Development 130, 901-915

doi:10.1242/dev.00315

Orbit/Mast, the CLASP orthologue of *Drosophila*, is required for asymmetric stem cell and cystocyte divisions and development of the polarised microtubule network that interconnects oocyte and nurse cells during oogenesis

Endre Máthé[1], Yoshihiro H. Inoue[2], William Palframan[1], Gemma Brown[1] and David M. Glover[1,*]

[1]Cancer Research UK, Cell Cycle Genetics Research Group, Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EN, UK
[2]Drosophila Genetic Resource Center, Kyoto Institute of Technology, Sagaippongi-cho, Ukyou-ku, Kyoto, 616-8354, Japan
*Author for correspondence (e-mail: dmg25@mole.bio.cam.ac.uk)

Accepted 29 Novermber 2002

SUMMARY

Drosophila **oocyte differentiation is preceded by the formation of a polarised 16-cell cyst from a single progenitor stem cell as a result of four rounds of asymmetric mitosis followed by incomplete cytokinesis. We show that the Orbit/Mast microtubule-associated protein is required at several stages in the formation of such polarised 16-cell cysts. In wild-type cysts, the Orbit/Mast protein not only associates with the mitotic spindle and its poles, but also with the central spindle (spindle remnant), ring canal and fusome, suggesting it participates in interactions between these structures. In** ***orbit*** **mutants, the stem cells and their associated fusomes are eventually lost as Orbit/Mast protein is depleted. The mitotic spindles of those cystocytes that do divide are either diminutive or monopolar, and do not make contact with the fusome. Moreover, the spindle remnants and ring canals fail to differentiate correctly in such cells and the structure of fusome is compromised. The Orbit/Mast protein thus appears to facilitate multiple interactions of the fusome with mitotic spindles and ring canals. This ensures correct growth of the fusome into a branched asymmetrically distributed organelle that is pre-determinative of 16-cell cyst formation and oocyte fate specification. Finally the Orbit/Mast protein is required during mid-oogenesis for the organisation of the polarised microtubule network inside the 16-cell cyst that ensures oocyte differentiation. The localisation of CLIP-190 to such microtubules and to the fusome is dependent upon Orbit/Mast to which it is complexed.**

Key words: Orbit, Mast, CLIP-190, Anillin, Pavarotti-KLP, Fusome, Asymmetric division, CLASP, Cytokinesis, Oogenesis, *Drosophila*

INTRODUCTION

The *orbit/mast* gene of *Drosophila* encodes a microtubule-associated protein required for correct mitotic spindle organisation in embryos, larval neuroblasts and cultured cells (Inoue et al., 2000; Lemos et al., 2000). The Orbit/Mast protein is required to maintain spindle bi-polarity and to ensure the attachment of kinetochores to microtubules to facilitate chromosome congression (Maiato et al., 2002). The mammalian counterpart of Orbit is known as CLASP and, together with its interacting partner CLIP-170, has been proposed to contribute to the stabilisation of the plus ends of microtubules at their cortical attachment sites in interphase cells (Akhmanova et al., 2001). Ultimately, this leads to the establishment of cellular asymmetry of crucial importance in processes such as wound healing-induced fibroblast motility (Akhmanova et al., 2001; Allan and Näthke, 2001; McNally, 2001). The emerging picture is that CLASP mediates microtubule based cellular processes in both interphase and mitosis and yet little is known of how the CLASPs, either alone and/or in association with other proteins, assist in establishing polarity.

Oogenesis in *Drosophila* is an attractive developmental process for studying microtubule-based polarisation events, as it involves asymmetric cell divisions that are important in establishing cell fate, and also polarised inter- and intracellular transport phenomena required for the oocyte to differentiate. A specialised organelle, the fusome (reviewed by Telfner, 1975; Büning, 1994; de Cuevas et al., 1997; McKearin, 1997) plays a determinative role in imposing asymmetry upon dividing stem cells and cysts, as it associates with only one pole of the mitotic spindle at every mitosis (Storto and King, 1989; Lin and Spradling, 1995; McGrail and Hays, 1997) and later is partitioned unequally between daughter cells (de Cuevas and Sradling, 1998). The fusome comprises membrane skeletal proteins such as the α- and β-spectrins, ankyrin, the adducin-like HtsF (the fusome specific product of the *hu-li tai shao* gene) (Yue and Spradling, 1992), Bam (bag-of-marbles)

