT organization in \textit{hts}\textsuperscript{1} mutants is disrupted by a fusome-independent mechanism. A processed fragment of HTS protein is incorporated into maturing ring canals in region 2b (Robinson et al., 1994), raising the possibility that ring canals, rather than the fusome, mediate MT polarization. Our observations do not support this interpretation, however. The MT network associates with the fusome and not the ring canals, and this association is detected in late interphase of cyst divisions and in meiotic cysts in region 2a, long before HTS proteins are incorporated into ring canals. All these associations, not just those in region 2b, are disrupted in \textit{hts}\textsuperscript{1} mutants. Consequently, the fusome rather than the ring canals is responsible for organizing MTs during early germ-cell development.

Genetic studies of three molecules that mediate transport along microtubules provide further evidence for a connection to the fusome. Mutations in cytoplasmic DHC64C disrupt cyst production and block production of a normally structured fusome, at least in part by disrupting the normal orientation of the mitotic spindle with the fusome during cyst divisions (McGrail and Hays, 1997). Unfortunately, it has remained unclear whether DHC64C is localized to the centrosomes and the fusome during prophase of meiotic cysts. It is, however, tempting to speculate that DHC64C plays a critical role in organizing the MTs of polarizing cysts, as there is a genetic requirement for DHC64C in oocyte formation (Li et al., 1994) and DHC64C staining is lost in \textit{hts}\textsuperscript{1} mutant cysts (Deng and Lin, 1997). In addition, \textit{Drosophila} LIS1 is involved in MT dynamics. LIS1 mutants have defects not only in MT organization but also in fusome formation (Liu et al., 1999; Swan et al., 1999). Finally, mutations in the kinesin-like protein KLP61F interfere with the production of a normal fusome in \textit{Drosophila} males (Wilson, 1999). Whether these last two proteins have a specific role during polarization or only during cyst formation remains unclear.

The fusome may mediate centrosome migration

Our observations reveal that a novel mechanism, directed centrosome migration, is likely to be involved in cyst polarization. Foci rich in \(\gamma\)-tubulin move onto and along the fusome in region 2a and 2b cysts, indicating that the nurse-cell centrosomes migrate along the fusome on their way to the oocyte. The total number of \(\gamma\)-tubulin foci in region 2a cysts ranged between 13 and 20, consistent with the number expected if most of the cystocyte centrosomes were labeled. The developmental changes in the timing and the approximate number of \(\gamma\)-tubulin foci were indistinguishable from the behavior of migrating centrioles (Mahowald and Strassheim, 1970). The association of the fusome with this process of directed movement provides further evidence that the fusome is itself a polarized structure, not only during its formation in region I, but also in region 2 cysts (Lin and Spradling, 1995; deCuevas and Spradling, 1998). It will be important to look for specific molecules that link centrosomes to the fusome and move them in a directional manner in response to fusome polarity.

Previously, only the movement of centrioles, not centrosomes, was reported (Mahowald and Strassheim, 1970). It was suggested that the migrating centrioles are inactive and break down in the oocyte so that centriolar constituents can be re-used in the early embryo. The \(\gamma\)-tubulin staining we observed argues that the migrating centrioles are accompanied by pericentriolar material and can actively nucleate microtubules. The preferential accumulation of active centrosomes in the central region of the fusome would probably contribute to polarizing the MT network. Later movements of the centrosomes probably contribute to subsequent stages of polarization. These include the focusing of the MT minus ends within a single cyst cell, and the movement of MTs to the posterior of the oocyte. The existence of 13-20 actively migrating centrosomes is not consistent with the suggestion that MTOC formation and oocyte determination are controlled by the selective inheritance of a single active centrosome (Theurkauf, 1994).

The fusome may act by binding or nucleating microtubules

At least some of the microtubule organizing activity of the
fusome is not due to associated centrosomes. During late interphase of the cystocyte divisions, the main part of the fusome strongly interacts with microtubules. Many MTs and/or MT bundles exit the fusome at this time, and it appears unlikely from their number and distribution that associated centrosomes could account for all this activity. Even though many discrete γ-tubulin spots/centrosomes were localized on or close to the fusome arms in region 2a, the overall MT polarity was not uniformly oriented towards the fusome, based on the NOD-lacZ staining. Consequently, the fusome may transiently acquire the ability to bind or bundle MTs, rather than nucleating them.

