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The microtubule motor cytoplasmic dynein is required for spindle orientation during germline cell divisions and oocyte differentiation in *Drosophila*

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SUMMARY

During animal development cellular differentiation is often preceded by an asymmetric cell division whose polarity is determined by the orientation of the mitotic spindle. In the fruit fly, *Drosophila melanogaster*, the oocyte differentiates in a 16-cell syncytium that arises from a cystoblast which undergoes 4 synchronous divisions with incomplete cytokinesis. During these divisions, spindle orientation is highly ordered and is thought to impart a polarity to the cyst that is necessary for the subsequent differentiation of the oocyte. Using mutations in the *Drosophila* cytoplasmic dynein heavy chain gene, *Dhc64C*, we show that cytoplasmic dynein is required at two stages of oogenesis. Early in oogenesis, dynein mutations disrupt spindle orientation in dividing cysts and block oocyte determination. The localization of dynein in mitotic cysts suggests spindle orientation is mediated by the microtubule motor cytoplasmic dynein. Later in oogenesis, dynein function is necessary for proper differentiation, but does not appear to participate in morphogen localization within the oocyte. These results provide evidence for a novel developmental role for the cytoplasmic dynein motor in cellular determination and differentiation.

Key words: dynein, cell fate, asymmetric division, *Drosophila*, spindle orientation, oogenesis

INTRODUCTION

A central question in the fields of cell biology and developmental genetics concerns how a cell chooses to adopt a particular cell fate and differentiate into a specific cell type. While the mechanisms that contribute to the decision process are still emerging, it is known that one pathway to cellular differentiation involves asymmetric cell division in which cell fate determinants are partitioned unequally between daughter cells. The orientation of the mitotic spindle has long been known to be important in directing asymmetric cell divisions during embryogenesis (reviewed by Strome, 1993). More recently, molecular genetic analyses in *C. elegans* (Kemphues et al., 1988; Cheng et al, 1995; Guo and Kemphues, 1995; Etemad-Moghadam et al., 1995) and *Drosophila* (Kraut et al., 1996) have identified molecules that couple spindle orientation to the asymmetric localization of cell fate determinants (reviewed by Rhyu and Knoblich, 1995; White and Strome, 1996; Doe, 1996). Members of the myosin family of actin-based molecular motors have been implicated in the asymmetric segregation of factors necessary for cell fate determination in yeast (Jansen et al., 1996; Bobola et al., 1996) and *C. elegans* (Guo and Kemphues, 1996). Less is known about the pathways which establish the initial cellular asymmetry that directs spindle orientation and polarized transport. Given the importance of the microtubule cytoskeleton in cytoplasmic organization and intracellular transport in polarized cell types, microtubules and their associated motor molecules may be part of the primary mechanism that establishes this cytoplasmic asymmetry.

Oogenesis in *Drosophila melanogaster* is an attractive system for studying the role of microtubule motors in cellular differentiation (for a comprehensive review of oogenesis, see Spradling, 1993). Cytological and pharmacological studies have suggested that the microtubule cytoskeleton is essential for oocyte differentiation through its roles in intercellular transport and in the asymmetric localization of determinants within the developing oocyte that influence axis specification (reviewed by Theurkauf, 1994; St Johnston, 1995). Oocyte development occurs in a cyst of 16 interconnected cells that arises from a single cystoblast which undergoes 4 synchronous rounds of division with incomplete cytokinesis. One cell differentiates as the oocyte, while the remaining 15 become highly polyploid nurse cells. The nurse cells synthesize materials which are transferred through the cytoplasmic connections (ring canals) into the differentiating oocyte (reviewed by Mahajan-Miklos and Cooley, 1994). The asymmetric transport of materials is thought to occur along a polarized microtubule array established shortly after the 16-cell cyst is formed (Mahowald and Strassheim, 1970; Theurkauf et al., 1993). Microtubules originate in the pro-oocyte and extend their plus ends through the ring canals into the nurse cells, creating a polarized transport system on which a minus-end directed microtubule motor could function.

Within the germline cyst a polarity must be established in

order to define the pro-oocyte as the site of microtubule nucleation in the assembly of the polarized microtubule array. This polarity may be reflected in the asymmetric segregation of the spectrin-rich, multivesicular fusome during the four mitotic divisions (Lin and Spradling, 1995) and in the resulting fixed pattern of cytoplasmic connections among the 16 cystocytes (King et al., 1982). The asymmetric cystocyte divisions result from the behavior of the fusome and its interaction with spindle poles during mitotic division. How anchorage of the mitotic spindles to the fusome ensures the stereotypical pattern of cyst cell divisions, which creates a polarized cyst leading to oocyte determination, reorganization of the microtubule cytoskeleton, and oocyte differentiation, is still unclear.

The dynein ATPases constitute a family of minus-end directed microtubule-based motors. The best characterized members of the dynein family are the axonemal dyneins which power ciliary and flagellar motility (reviewed by Porter, 1996). Since the identification of a cytoplasmic form of dynein in non-ciliated cells (Paschal and Vallee, 1987), the function, regulation, and subcellular targeting of the cytoplasmic dynein motor have been the subject of intense study (reviewed by Schroer, 1994; Holzbaur and Vallee, 1994; Vallee and Sheetz, 1996). Our previous characterization of the asymmetric localization of cytoplasmic dynein to and within the developing oocyte suggested that cytoplasmic dynein might function in multiple microtubule-dependent processes in *Drosophila* oogenesis (Li et al., 1994). The identification of mutations in the *Drosophila* cytoplasmic dynein heavy chain gene *Dhc64C* (Gepner et al., 1996) has allowed us to investigate the role of cytoplasmic dynein in oocyte determination and differentiation. Our results provide evidence for a novel role of the cytoplasmic dynein in asymmetric cell division and cellular differentiation.

MATERIALS AND METHODS

Fly stocks and genetic analyses

The mutations in the *Drosophila* cytoplasmic dynein heavy chain *Dhc64C* used in this study are described by Gepner et al. (1996). Adult flies, heteroallelic for *Dhc64C* mutations, were generated at the expected frequency relative to sibling classes. The female-sterile phenotype of the dynein mutants was shown to be rescued by the wild-type *Dhc64C* transgene by introducing $P[Dhc^{+}]^{X}$ (Gepner et al., 1996) into the mutant background. Examination of the localization of the *Drosophila* kinesin heavy chain *khc* motor domain-β-galactosidase fusion protein was performed by introducing the second chromosome kinesin-β-gal insert *KZ32* (Clark et al., 1994), provided by I. Clark (Princeton University), into a *Dhc64C* mutant background using the appropriate crosses. Flies heterozygous for *KZ32* and heteroallelic for *Dhc64C*, ie., *KZ32*/+; $Dhc64C^{6\text{-}6}$/$Dhc64C^{6\text{-}12}$, were selected for ovary dissection and analysis.