(McKearin and Ohlstein, 1995), TER94 (León and McKearin, 1999), and motor molecules such as cytoplasmic dynein encoded by the *Dhc64C* gene (McGrail and Hays, 1997). Mutations in the genes *hts* (Yue and Spradling, 1992), α-spectrin (de Cuevas et al., 1996), *ovarian tumour* (King et al., 1978) and *Dhc64C* (McGrail and Hays, 1997) disrupt the fusome, leading to formation of a cyst with an abnormal number of germ cells and the failure of any cystocyte to acquire oocyte identity. Thus, the fusome is required for the formation of a polarised 16-cell cyst and for oocyte specification, and fulfils this function by its regular and polarised growth throughout the stem cell and cyst cell cycles (Lin et al., 1994; Knowles and Cooley, 1994; Deng and Lin, 1997; de Cuevas and Spradling, 1998; Grieder et al., 2000). During each growth cycle, fusome – spindle interactions specify the cleavage plane. Cleavage is incomplete, with the furrow arresting upon contact with the central spindle (spindle remnant). Concomitantly, fusome material is recruited to lie between the arrested cleavage furrow (nascent ring canal) and spindle remnant, and will transform into a fusome plug. The fusome plug, together with its ring canal, moves centripetally, most likely facilitated by microtubules, and gradually fuses with the pre-existing fusome, so changing the geometry of the cyst. As a consequence, the fusome remains asymmetrically distributed within the cyst: the older cells retain bigger, while the younger cells retain smaller parts of fusome at the end of each cycle of cyst division. Upon completion of cyst divisions, the fusome continues to play a crucial role in polarising the 16-cell cyst by interacting with interphase-specific microtubules that span across the ring canals, and as a result a selective, microtubule-dependent transport is initiated (reviewed by Mahajan-Miklos and Cooley, 1994; Navarro et al., 2001; Riechmann and Ephrussi, 2001). Several mRNAs and proteins are transported along microtubules, whose polarity is determined by the microtubule-organising centre (MTOC) of the cyst situated in the oocyte. During mid-oogenesis, this MTOC is disassembled, a process requiring the *par-1* gene. This is a precondition of the oocyte-specific anteroposterior and dorsoventral polarisation events (Tomancak et al., 2000; Shulman et al., 2000). Thus, a plethora of cytoskeletal polarisation events contribute to the differentiation of the oocyte.

As CLASPs and CLIP-170 had been shown to mediate interactions between microtubules and the cortical cytoskeleton in cultured mammalian cells, we wondered whether their *Drosophila* counterparts Orbit/Mast and CLIP-190 (Lantz and Miller, 1998) could be involved in cytokskeletal polarisation events during oogenesis. To this end, we have characterised the phenotype of newly isolated *orbit* mutants (*orbit*^{5} and *orbit*^{6}) in oogenesis. Our findings point to the importance of Orbit/Mast for many aspects of stem cell, cytoblast and cystocyte divisions, and in the generation of polarised arrays of microtubules in the 16-cell cyst. We show that the Orbit/Mast and CLIP-190 proteins follow specific localisation patterns in dividing germline cysts. Orbit/Mast is initially found on the mitotic spindle, being concentrated at the poles, while CLIP-190 accumulates on the spindle and at higher levels on fusome. Orbit/Mast progresses onto the spindle remnant, from where it moves to the arrested cleavage furrow and fusome. Concomitantly, CLIP-190 will show a uniform distribution across the cyst as mitosis is completed. A role for the Orbit/Mast protein in facilitating interactions between the organelles of the cystocytes is indicated by the failure of the fusome to contact the mitotic spindles, severe ring canal differentiation defects and disrupted fusome organisation in newly isolated *orbit* mutant alleles. Moreover, in mid-oogenesis, the polarised microtubule network that interconnects the oocyte with the remaining nurse cells of the 16-cell fails to develop in the *orbit* mutants.