At other times, the fusome might interact directly with MT minus ends. Fusomal material may acquire the ability to nucleate MT minus ends by associating with peri-centriolar material. The fusome in polarizing cysts appears most likely to exhibit such an activity, as MT-fusome interaction during polarization involves MT minus end interactions. Alternatively, the fusome might interact with motor proteins that bridge it to MTs and specifically interact with minus ends. Polarization of the MT network into the differentiating oocyte could involve both minus end interaction as well as bundling. Both of these mechanisms would probably be amplified through MT-MT interactions outside the fusome.

Previously, it has been unclear when and if the fusome contained polymerized microtubules. The retention of such MTs was proposed to store polarity information from the fusome for later use in developing 16-cell cysts (Lin and Spradling, 1995). GFP-α-tubulin staining did reveal MTs that ran parallel to the fusome in region 2b cysts. However, there is no evidence for such MTs in aligning cysts within region 2a, or within the main body of the fusome in early interphase of the cystocyte divisions. Consequently, polymerized MTs within the fusome that persist from the time of cyst formation do not appear to be the basis of the fusomal polarity.

Developmental timing of polarization

The use of a highly sensitive GFP-α-tubulin fusion protein revealed a more detailed picture than previously available of the dynamic process of MT polarization in developing 16-cell cysts. At the beginning of the polarization process in oblique meiotic cysts, MTs extend equally into all cystocytes and embedded the fusome. Subsequently, MT minus ends coalesce towards the central region of the fusome, which resides within both four-ring canal cells and their immediate neighbors. Gradually, the MT network continues to polarize until it becomes clearly focused in a single cell, the future oocyte, late in region 2b. Thus, an organized microtubule cytoskeleton does not arise spontaneously within region 2a cysts in response to the appearance of a new MTOC in one of the cyst cells. Rather, the MT network polarizes progressively over an extended period of time and the new MTOC develops gradually.

Our observations clarify the likely relationship between cyst polarization and the asymmetric accumulation of specific molecules. The exact stage at which specific RNAs and proteins accumulate has remained unclear in previous studies, as reagents differing in sensitivity were often used. Only after a significant fraction of the MT minus ends become concentrated would mRNAs and proteins whose transport is MT dependent be able to accumulate in their vicinity. MT minus ends begin to cluster in mid- to late-region 2a cysts; we propose that this event determines when specific molecules begin to accumulate unequally. This is consistent with the observation that orb, hts and probably other mRNAs become clearly localized within a small region of the cyst as early as the middle of region 2a in some germaria.

Germline cysts are models for the acquisition of intracellular asymmetry

The origin of cellular asymmetry is being studied in diverse systems, including the formation of yeast buds, the development of embryonic axes in C. elegans and Drosophila embryos, the segregation of Drosophila neuroblasts and the polarization of epithelial layers (Takizawa et al., 1997; Bowerman and Shelton, 1999; Lu et al., 1998; Palmer et al., 1992). In all these systems, there is evidence that the microtubule cytoskeleton interacts with other cytoskeletal components, particularly actin, to orient divisions and mediate intracellular asymmetry. For example, localization of the MT-orienting KAR9 protein in yeast depends on actin (Miller et al., 1999). The asymmetric distribution of proteins after the first division of the C. elegans embryo requires actin, and some localized gene products such as PAR1 appear to act on microtubules. In at least one case, the requirement for actin in spindle orientation exists during only a portion of the cell cycle (Theesfeld et al., 1999).

Our results indicate that Drosophila germline cysts provide another system where the development of polarity depends on interactions between the microtubule cytoskeleton and another cytoskeletal system, the fusome. The fusome arises early in germ cell development at the time germ cells migrate toward the gonadal mesoderm (Lin and Spradling, 1997), and subsequently is often located near the centrioles. Interactions, between the fusome and the MT cytoskeleton may take place in some form during much of germ-cell development, and be elaborated as cysts form and polarize. Further study of the molecular basis of fusome-microtubule interactions is likely to advance our understanding of many aspects of cell and tissue polarization.

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