Germline clonal analysis was performed using the FRT/FLP recombinase method (Golic and Lindquist, 1989; Golic, 1991) together with the ovo^{D1}-Dominant Female Sterile technique (Chou et al., 1993). The third chromosome $P[ovo^{D1}]^{2X48}$ $P[w^{+}\text{FRT}]^{2A}$ (Chou et al., 1993) was provided by N. Perrimon (Harvard University). The X chromosome FLP recombinase stock $P[ry^{+}$, hsFLP] (Golic and Lindquist, 1989), and the third chromosome FRT stock $P[w^{+}$ FRT$]^{2A}$ (Chou et al., 1993), were obtained from the Bloomington Stock Center (Bloomington, IN). A $Dhc64C^{4\text{-}19}$ $P[w^{+}$ FRT$]^{2A}$ recombinant chromosome was constructed using standard recombination techniques. The recombinant chromosome was shown to be free of secondary lethal mutations by rescue of the $Dhc64C^{4\text{-}19}$ recessive lethal phenotype by the wild-type $P[Dhc^{+}]^{X}$ transgene. For clonal analysis, 0-24 hour eggs were collected from the appropriate crosses, aged for 4 days to the third instar larval stage, and the larvae heat shocked in a 37°C water bath for 2 hours to induce expression of the FLP recombinase. Under these conditions the frequency of control females that contained clones in at least one ovary was between 70-90%. Ovaries were examined under the dissecting microscope and clones were identified by the presence of mature eggs and vitellogenic egg chambers. To determine the number of germline cells/mosaic egg chamber, the clonal analysis with the $Dhc64C^{4\text{-}19}$ was repeated in the absence of ovo^{D1}, and ovaries were then prepared for immunofluorescence and probed with the dynein heavy chain antibody P1H4 and the nuclear stain OliGreen (Molecular Probes, Inc., Eugene, OR).

Antibodies and immunofluorescence localization

A purified, bacterially expressed fusion protein from *Dhc64C* cDNA clone pTR13 (Li et al., 1994), encoding amino acid residues 128-422 of the cytoplasmic dynein heavy chain, was used as antigen in the preparation of hybridoma cell lines and the P1H4 ascites (Immunological Resource Center, U of IL Urbana/Champaign). Microtubule-associated proteins were prepared from wild-type OregonR ovaries, and SDS-PAGE and blotting were carried out essentially as described by McGrail et al. (1995) except that 15 μg total protein, and approx. 2 μg ATP-MAPS, was loaded per lane. For blotting the P1H4 monoclonal was used at a dilution of 1:10,000.

Ovaries were dissected, fixed and processed for immunocytochemistry as described by McGrail et al. (1995). For immunofluoresence, the P1H4 anti-dynein heavy chain mouse monoclonal was diluted 1:500, the anti-Bicaudal-D mouse monoclonal (Suter and Steward, 1991) was diluted 1:3, the rabbit polyclonal anti-β-galactosidase antibody (Cappel/Organon Teknika Corp., Westchester, PA) was diluted 1:200, and ring canals were detected with the anti-phosphotyrosine monoclonal antibody (ICN Biomedical, Inc., Aurora, OH) diluted 1:50. Dynein and staufen localization were examined in ovaries double-labelled with the anti-dynein heavy chain rabbit polyclonal antibody PEP1 (Li et al., 1994) diluted 1:50, the rat polyclonal anti-staufen antiserum diluted 1:100, and FITC-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-rat secondary antibodies. Mitotic spindles and fusomes were detected in ovaries double labelled with a rat monoclonal anti-α-tubulin antibody (Accurate Chemical Co., Westbury, NY) diluted 1:10, the rabbit polyclonal anti-α-spectrin antibody (Dubreuil et al., 1987; Byers et al., 1987) diluted 1:200, and Texas Red-conjugated goat anti-rat and FITC-conjugated goat anti-rabbit secondary antibodies. For triple labelling ovaries were incubated with a rabbit polyclonal anti-dynein heavy chain antibody (Hays et al., 1994) diluted 1:3, the rat monoclonal anti-α-tubulin antibody diluted 1:10 (Accurate Chemical Co., Westbury, NY), the chromatin marker OliGreen diluted 1:200 (Molecular Probes, Inc., Eugene, OR), and Cy5-conjugated goat-anti-rabbit and Texas Red-conjugated goat-anti-rat secondary antibodies. A similar pattern of dynein localization in mitotic cysts was observed with the P1H4 monoclonal and colocalized with α-spectrin on the fusome. The secondary antibodies Texas Red-conjugated goat anti-rat and Texas Red-conjugated goat anti-mouse (Jackson ImmunoResearch Labs, West Grove, PA), FITC-conjugated goat-anti-rabbit (Boehringer Mannheim, Indianapolis, IN) and Cy5-conjugated goat anti-rabbit (Amersham, Arlington Heights, IL) were diluted 1:100. Confocal z series image files of labelled tissues were acquired with a BioRad 1000 laser scanning confocal microscope, and processed using NIH Image 1.59 and Adobe Photoshop 3.0 (Adobe Systems, Inc., Mountain View, CA) software.

In situ hybridization

Whole-mount in situ hybridization of digoxigenin-labelled antisense RNA probes to egg chambers was performed according to the method of Tautz and Pfeifle (1989) with modifications. The *oskar* cDNA (Ephrussi et al., 1991) was provided by R. Lehmann (Skirball

Institute, New York University Medical School), the *bicoid* cDNA (Berleth et al., 1988) by E. Stephenson (University of Alabama), and the *gurken* cDNA (Neuman-Silberberg and Schüpbach, 1993) by T. Schüpbach (Princeton University). Digoxigenin-labelled antisense RNA probes were synthesized from cDNAs using the RNA Transcription Kit (Stratagene, La Jolla, CA) and digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, IN). Probes were hydrolyzed to approximately 200 nucleotides in length by incubation in 60 mM Na_2CO_3/40 mM $NaHCO_3$ pH 10.2 (Cox et al., 1984). Ovaries were dissected in EBR buffer (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes pH 6.9) and fixed for 20 minutes in 200 μl 4% paraformaldehyde in PBS containing 10% DMSO plus 600 μl heptane. The fixed ovaries were rinsed 3 times in PBS/0.1% Tween-20 (PBTw), washed in PBTw for 2 hours at room temperature, and dissected into individual ovarioles. Ovarioles were treated with 10-50 μg/ml Proteinase K at room temperature for 30 minutes up to 2 hours, depending on the probe. Prehybridization, hybridization and washes were carried out at 55°C. Hybridized tissues were incubated with an anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim Co., Indianapolis, IN) and the color reaction performed with BCIP and NBT (Sigma Chemical Co., St. Louis, MO) in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris pH 9.5). After staining, tissues were washed in PBTw, mounted on slides in PBTw, and examined on a Zeiss Axioskop 10 microscope using 20×/0.5 and 40×/0.75 plan-neofluar lenses and differential interference contrast optics. Images were recorded on Kodak Ektachrome 160T color slide film. Composites were made from slide images scanned into a Macintosh Power PC 8100 (Apple Computer Co., Cupertino, CA) using a Polaroid Sprintscan 35 film scanner (Polaroid Co., Cambridge, MA) and Adobe Photoshop 3.0 (Adobe System, Inc., Mountain View, CA) software.

RESULTS

Cytoplasmic dynein is required in the germline for oocyte differentiation

To investigate whether dynein function was specifically required in the germline for oocyte development, we made mosaic egg chambers whose germline cyst lacked wild-type dynein. We used the FRT/FLP recombinase method (Golic and Lindquist, 1989; Golic, 1991) together with the ovo^{D1} Dominant-Female Sterile technique (Chou et al., 1993) to generate dynein mutant germline clones. We previously determined that the recessive lethal dynein heavy chain allele $Dhc64C^{4-19}$ is a strong loss-of-function mutation (Gepner et al., 1996). Mosaic egg chambers whose germline was homozygous for the $Dhc64C^{4-19}$ allele failed to produce mature eggs. Out of 590 ovaries examined from females that could potentially contain clonally derived eggs, only 12 mature eggs were found and none of the ovaries contained egg chambers with developing oocytes (Table 1). The clonal $Dhc64C^{4-19}$ egg chambers contained 16 differentiated nurse cells (see below) and were distinguished from the undifferentiated cells contained within the $ovoD^1$ egg chambers. In contrast, 96% of the 267 ovaries examined from control females, which carried one copy of the wild-type *Dhc64C* transgene $P[Dhc^+]^X$ (Gepner et al., 1996), contained many mature eggs and egg chambers with developing oocytes (Table 1) and produced viable adult progeny. The ability of the wild-type *Dhc64C* transgene to rescue the production of normal, fertilized eggs demonstrates that cytoplasmic dynein is required in the germline for oocyte development.