MATERIALS AND METHODS

Fly strains and culture conditions

Wild-type and mutant strains were maintained on standard yeast-agar-cornmeal medium and all experiments were performed at 25°C. All genetic markers and mutations used are described in FlyBase. Deficiency stocks *Df(3L)Pc-9a* and *Df(3L)Pc-12h* were obtained from Adelaide Carpenter. The *orbit*^{1} and *orbit*^{5} mutants were originally isolated as P-lacW insertion lines (Deák et al., 1997). The *orbit*^{6} mutant allele was isolated by the remobilisation of the P-lacW element in the *orbit*^{5} line. All the stocks used were on a *w*^{1118} background.

Remobilisation of the P-lacW element

In order to revert the *orbit*^{5} mutation, the P-lacW element was remobilised under dysgenic conditions. About 250 jump starter males of genotype w^{1118}/Y; o*rbit*^{5}*/TM3,Sb,ry,[Δ2–3, ry*$^{+}$*]* were crossed individually to w^{1118}/w^{1118}; *TM3,Sb,Ser/TM6b,Tb* virgins. From their progeny the *orbit*^{5}*/TM3* and *orbit*^{5}*/TM6b* flies were scored for *w*$^{-}$ or modified *w*$^{+}$ expression when compared with original *w*$^{+}$ expression level seen in the eyes of *orbit*^{5} flies. For each jump starter male, only one fly was selected, showing the *w*$^{-}$ or the modified *w*$^{+}$ phenotype and for these revertants strains were established over *TM6b,Tb*. A complementation test was performed with *orbit*^{1}, *orbit*^{5} and their revertant alleles.

Mutant phenotype analysis

The phenotypes of homozygous, hemizygous and transheterozygous *orbit*^{5} and *orbit*^{6} mutants were determined on a *w*^{1118}*;TM6b,Tb* or *y,w;TM6c,Tb,Sb* background (Table 1). Individual sterility tests were performed for homozygous, hemizygous and transheterozygous *orbit*^{5} and *orbit*^{6} females by crossing them to wild-type Canton-S males. The [*orbit*$^{+}$] transgene (Inoue et al., 2000) was used to rescue the *orbit*^{5}- and *orbit*^{6}-associated homozygous mutant phenotypes.

Germline autonomy of the *orbit*^{6} phenotype

To decide whether the *orbit*^{6} mutation affects the female germline or soma, two types of germline chimeras were constructed (Tables 2, 3). In the first experiment, *orbit*^{6}*/orbit*^{6} pole cells had been transplanted into *Fs(1)K1237/+* host females. The *Fs(1)K1237* mutation blocks germline function without affecting the soma (Komitopoulou et al., 1983). In the reciprocal experiment, wild-type pole cells, marked with *y, v, f, mal* mutations, were transplanted into *orbit*^{6}*/orbit*^{6} host females, then mated with *y, v, f, mal* males and the progeny scored for these marker mutations. In both experiments, the *orbit*^{6}*/TM3, orbit*^{6}*/TM6b and TM3/TM6b* sibling chimeras served as internal controls.

Western blot analysis of ovarian extracts

Total ovarian protein extracts were prepared from 15 wild-type, *orbit*^{1}*/orbit*^{1}, *orbit*^{5}*/orbit*^{5} or *orbit*^{6}*/orbit*^{6} females in standard 2× Sigma protein loading buffer. Equal volumes of ovarian protein samples were loaded onto 7.5 or 10% polyacrylamide gels and after electrophoresis electroblotted onto Hybond ECL (Amersham-Pharmacia) nitrocellulose membranes. Tubulin was used as a loading standard and detected using mouse monoclonal α-tubulin antibody (diluted 1:10; Amersham-Pharmacia). The Orbit protein was detected with the affinity purified rabbit antibody described by Inoue et al. (Inoue et al., 2000), diluted 10^{-3}. Horseradish peroxidase-labelled

anti-rabbit and anti-mouse secondary antibodies were purchased Jackson Immunoresearch Laboratories. The blots were developed using ECL or ECL plus kits (Amersham-Pharmacia).

Immunoprecipitation

Ovarian lysates were prepared by dissecting and grinding 50 pairs of ovaries in 150 ml of lysis buffer (0.3M sorbitol; 10 mM HEPES, pH 7.5; 10 mM sodium azide; 1 mM PMSF; 0.5 mg/ml leupeptin; 0.7 mg/ml pepstatin). Two volumes of IP dilution-1 buffer (125 mM Tris-HCl, pH 6.8; 20% glycerol; 20 mM DTT; 0.02 Bromophenol Blue) were added. After 1 hour of incubation at room temperature, four volumes of IP dilution-2 buffer (1.25% Triton X-100; 190 mM NaCl; 6 mM EDTA; 60 mM Tris-HCl, pH 7.5) were added and the lysate spun for 5 minutes in a microcentrifuge at maximum speed. The supernatant was incubated with either anti-Orbit or anti-CLIP-190 antibodies at 4°C overnight, after which 100 ml of Dynabeads M-280 sheep anti-rabbit IgG (Dynal Biotech) was added and the incubation continued for 3 more hours at room temperature. The samples were washed three times in IP buffer (1% Triton X-100; 0.2% SDS; 150 mM NaCl; 5 mM EDTA; 50 mM Tris-HCl, pH 7.5) before using the magnetic particle concentrator. After the last wash, the Dynabead particles were resuspended in two volumes of protein gel loading buffer (4% SDS; 125 mM Tris-HCl, pH 6.8; 20% glycerol; 100 mM DTT; 0.02% Bromophenol Blue) and incubated for 1 hour at room temperature. The cleared lysate was removed, boiled and loaded onto 7.5% SDS-PAGE gels. Blotting and detection of proteins was carried out as described above.

Immunocytochemistry and confocal microscopy

To study the germarial divisions in *orbit* mutants, ovaries of newly eclosed, 1-2 and 3-day-old females were dissected in EBR buffer and fixed as described by McGrail et al. (McGrail et al., 1995). For the phenotypic analysis of the *orbit* egg chambers, the ovaries were dissected out from 2- and 3-day-old females that were mated to wild-type males and fed with yeast paste, while the fixation was carried out as described by Minestrini et al. (Minestrini et al., 2002). For rhodamine-phalloidin staining, the ovaries were dissected out from females in EBR buffer and fixed according to the protocol of McGrail et al. (McGrail et al., 1995), but the fixative and washing solutions (that were used prior antibody incubation) were supplemented with 5 units/ml rhodamine-phalloidin (Molecular Probes). All the samples were incubated overnight with the primary antibodies at 4°C, while the incubations with the secondary antibodies were for 4 hours at room temperature. Orbit/Mast protein was detected with the affinity purified rabbit antibody described by Inoue et al. (Inoue et al., 2000), and was diluted 1:200. Tubulins were detected with YL1/2 rat monoclonal anti-α-tubulin antibody (1:50; Sera Lab) or the Bx69 mouse monoclonal anti-β-tubulin antibody (1:2) and mouse monoclonal anti-γ-tubulin (1:50; Sigma GTU88). The T47 mouse monoclonal anti-lamin antibody was used to visualise the nuclear lamina (1:10) (Paddy et al., 1990). The fusome was stained using rabbit affinity-purified anti-α-spectrin (1:200, Sigma), mouse monoclonal anti-HtsF antibody (1B1; 1:10; DSHB of Iowa University). Ring canals had been stained with mouse monoclonal anti-HtsRC, mouse anti-Kelch (1B, 1:1) (Xue and Cooley, 1993), rat anti-Filamin (1:200) (Sokol and Cooley, 1999), PY20 anti-phosphotyrosine (1:1000; ICN), rabbit affinity-purified anti-Pavarotti-KLP antibody (1:200) (Adams et al., 1998) and the rabbit affinity-purified anti-Anillin (1:2000) (Field and Alberts, 1995). The rabbit anti-Inscuteable (1:100) (Huynh and St Johnston, 2000) was used to visualise the synaptonemal complexes and the affinity purified rabbit anti-CLIP-190 (1:1000) antibody was provided by K. Miller. The Alexa 488, 594 and 624-conjugated anti-rat, anti-mouse and anti-rabbit secondary antibodies were obtained from Molecular Probes. DNA was visualised through TOTO3 staining purchased from Molecular Probes. Digital images of serial optical sections were collected with a BioRad 1024 confocal microscope, merged, and then processed using Adobe Photoshop 5.5 (Adobe Systems).