The mosaic analysis was repeated in the absence of ovo^{D1} to further characterize the germline requirement for dynein function in oocyte determination. To identify the mosaic egg chambers, we used an anti-dynein heavy chain monoclonal antibody (P1H4) that specifically detects the cytoplasmic dynein heavy chain in extracts and microtubule-associated proteins isolated from ovaries (Fig. 1). The egg chambers were double-labelled with the chromatin marker OliGreen to determine the number and size of nuclei. Immunofluorescence

Table 1. Cytoplasmic dynein function is required in the germline for oocyte development

Genotype	No. of females analyzed	No. of ovaries with eggs* Total no. of ovaries scored (% of ovaries with eggs)
$Dhc64C^{4-19}/P[ovo^{D1}]$	295	12/590 (2)
$P[Dhc^+]$; $Dhc64C^{4-19}/P[ovo^{D1}]$	139	267/278 (96)

*Mosaic egg chambers which lack dynein function in the germline fail to develop an oocyte or mature egg. Egg chambers with germline cells homozygous mutant for the dynein allele $Dhc64C^{4-19}$ (Gepner et al., 1996) were produced with the FRT/FLP recombinase system (Golic, 1991; Golic and Lindquist, 1989). On the homologous third chromosome was the transgene $P[ovo^{D1}]^{2X48}$ (Chou et al., 1993). ovo^{D1} is a dominant female-sterile allele of the *ovo* locus which prevents the germline from differentiating, therefore eggs are not normally produced in the ovaries of females carrying the ovo^{D1} transgene (Chou et al., 1993). Consequently, any eggs present must have arisen from a mitotic recombination event which produced a stem cell that has lost the chromosome bearing the $P[ovo^{D1}]$ transgene and is now homozygous mutant for the $Dhc64C^{4-19}$ allele. In test females of the genotype $Dhc64C^{4-19}/P[ovo^{D1}]$, a total of 12 eggs were found in 590 ovaries scored. None of the ovaries contained egg chambers with developing oocytes. In contrast, in control females of the genotype $P[Dhc^+]^X$; $Dhc64C^{4-19}/P[ovo^{D1}]$, which carried a copy of the wildtype *Dhc64C* transgene $P[Dhc^+]$ on the X chromosome, 96% of the ovaries had at least one ovariole with developing oocytes and many mature eggs. The almost complete absence of oocytes and eggs in the test ovaries demonstrates that cytoplasmic dynein function is necessary in the germline for oocyte development.

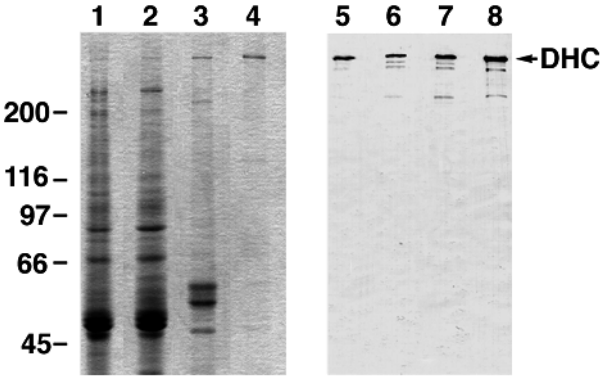

Fig. 1. Specificity of the mouse monoclonal anti-dynein heavy chain antibody P1H4. A Coomassie blue-stained gel (lanes 1-4), and duplicate gel blotted and probed with the P1H4 monoclonal (lanes 5-8), of fractions from a microtubule-associated proteins (MAPS) preparation from ovary extracts. Lanes 1 and 5, homogenate; lanes 2 and 6, 125,000 ***g*** supernatant; lanes 3 and 7, taxol-stabilized microtubule pellet; lanes 4 and 8, cytoplasmic dynein enriched ATP-eluted MAPS. The P1H4 monoclonal antibody specifically detects the dynein heavy chain (arrow, DHC) in the homogenate and high speed supernatant, and reveals the enrichment of cytoplasmic dynein in the extraction of ATP-dependent MAPS from the microtubule pellet. Molecular mass standards are indicated to the left of lane 1.

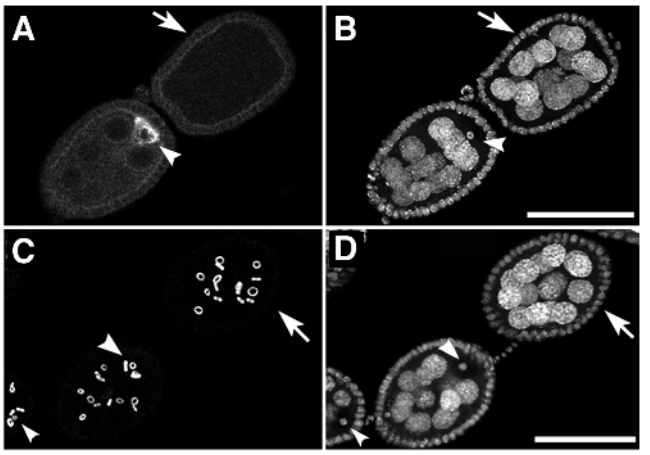

Fig. 2. Cytoplasmic dynein is required in the germline for oocyte differentiation. (A,B) The same egg chambers probed with the anti-dynein heavy chain monoclonal antibody P1H4 (A) and stained with the chromatin marker OliGreen (B). (C,D) Identical egg chambers probed with a monoclonal antibody specific for phosphotyrosine epitopes to label ring canals (C) and stained with the chromatin marker OliGreen (D). (A-D) In mosaic egg (top right in each panel) chambers in which the germline is homozygous mutant for the dynein allele *Dhc64C*$^{4-19}$ (arrows, A-D), the oocyte fails to differentiate and each of the cells adopt the nurse cell fate. In wild-type egg chambers (bottom left in each panel) the oocyte is identified by the reduced amount of chromatin in the nucleus (arrowheads, B,D), the accumulation of dynein within the oocyte (arrowhead, A), and the presence of 4 ring canals at the anterior surface (arrowheads, C). In contrast, in the mosaic chambers dynein (as well as, Bic-D; not shown) failed to accumulate (arrow, A) and contained a polyploid nucleus in each germline cell (arrows, B,D), indicating the failure of the oocyte to differentiate. The disorganized pattern of ring canals in the mosaic egg chambers (arrow, C) indicates that the initial polarity of the mosaic germline cyst is disrupted. Magnification is identical in all panels. Scale bars in B and D, 50 μm.

localization with the P1H4 monoclonal antibody revealed that the mosaic egg chambers lacked dynein signal in the germline (arrow Fig. 2A). 96% of the 85 mosaic egg chambers examined contained the normal number of 16 cells, however, each of the cells appeared to develop as nurse cells, as indicated by their polyploid nuclei (arrows, Fig. 2B,D). In addition, the Bicaudal-D protein (Bic-D; Suter and Steward, 1991) did not accumulate in a single cell in the mosaic chambers.