RESULTS

Orbit/Mast shows a dynamic pattern of localisation during germline divisions

To examine the spatial distribution of the Orbit/Mast protein during the division cycles of stem cells, cystoblasts and cystocytes we carried out immunostaining of wild-type ovaries with antibodies against the Orbit/Mast protein and the fusome component HtsF (see Materials and Methods). We assessed cell cycle stages from the spindle morphology revealed by Orbit/Mast staining and the fusome cycle described by de Cuevas and Spradling (de Cuevas and Spradling, 1998). During stem cell mitosis, the Orbit/Mast protein accumulated on the microtubules of the asymmetrically positioned spindle being concentrated at the spindle poles (see Fig. 5A,D). After mitosis of the stem cell, when fusome plug formation is initiated in the cleavage furrow, Orbit/Mast protein was seen on the spindle remnant (Fig. 1A). As interphase progressed, the original fusome appeared to elongate along the anteroposterior axis of the stem cell, eventually contacting the fusome plug and extending through the transient ring canal that contains Orbit/Mast protein (Fig. 1B). At this stage, Orbit/Mast protein was not only associated with the ring canal but also with the fusome itself. During the following mitoses of cytoblasts and cytocytes, Orbit/Mast protein associated with spindle microtubules as has been seen in other cell types, and also with the fusome that at this stage is linked to one of the poles of each spindle (Fig. 1C). Upon the completion of mitosis in these cells, the spindle associated Orbit/Mast protein accumulated in the spindle remnants as it did in stem cells (Fig. 1D), and then appeared to concentrate in the ring canals around the newly formed fusome plugs in addition to in the fusome itself (Fig. 1E,F). The fusome plugs appeared to fuse with the pre-existing fusome before the next round of mitosis. Thus, the Orbit/Mast protein has a distinct localisation pattern during germline divisions, being located initially on the mitotic spindle and spindle remnant, becoming incorporated into the newly formed ring canals, their fusome plugs and finally into the mature fusome (Fig. 1G).

An *orbit* allele that disrupts oogenesis

In order to determine whether the distribution of Orbit/Mast protein on the spindle, fusome and ring canal reflected a role in organising these structures, we sought mutant alleles that might specifically affect these structures. The original P-element insertion line *orbit*^{1} is hypomorphic and leads to maternal effect lethality as a result of the abnormal mitotic spindles that form in syncytial embryos (Inoue et al., 2000). Other amorphic *orbit* alleles show late larval lethality with hyperploid mitotic cells. We have identified a new P-lacW insertion line from the collection of Deák et al. (Deák et al., 1997) that had a P-element inserted in the opposite direction to that in the original allele and 690 bp upstream from the first ATG of the *orbit* open-reading-frame. As the associated mutant phenotype was completely rescued by an [*orbit*$^{+}$] transgene (Table 1) we have named this new allele *orbit*^{5}. Through the remobilisation of the P-element from the *orbit*^{5} line, we

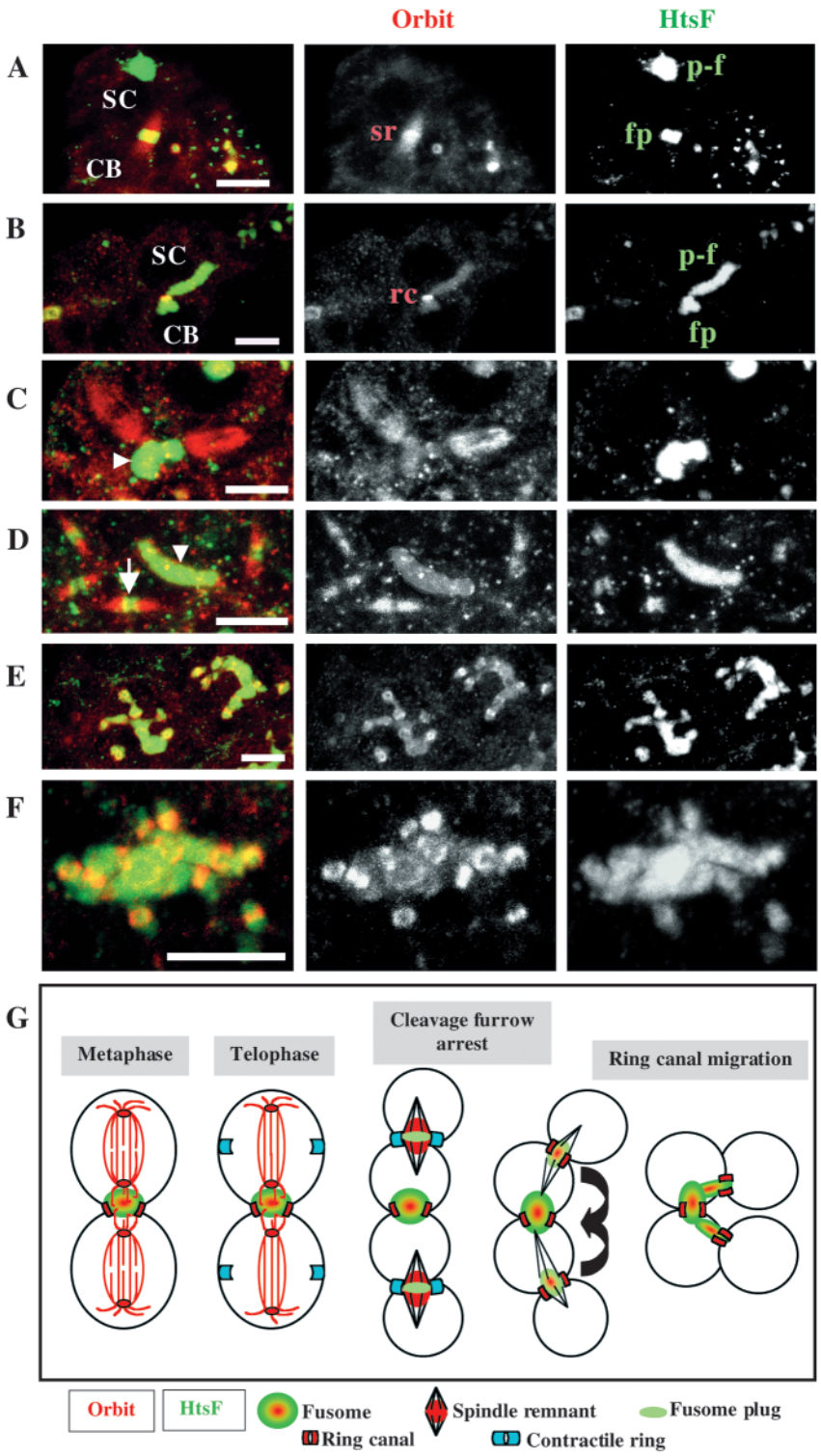

Fig. 1. Subcellular localisation of Orbit/Mast during germline cyst divisions. Wild-type germaria were stained with anti-Orbit/Mast (red), anti-HtsF (green) antibodies and TOTO3 to reveal DNA (not shown). (A) Orbit/Mast locates on the spindle remnant (sr) during stem cell cytokinesis. The HtsF associates with the nascent fusome plug (fp), which is situated at the mid-zone of the spindle remnant. The pre-existing fusome (p-f) is spherical and resides at the anterior tip of the stem cell. (B) The stem cell (SC) and cystoblast (CB) are interconnected through an Orbit/Mast containing ring canal (rc) that divides the fusome into two unequal pieces. (C) Metaphase cyst, showing the Orbit/Mast on the spindles and pre-existing fusome (arrowhead). Only two out of four spindles in this cyst are shown. (D) Cyst featuring Orbit/Mast on spindle remnants with fusome plugs (arrow). The pre-existing fusome (arrowhead) is situated in the middle of the cyst. (E,F) Orbit/Mast accumulates in the ring canals surrounding the newly formed fusome plugs. Along the pre-existing fusome, Orbit/Mast shows a more diffuse distribution in addition to its presence in ring like structures. Some fusome plugs have already fused with the pre-existing fusome, while others are still 2-3 μm distant. Scale bar: 5 μm. (G) The dynamic changes in the subcellular localisation of Orbit/Mast and HtsF during a division cycle of a germline cyst. Two-cell cyst at metaphase: the asymmetric nature of this division is determined by the fusome-spindle interaction, as only one of the poles of each spindle is fusome anchored. Orbit/Mast accumulates at the spindle poles and decorates the spindle MTs. It is also present in the fusome and the fusome associated ring canal. Note that one of the cells contains a bigger part of the fusome. Two-cell cyst at telophase: central spindle and contractile ring assembly marks the onset of cytokinesis. Cleavage furrow arrest takes place upon contact with spindle remnants in a four-cell cyst. Orbit/Mast associates with central region of spindle remnants. Concomitantly, the fusome plug formation is initiated as inferred from the accumulation of HtsF between the spindle remnant and contractile ring. Ring canal migration: Orbit/Mast accumulates in the ring canals and to a less extent associates with the fusome plugs. The spindle remnants reorient towards the fusome and the fusome plugs together with their ring canals move towards the pre-existing fusome, changing the cyst geometry from a linear into a rosette configuration. Eventually, the pre-existing fusome fuses with fusome plugs and the spindle remnants disassemble. Once again the bigger fusome part will be retained by the cell that has inherited it during the previous division cycle.