The polarity of the germline cyst is disrupted in cytoplasmic dynein mutants

To understand how oocyte determination is blocked in dynein mutant egg chambers, we first examined the polarity of the egg chambers in germline clones. An antibody against a phosphotyrosine epitope present in ring canals was used to reveal the size and position of the intercellular bridges in wild-type and mutant egg chambers. In the mosaic egg chambers the pattern (e.g., size and location) of the ring canal connections appeared abnormal. The most posterior cell in the egg chamber did not have the typical cluster of the 4 largest ring canals (Fig. 2C,D arrow). One explanation of this phenotype is based on the disruption of spindle orientation and division pattern (see below), but we do not exclude the possibility that a cell containing four ring canals is mislocalized within the 16-cell cyst. Nonetheless, the mosaic egg chambers always lacked an oocyte nucleus, and instead contained 16 polyploid nurse cell nuclei. By contrast, in wild-type egg chambers an oocyte invariably was positioned at the posterior of the egg chamber, and was easily distinguished by the four largest ring canals which reside at the anterior margin of the oocyte (arrowheads, Fig. 2C,D). These results suggest that dynein function is required early in germline cyst formation to establish cyst polarity. The disruption of cyst polarity can also explain the failure of Bic-D to accumulate in a single cell in the germline clones. The absence of polarity in the cyst correlates with a failure in oocyte fate determination and the differentiation of 16 polyploid nurse cells.

Table 2. Female-sterile phenotypes of *Dhc64C* mutants analyzed in this study

Genotype	Phenotype
Dhc64C$^{3-2}$/*Dhc64C*$^{6-12}$	The ovaries do not produce mature eggs; the egg chambers do not contain a developing oocyte and each cell develops with a polyploid nucleus like a nurse cell
Dhc64C$^{6-6}$/*Dhc64C*$^{6-12}$	The ovaries produce mature eggs, however, the eggs are fragile and show variable defects in size, shape, and number and orientation of the chorionic appendages

Genotype refers to the genotype of the adult female doubly heterozygous for the two *Dhc64C* mutations. The *Dhc64C* alleles *Dhc64C*$^{3-2}$, *Dhc64C*$^{6-6}$ and *Dhc64C*$^{6-12}$ are described by Gepner et al. (1996).

The polarity of the germline cyst is also aberrant in ovaries from the female-sterile combination of *Dhc64C* alleles, *Dhc64C*$^{3-2}$/*Dhc64C*$^{6-12}$. Females doubly heterozygous for the recessive lethal alleles *Dhc64C*$^{3-2}$ and *Dhc64C*$^{6-12}$ are viable but sterile (Gepner et al., 1996; Table 2). In contrast to the mosaic egg chambers, only 15% of 700 *Dhc64C*$^{3-2}$/*Dhc64C*$^{6-12}$ chambers scored contained 16 cells. The remaining egg chambers contained predominantly 8 (approx. 45%), 4 (approx.32%), or 2 (approx.5%) cells, while a small percentage (>5%) of single cell chambers were also noted. However, regardless of cell number an oocyte was never observed in the mosaic or transheterozygous mutant egg chambers and each cell appeared to develop along the nurse cell pathway. The reduced and even number of cells in the transheterozygous egg chambers suggests an additional functional contribution of dynein to stem cell division. The predominant 16-cell cysts in clonal egg chambers may indicate a perdurance of wild type *Dhc64C* product that is sufficient for these mitotic divisions.

The monoclonal antibody P1H4 revealed a low level of cytoplasmic dynein evenly distributed throughout the germline compartment of the mutant egg chambers (Fig. 3A). Similar to the mosaic egg chambers, Bicaudal-D also failed to accumulate in a single cell within these mutant egg chambers (Fig. 3C). Moreover, Bicaudal-D accumulation was never observed to be enriched in a single cell within mutant germarial cysts. Recent molecular studies have identified the membrane skeletal proteins α- and β-spectrin and the *Drosophila* adducin-like *hu-li tai shao* gene product (Yue and Spradling, 1992; Lin et al., 1994b; de Cuevas et al., 1996) as components of the fusome. We examined fusomes in the *Dhc64C*$^{3-2}$/*Dhc64C*$^{6-12}$ germaria by immunofluorescence microscopy using an anti-α-spectrin antibody (Dubreuil et la., 1987; Byers et al., 1987). We found that fusomes were present in the mutant germline cysts (Fig.

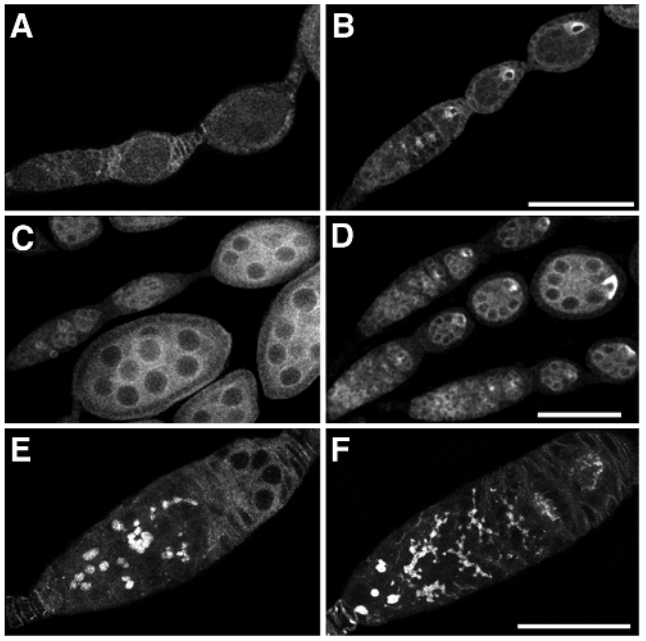

Fig. 3. The heteroallelic female-sterile combination of *Dhc64C* alleles $Dhc64C^{3\text{-}2}/Dhc64C^{6\text{-}12}$, blocks oocyte differentiation. In $Dhc64C^{3\text{-}2}/Dhc64C^{6\text{-}12}$ egg chambers the oocyte fails to differentiate as in $Dhc64C^{4\text{-}19}$ mosaic egg chambers. (A,B) Immunofluorescence localization with the anti-dynein heavy chain monoclonal antibody P1H4. (A) In $Dhc64C^{3\text{-}2}/Dhc64C^{6\text{-}12}$ chambers dynein signal was uniformly distributed throughout all cells in the germline cysts of the germarium and in the developing egg chambers.
(C,D) Immunofluorescence localization of Bicaudal-D. (C) Bicaudal-D did not accumulate in a single cell in the $Dhc64C^{3\text{-}2}/Dhc64C^{6\text{-}12}$ germline cysts or egg chambers, indicating the oocyte fails to differentiate in the dynein mutant cysts. (E,F) Fusome labelling with an anti-α-spectrin antibody (Dubreuil et al., 1987; Byers et al., 1987). (E) Fusomes are present in the germline cysts in the $Dhc64C^{3\text{-}2}/Dhc64C^{6\text{-}12}$ germaria, however, the fusomes appear less branched in comparison to control, wild-type fusomes (F).
(B,D,F) Control ovaries in which the localization of dynein (B) and Bicaudal-D (D) to the oocyte, and normal fusome morphology (F) were rescued by introducing the wild-type *Dhc64C* transgene $P[Dhc^{+}]^{X}$ into $Dhc64C^{3\text{-}2}/Dhc64C^{6\text{-}12}$ females. Magnification is identical in A and B; C and D; and E and F. Scale bars (A and B), (C and D) (E and F), 50 μm.

3E), although the branching of the fusomes was reduced compared to the control (Fig. 3F). The wild-type *Dhc64C* transgene was able to rescue each aspect of the mutant phenotype, including cyst cell number, dynein (Fig. 3B) and Bicaudal-D (Fig. 3D) localization to the pro-oocyte and oocyte, fusome morphology (Fig. 3F) and fertility (data not shown).

Spindle orientation during asymmetric cell division requires cytoplasmic dynein

The orientation and attachment of mitotic spindles to the fusome during cystocyte divisions is thought to ensure the fixed pattern of cell divisions necessary to create the polarized cyst (Telfer, 1975; King et al., 1982; Storto and King, 1989; Lin and Spradling, 1995). We found a striking defect in spindle orientation in the dynein mutant cysts. Ovaries from $Dhc64C^{3\text{-}2}/Dhc64C^{6\text{-}12}$ females, and control $P[Dhc^{+}]^{X}$; $Dhc64C^{3\text{-}2}/Dhc64C^{6\text{-}12}$ females carrying one copy of the wild-type *Dhc64C* transgene, were double-labelled with antibodies to α-tubulin and α-spectrin. As has been reported previously for wild-type cysts (Storto and King, 1989; Lin and Spradling, 1995), in control 2-cell (Fig. 4A,D), 4-cell (Fig. 4B,E) and 8-cell (Fig. 4C,F) mitotic cysts the spindles were arranged in compact clusters with one spindle pole in close contact with a single lobe or branch of the fusome. In contrast, in the mutant cysts the spindles frequently did not appear to contact the fusome, but instead were randomly oriented in the cyst (Fig. 4G-L). In the 2-cell mutant cyst shown in Fig. 4G, neither spindle has a pole in close contact with fusome. α-spectrin localization revealed that occasionally the fusome was less branched and failed to extend into every cell of the cyst (Fig. 4H,I). However, in those cells that contained a branch of the fusome, the spindles were not oriented with one pole close to the fusome (Fig. 4 arrows in H,L).

The defect in spindle orientation suggested that dynein function might be directly involved in the mechanism of spindle orientation. We examined dynein localization in wild-type mitotic cysts and detected an enriched, punctate pattern of dynein centrally located in a region of the cyst that would correspond to the position of the fusome (Fig. 5A-H). We did not detect an enrichment of dynein on a fusome-like structure in interphase cysts (data not shown). In the 2-cell anaphase cyst and the 4-cell metaphase cyst, each spindle (green, Fig. 5A,E) was oriented with one pole in close association to the area of dynein staining (red, Fig. 5A,E). The individual dynein (Fig. 5B,F), tubulin (Fig. 5C,G), and chromatin (Fig. 5D,H) images are shown. These data suggest that spindle attachment to the fusome is mediated by cytoplasmic dynein. This mechanism would ensure the fixed pattern of cystocyte divisions that lead to a polarized germline cyst.

Cytoplasmic dynein is required in late stage egg chambers for oocyte differentiation

In addition to its role in the early events of oocyte determination and differentiation, the microtubule cytoskeleton has been implicated in the establishment of axial polarity and the localization of morphogens within the developing oocyte (Pokrywka and Stephenson, 1991; Theurkauf et al., 1992; Lane and Kalderon, 1994). Female adults doubly heterozygous for the dynein alleles $Dhc64C^{6\text{-}6}$ and $Dhc64C^{6\text{-}12}$ produce late stage egg chambers but are female sterile (Table 2). We showed previously that the localization of dynein to the posterior of the stage 9 oocyte is disrupted in egg chambers derived from $Dhc64C^{6\text{-}6}/Dhc64C^{6\text{-}12}$ females. Instead dynein accumulates in a punctate pattern at the anterior margin of the oocyte (McGrail et al., 1995). We further demonstrated that the posterior accumulation of a dynein-activating complex, the Glued complex, is dependent on cytoplasmic dynein. The wild-type Glued complex is mislocalized along with the dysfunctional dynein to the oocyte anterior (McGrail et al., 1995). Using this mutant background, we have investigated whether the cytoplasmic dynein motor is required in later stages of oocyte differentiation for the positioning of known morphogens within the oocyte.

The *staufen* gene encodes a protein with a double-stranded RNA-binding motif which is required for localization of *oskar* mRNA to the posterior of the oocyte (St. Johnston et al., 1991). In egg chambers derived from control females, dynein and