isolated a new allele, *orbit*^{6}; the mutant phenotype of *orbit*^{6}/*orbit*^{5} was again completely rescued by the [*orbit*$^{+}$] transgene (Table 1). In this allele the P-element was inserted into the identical site but with the opposite orientation to the P-element in *orbit*^{5} and 60 bp of downstream sequence were deleted. Most homozygous *orbit*^{5} individuals died as pharate adults, but 1-5% reached adulthood with females and males being sterile. Homozygous *orbit*^{5} adults died shortly after eclosion and females did not lay eggs. Hemizygous *orbit*^{5} individuals showed third larval instar/pupal lethality indicating the hypomophic nature of the mutation and suggesting multiple somatic in addition to germline roles for the *orbit/mast* gene. The *orbit*^{6} individuals were viable when homozygous, hemizygous or transherozygous to *orbit*^{5} and both females and males were sterile, suggesting that the mutation affects the function of this gene in the germline. To confirm the female

Table 1. Phenotypes of the new *orbit* alleles

Alleles	*orbit*^{1}	*orbit*^{5}	*orbit*^{6}
Df(3L)PC-MK	Female sterile, male sterile	Larval-pupal lethal	Female sterile, male sterile
orbit^{1}	Female sterile, male sterile	Female sterile, male sterile	Female sterile, male sterile
orbit^{5}		Pharate adult, Female sterile, male sterile	Female sterile, male sterile
orbit^{6}			Female sterile, male sterile
orbit^{5} [*orbit*+]	Fertile	Fertile	Fertile

Table 2. Chimeras with wild-type (*K1237/+*) soma and *orbit*^{6}/*orbit*^{6} germline

Host genotype	Genotype of implanted germline cells	Number of chimeras	Phenotype
K1237/+	*orbit*^{6}/*orbit*^{6}	8	Sterile
K1237/+	*orbit*^{6}/*TM3*	10	Fertile
K1237/+	*orbit*^{6}/*TM6b*	11	Fertile
K1237/+	*TM3/TM6b*	6	Fertile

Table 3. Chimeras with *orbit*^{6}/*orbit*^{6} soma and wild-type (*y, v, f, mal*) germline

Host genotype	Genotype of implanted germline cells	Number of chimeras	Phenotype
orbit^{6}/*orbit*^{6}	*y v f mal*	7	Fertile
orbit^{6}/*TM3*	*y v f mal*	9	Fertile
orbit^{6}/*TM6b*	*y v f mal*	12	Fertile
TM3/TM6b	*y v f mal*	5	Fertile

germline dependency of the *orbit*^{6} mutation, we constructed two types of germline chimeras. First, *orbit*^{6}/*orbit*^{6} germline cells were transplanted into *K1237/+* hosts that have wild-type soma and non-functional germline (Table 2). Eight such chimeras were identified that each manifested the same mutant phenotype as *orbit*^{6}/*orbit*^{6} females. In the second experiment, wild-type (*y,v,f,mal*) germline cells were transplanted into *orbit*^{6}/*orbit*^{6} females, and seven chimeras were recovered that produced wild-type (*y,v,f,mal*) offspring (Table 3). Thus, classical germline clonal analysis suggests that the *orbit*^{6} mutation appears to affect only the female germline.