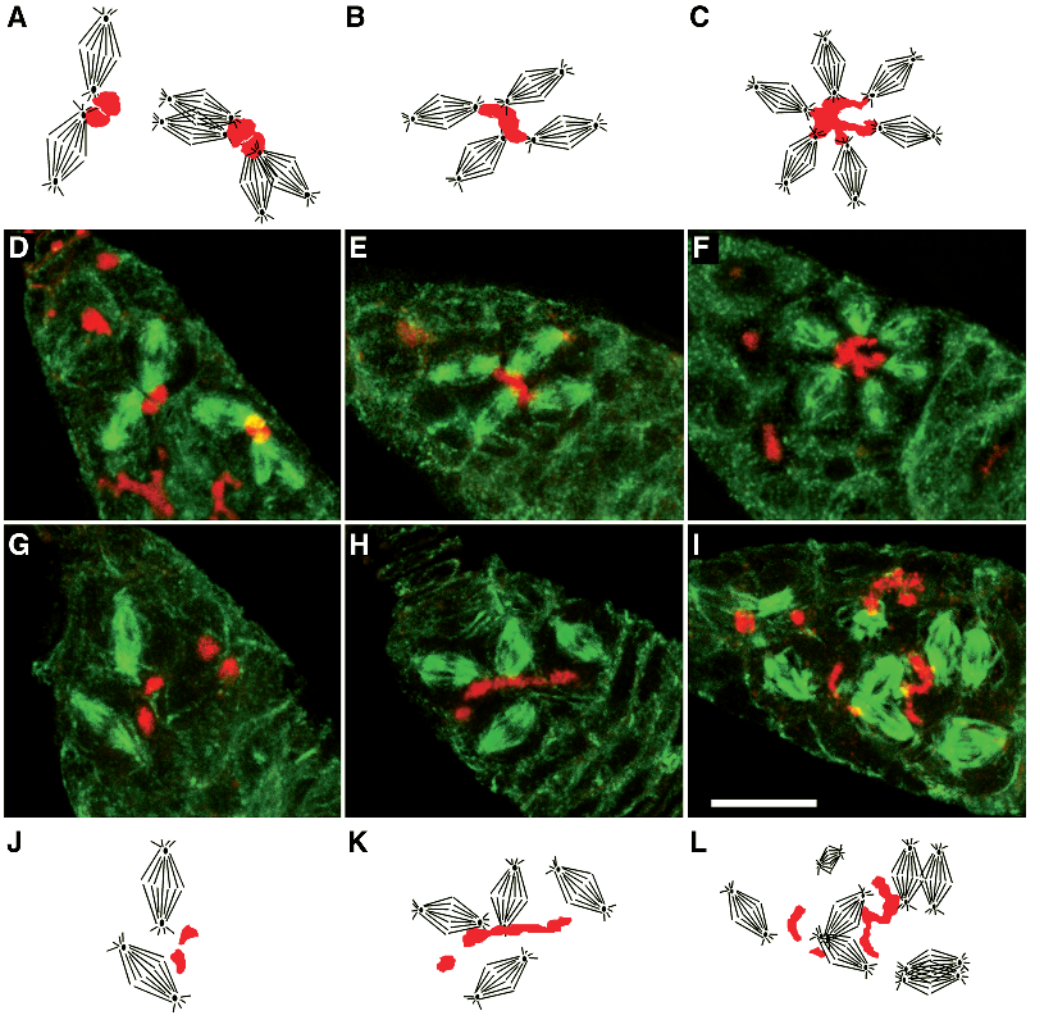

Fig. 4. Cytoplasmic dynein is required for spindle orientation in mitotic germline cysts. (A-C, J-L) Powerpoint (Microsoft Co.) drawings of D-I to illustrate the orientation of the spindles in relation to the fusome (red). (D-F) Control and (G-I) *Dhc64C*$^{3-2}$/*Dhc64C*$^{6-12}$ germaria double-labelled with antibodies to α-tubulin and α-spectrin (Dubreuil et al., 1987; Byers et al., 1987). (A-F) In control *P*[*Dhc*$^{+}$]^{X} ; *Dhc64C*$^{3-2}$/*Dhc64C*$^{6-12}$ females whose fertility has been rescued with the X chromosome-linked wild-type dynein transgene *P*[*Dhc*$^{+}$]^{X}, one pole of each spindle (green, α-tubulin) associates with the fusome (red, α-spectrin) during each synchronous mitotic division in the germline cysts. (A,D) The germarium in D contains a 2-cell and a 4-cell mitotic cyst. (B,E) A 4-cell mitotic cyst. (C,F) Only 6 of the 8 spindles in this 8-cell mitotic cyst are shown in order to clearly demonstrate the extension of the fusome into each cell of the cyst, and the association of one pole of each spindle with one branch of the fusome. (G-L) In *Dhc64C*$^{3-2}$/*Dhc64C*$^{6-12}$ females, the mitotic spindles appear randomly oriented within the cyst and frequently fail to become attached to one branch of the fusome. (G,J) A 2-cell mitotic cyst in which neither spindle has a pole closely associated with a fusome. (H,K) A 4-cell mitotic cyst in which 2 of the four spindles are oriented at random relative to the fusome. (I,L) An 8-cell mitotic cyst. The fusome does not appear to extend into every cell in the cyst. In those cells that contain a branch of the fusome, the spindles are not oriented with one pole in close contact with the fusome. Magnification is the same in D-I. Scale bar in I, 10 μm.

staufen colocalize to the posterior of the stage 9 oocyte (Fig. 6A,C). In contrast, in the *Dhc64C*$^{6-6}$/*Dhc64C*$^{6-12}$ mutant egg chambers dynein is mislocalized to the anterior margin of the oocyte (Fig. 6B). Regardless of dynein mislocalization, staufen protein was still concentrated at the posterior of the oocyte (Fig. 6D) as in wild type. Given the normal distribution of staufen, it was not surprizing to find that *oskar* mRNA also remained concentrated at the posterior cortex of the stage 10 oocyte in *Dhc64C*$^{6-6}$/*Dhc64C*$^{6-12}$ egg chambers (Fig. 6F) similar to control chambers (Fig. 6E).

The TGF-α-like molecule encoded by the *gurken* gene (Neuman-Silberberg and Schüpbach, 1993), together with the EGF-receptor homolog *torpedo* (Price et al., 1989; Schejter and Shilo, 1989), are involved in signalling processes between the oocyte and the overlying somatic follicle cells necessary for both anterior-posterior and dorsal-ventral polarity within the oocyte (Schüpbach, 1987; Gonzalez-Reyes and St. Johnston, 1995; Roth et al., 1995). In stage 9 and 10 egg chambers, *gurken* mRNA normally acquires a dorsal-anterior location over the oocyte nucleus (Neuman-Silberberg and Schüpbach, 1993). In the dynein mutant egg chambers, we find that in stages 9 and later, the oocyte nucleus and *gurken* mRNA localize normally to the dorsal-anterior margin (Fig. 6H), as in control egg chambers (Fig. 6G).

Our observations suggest that cytoplasmic dynein is not directly involved in the final positioning of several known morphogens within the oocyte. However, the dynein alleles used in these studies are not null mutations and therefore we cannot exclude a residual level of dynein activity that is insufficient for the proper localization of the Glued complex, but which may be sufficient for directing other mRNA and protein localizations. It will be important to identify the cargoes transported by dynein at this later stage of oogenesis. In this regard, we did find that the posterior accumulation of a predicted plus-end motor molecule, the kinesin-β-gal fusion protein (Clark et al., 1994), was markedly reduced (Fig. 6I,J). Moreover, the fragile nature of the egg shell and the variable defects in the egg shape and chorionic appendages of the mutant eggs suggest another potential role for dynein in the polarized secretion of the egg shell components by the somatic follicle cells.

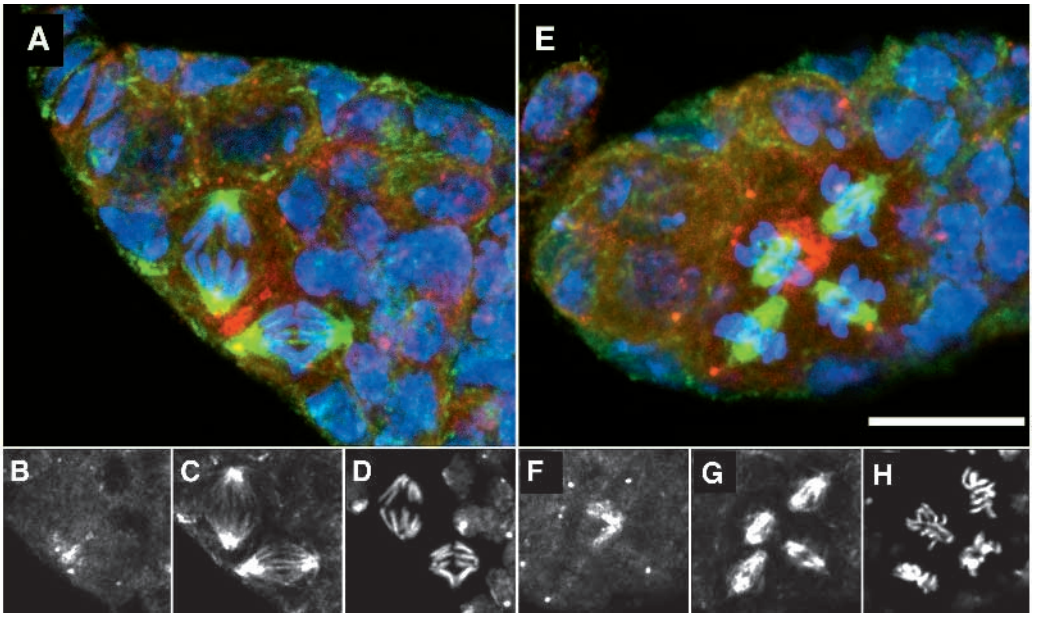

Fig. 5. Cytoplasmic dynein is enriched on a structure resembling the fusome in mitotic cysts. (A,E) Wild-type Oregon R germaria triple labelled with antibodies to the cytoplasmic dynein heavy chain (Hays et al., 1994) and α-tubulin, and the chromatin stain OliGreen. In wild-type mitotic cysts cytoplasmic dynein (red) accumulates in an area of the cyst where the fusome normally resides. One pole of each spindle (green) in the 2-cell anaphase cyst (A) and the 4-cell metaphase cyst (E) appears close to the area of enriched cytoplasmic dynein staining. Chromatin is shown in blue. Below panels A and E the individual dynein heavy chain (B,F), α-tubulin (C,G), and chromatin (D,H) images are shown. Magnification is the same in A and E. Scale bar in E, 10 μm.

DISCUSSION

In this report we have presented evidence that the microtubule motor cytoplasmic dynein is required at two stages of *Drosophila* oogenesis. Early in oogenesis dynein function appears to mediate spindle orientation during the asymmetric cell divisions that give rise to the germline cyst. The disruption of spindle orientation in dynein mutant backgrounds correlates with a defect in cyst polarity and a failure to differentiate an oocyte in the dynein mutant cyst. Other dynein alleles do not block these early stages of oocyte determination, but still result in female sterility and demonstrate a second dynein function in the subsequent differentiation of the oocyte. Our results provide a direct demonstration that the microtubule

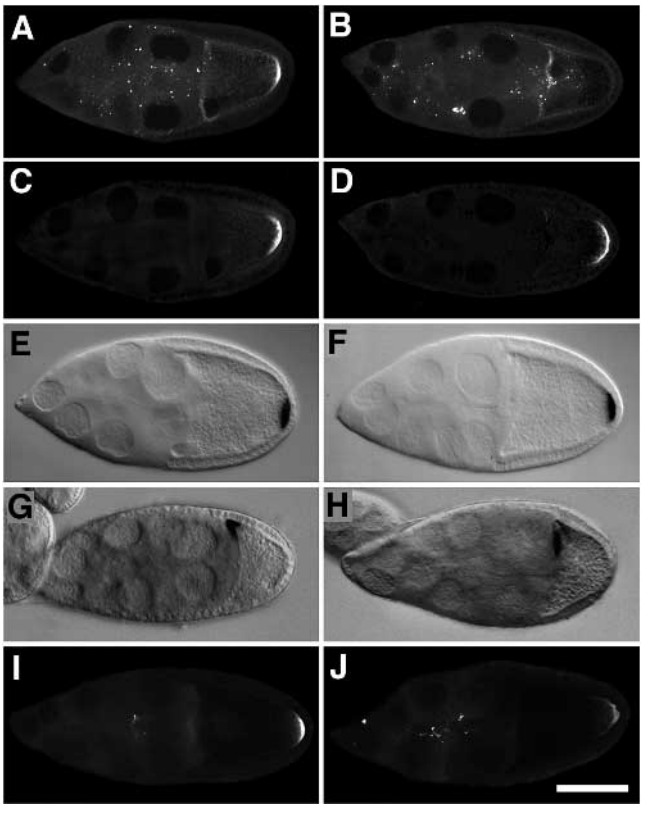

Fig. 6. Cytoplasmic dynein is not directly involved in morphogen localization. In heteroallelic dynein mutant *Dhc64C*$^{6\text{-}6}$/*Dhc64C*$^{6\text{-}12}$ females the localization patterns of mRNAs and proteins required for axis specification are not disrupted. (A,C,E,G,I) Control and (B,D,F,H,J) mutant *Dhc64C*$^{6\text{-}6}$/*Dhc64C*$^{6\text{-}12}$ egg chambers labelled with probes to dynein (A,B) and staufen (C,D), *oskar* mRNA (E,F), *gurken* mRNA (G,H), and β-galactosidase (I,J). (A-D) Stage 9 egg chambers double-labelled with the dynein antibody PEP1 (A,B; Li et al., 1994) and an antibody to staufen (C,D). In the mutant egg chambers dynein is mislocalized to the anterior margin (B) while staufen is enriched at the posterior pole of the oocyte (D). In control egg chambers, dynein (A) and staufen (C) co-localize at the oocyte posterior. (E, F) *oskar* mRNA is enriched at the oocyte posterior in *Dhc64C*$^{6\text{-}6}$/*Dhc64C*$^{6\text{-}12}$ stage 10 egg chambers (F) as in control egg chambers (E). G, H *gurken* mRNA is localized in a dorsal anterior position over the oocyte nucleus in the mutant egg chambers (H) as in control chambers (G). I, J In the *Dhc64C*$^{6\text{-}6}$/*Dhc64C*$^{6\text{-}12}$ egg chambers the kinesin-β-gal fusion protein is localized to the oocyte posterior (J), however the amount of staining is reduced compared to control chambers (I). Magnification is the same in all panels. Scale bar in H, 50 μm.

motor cytoplasmic dynein is required for oocyte differentiation. These studies significantly extend the evidence that microtubule-based transport is central to the process of oocyte differentiation.

Cytoplasmic dynein is required for oocyte differentiation

Microtubule-based transport has been implicated in the determination of oocyte fate and the subsequent specification of axial polarity within the oocyte (reviewed by St Johnston, 1995; Lehmann, 1995; Theurkauf, 1994). The establishment of oocyte fate and axial polarity can both be characterized as multistep processes. In the case of oocyte fate determination, the initial step may be the asymmetric segregation of the fusome precursor, or spectrosome, during the first division of the cystoblast (Lin and Spradling, 1995). In a second step, asymmetric cell division generates a polarized 16 cell germline cyst. The spectrin-rich fusome organelle serves to anchor a single mitotic spindle pole at each of four divisions and ensures a fixed pattern of interconnections between the 16 cells in a cyst (Telfer, 1975; Storto and King, 1989). The pattern of interconnections within the cyst is reflected in the branching of the fusome material that extends into and connects each cell of the cyst. As a final step, a polarized array of microtubules is nucleated from a single microtubule organizing center (MTOC) within the pro-oocyte and extends into the nurse cell complex (Mahowald and Strassheim, 1970; Theurkauf et al., 1993). One hypothesis is that the assembly of this polarized microtubule array may depend on the fusome (Lin and Spradling, 1995). Directed transport of specific mRNAs and cytoplasmic constituents along these microtubules and into the pro-oocyte is required for the continued differentiation of the oocyte (reviewed by Spradling, 1993).

Our previous studies on cytoplasmic dynein led us to propose that the minus-end motor might participate in the transport of determinants along the polarized microtubule array from the nurse cell cytoplasm to the pro-oocyte (Li et al., 1994). This was based on the observation that the dynein heavy chain accumulated in the pro-oocyte, in contrast to the predominant expression of the *Dhc64C* dynein heavy chain transcript in the nurse cell complex (Li et al., 1994). In addition, the temporal and spatial pattern of dynein accumulation to the pro-oocyte was similar to Bicaudal-D, and was dependent on the products of both the *Bicaudal-D* and *egalitarian* genes. Given that mutations in *Bicaudal-D* and *egalitarian* appear to disrupt the establishment and maintenance of the polarized microtubule array in the 16-cell cyst (Theurkauf et al., 1993), the failure to accumulate dynein in a single cell might reflect the absence of a microtubule network along which the dynein motor would translocate. In this case, the block in oocyte determination by mutations in *Dhc64C*, *egl*, or *Bic-D* would reflect a disruption of the polarized transport system and the failure to accumulate some necessary determinant in the pro-oocyte.

The analyses reported here indicate that dynein function is required within the germline of the 16-cell cyst, but at an earlier step in oocyte determination. Our observation that Bicaudal-D does not accumulate in a single cell in dynein mutant cysts, together with the absence of a clearly defined pro-oocyte positioned at the posterior of the mutant cysts, suggests that cytoplasmic dynein is required to generate a polarized germline cyst. The aberrant pattern of ring canals that we observed in mosaic egg chambers can be explained by disruption of the fixed pattern of cystocyte divisions. In support of this explanation, we observed disrupted spindle orientations in germarial mitotic cysts from dynein mutant ovaries and suggest that one function of dynein during cyst formation is to mediate proper spindle orientation. Spindle orientation is important for directing the fixed pattern of cell divison and the asymmetric segregation of existing ring canals as well as fusome material during the formation of the polarized germline cyst. In the absence of the proper polarity within the cyst, the transport system may fail to assemble or assemble incorrectly and oocyte determination could be blocked. Our studies have not eliminated the possibility that dynein also functions shortly after cyst formation as part of a transport system that delivers factors into the pro-oocyte which are necessary for maintaining the pathway toward oocyte differentiation. However, if Bic-D is required to establish the polarized microtubule substrate along which dynein is proposed to translocate, then it is not clear why Bic-D accumulation is blocked in dynein mutant egg chambers. In the absence of motor function, why should microtubule assembly be disrupted? Perhaps dynein is required together with Bic-D and egl to organize the polarized microtubule array? Further work is necessary to address this question directly and to identify potential determinants as cargoes of the dynein motor.

Once oocyte fate is established, polarized transport along microtubules could direct the positioning of molecules within the oocyte in later stages of differentiation. For example, microtubules have been implicated in the positioning of the oocyte nucleus to the dorsal-anterior corner of the oocyte, the localization of *oskar* mRNA and staufen protein to the posterior pole plasm, and the enrichment of *bicoid* mRNA at the anterior margin of the oocyte (reviewed by Theurkauf, 1994; St. Johnston, 1995). We previously showed that dynein accumulates at the posterior end of the growing oocyte at a time when other morphogenetic proteins and mRNAs also become enriched in the posterior pole plasm (Li et al., 1994). Moreover, dynein accumulation is disrupted by microtubule inhibitors (J. T. Robinson and T. S. Hays, unpublished observations) and exhibits gene product requirements (e.g., capu and spire) similar to those observed for the posterior localization of known determinants (Li et al., 1994).

The functional significance of the posterior accumulation of the dynein motor is suggested by a female-sterile heteroallelic combination of *Dhc64C* alleles, $Dhc64C^{6\text{-}6}/Dhc64C^{6\text{-}12}$, in which dynein is enriched at the anterior, rather than posterior, pole of the oocyte. This mislocalization of the mutant dynein motor disrupts the posterior positioning of the associated complex, Glued (McGrail et al., 1995). Despite these observations, in the present study we found no evidence for mislocalization of staufen protein, *oskar* mRNA, or *gurken* mRNA in the $Dhc64C^{6\text{-}6}/Dhc64C^{6\text{-}12}$ background. One interpretation of these results is that dynein motor function may not be critical to the positioning of morphogens during oogenesis. Alternatively, given that the *Dhc64C* alleles $Dhc64C^{6\text{-}6}$ and $Dhc64C^{6\text{-}12}$ are not null mutations, our results cannot exclude the possibility that a residual level of dynein function is sufficient for *oskar* mRNA, *gurken* mRNA, and staufen protein localization, but insufficient for Glued localization. Interestingly, we did observe an apparent reduction in the accumulation of the kinesin-β-gal fusion protein at the posterior pole of

the oocyte in the dynein mutant egg chambers. This observation raises the possibility that dynein may be required for some other aspect of cytoplasmic organization (e.g., microtubule architecture) within the oocyte. While the underlying basis of the sterile phenotype remains to be elucidated, sterility is not apparently related to a disruption of the proper accumulation of *oskar* mRNA, *gurken* mRNA, or staufen protein in the developing oocyte. Further studies will need to address whether a somatic function for dynein in follicle cell secretion may contribute to the late defect in oogenesis. The present work provides evidence that cytoplasmic dynein may serve multiple functions during distinct stages of oocyte differentiation. Future studies of cytoplasmic dynein function in *Drosophila* oogenesis may help to reveal how a single motor accomplishes multiple tasks.

A novel role for cytoplasmic dynein in asymmetric cell division

Storto and King (1989) proposed that spindle positioning ensures the unequal inheritance of the ring canals and associated fusome material at each round of mitosis. This mechanism is believed to account for the production of a branched chain of interconnected cystocytes, in which the two cells with four ring canals and the largest amount of fusome material become pro-oocytes. How the selection is made for one of the two cells to continue to differentiate as the oocyte, while the other takes up a nurse cell fate is not clear. However, a recent model has addressed this issue and proposed that the future single pro-oocyte is initially determined by the asymmetric segregation of the spectrosome at the first cystoblast division. The identity of the pro-oocyte is maintained at each division by the asymmetric inheritance of the fusome material, which leads to a polarized fusome (Lin and Spradling, 1995). The polarized fusome may provide the information necessary to subsequently assemble a polarized microtubule array and establish directed transport to the pro-oocyte. Mutations that disrupt the assembly of the fusome and oocyte differentiation, including mutations in the fusome components α-spectrin and the adducin-like *hu-li tai shao* gene product, foster the view that fusome function is critical in oocyte differentiation (Yue and Spradling, 1992; Lin et al., 1994b; de Cuevas et al., 1996). Our analysis of mitotic germline cysts in the dynein mutant germaria revealed that mitotic spindles fail to acquire the proper orientation with respect to the fusome, and the normal pattern of ring canal connections and cyst polarity is disrupted. These results support the hypothesis that spindle orientation and asymmetric cell division are critical for oocyte determination.

Localization of cytoplasmic dynein to the fusome and the mechanism of spindle orientation

The localization of cytoplasmic dynein to the fusome suggests a simple model for the mechanism of asymmetric cell division in *Drosophila* germline mitotic cysts. Anchored to the fusome through its interaction with spectrin and adducin, the dynein motor acts upon astral microtubules to rotate the mitotic spindles into alignment. An analogous role has been proposed for cytoplasmic dynein in the positioning of the nucleus and spindle into the bud neck during mitosis in *S. cerevisae* (Li et al., 1993; Eschel et al., 1993; Yeh et al., 1995). Moreover, recent studies have suggested that dynein attachment to membrane vesicles in mammalian cells is mediated through the binding of dynactin to a spectrin and adducin-containing membrane skeleton (Holleran et al., 1996). The observation that cytoplasmic dynein appeared enriched on the fusome only in mitotic cysts suggests a cell cycle-dependent regulation of dynein association with the fusome. Previous investigators have reported that levels of phosphorylation correlate with the cell cycle-dependent association of dynein with membrane vesicles (Niclas et al., 1996), as well as the redistribution of dynein from a vesicular to a diffuse cytoplasmic pool (Lin et al., 1994a).

An alternative interpretation of dynein enrichment on the fusome is that dynein participates in the assembly and function of the fusome during cell division and cyst formation. Defects in the assembly or integrity of the fusome could also result in aberrant spindle orientation and an altered pattern of cell divison. Dynein association with the fusome could reflect dynein attachment to microtubules within the fusome and minus-end motor activity could act to organize the polarized microtubule array during cyst formation. Mutations in the fusome components α-spectrin (de Cuevas et al., 1996) and the *Drosophila* adducin homolog hu-li tai shao (Yue and Spradling, 1992; Lin et al., 1994b) disrupt cyst formation and oocyte differentiation. However, in these mutant cysts the fusome appears absent, or nearly absent. In dynein mutant cysts the morphology of the fusome was frequently abnormal, however spindle poles present in proximity to branched arms of the fusome still often failed to attach. This result suggests that while dynein association with the fusome may serve other functions, it appears to be required for attachment of the mitotic spindle pole to the fusome.

Our previous analyses of cytoplasmic dynein in *Drosophila* demonstrated the requirement for dynein function in cell viability during development (Gepner et al., 1996). The results presented here demonstrate a specific developmental role for cytoplasmic dynein in cellular differentiation, and provide evidence for a novel function for cytoplasmic dynein in asymmetric cell division that is critical to the differentiation of the *Drosophila* oocyte. Together with recent studies on the role of myosins in asymmetric cell division in yeast and *C. elegans* (Jansen et al., 1996; Bobolo et al., 1996; Guo and Kemphues, 1996) our observations on dynein function in *Drosophila* oogenesis underscore the importance of cytoskeletal motors in cellular differentiation during development.

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