To characterise the ovarian expression levels of the Orbit/Mast protein in the new mutant alleles, we performed Western blot analysis on total protein extracts from wild-type, *orbit*^{1}/*orbit*^{1}, *orbit*^{5}/*orbit*^{5} and *orbit*^{6}/*orbit*^{6} ovaries (Fig. 2A). Although in *orbit*^{1}/*orbit*^{1} ovaries the level of Orbit/Mast protein was slightly reduced when compared with the wild-type, in *orbit*^{5}/*orbit*^{5} ovaries it was reduced to 30%, while in *orbit*^{6}/*orbit*^{6} ovaries only traces of protein were detectable. In all three mutants, the residual Orbit/Mast protein had identical mobility to wild type. Disruption of oogenesis in *orbit*^{5}/*orbit*^{5} and *orbit*^{6}/*orbit*^{6} ovaries was further suggested by the absence of Hts (Hu-li tai shao) isoform usually expressed during mid to late oogenesis (Zaccai and Lipshitz, 1996) (Fig. 2B). In agreement with this observation, we found that oogenesis never proceeds beyond mid-oogenesis in the mutant *orbit*^{5}/*orbit*^{5}, *orbit*^{6}/*orbit*^{6}, *orbit*^{6}/*orbit*^{5} and *orbit*^{6}/*Df(3L)PC-MK* ovaries. Moreover, in such *orbit* mutant ovaries the levels of Orbit/Mast appeared to decrease as the females aged. It was the highest at the time females emerged from pupae, while from the third day onwards hardly any protein was found (Fig. 2C). The ovaries of *orbit*^{5}/*orbit*^{5}, *orbit*^{6}/*orbit*^{6}, *orbit*^{6}/*orbit*^{5} and *orbit*^{6}/*Df(3L)PC-MK* females were small and only a few egg

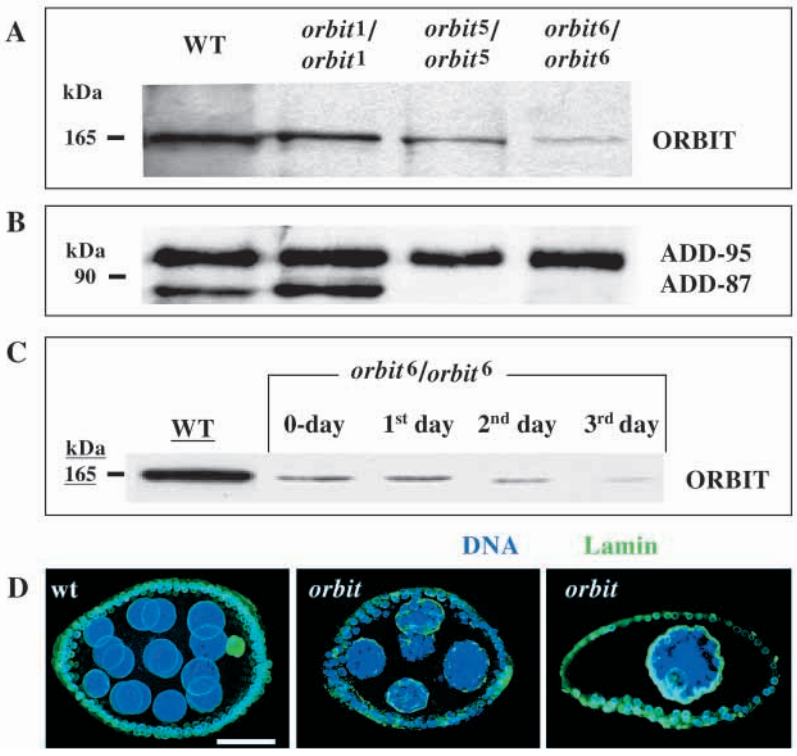

Fig. 2. Diminished levels of Orbit/Mast lead to egg chambers with a reduced number of germline cells. (A) Western blot indicating the reduced levels of Orbit/Mast in total ovarian protein extracts of the indicated genotypes. (B) Western blot showing levels of Hts (Hu-li tai shao) ADD-95 and ADD-87 isoforms in ovarian total protein extracts. The ADD-95 isoform is present at similar levels in all four extracts, while the ADD-87 isoform is absent from *orbit*^{5}/*orbit*^{5} and *orbit*^{6}/*orbit*^{6} ovaries. Equal amount of samples were loaded on both Western blots and confirmed by staining with an anti-tubulin antibody. (C) Western blot indicating the age-dependent reduction of the expression level of Orbit/Mast in total ovarian protein extracts of the *orbit*^{6}/*orbit*^{6} ovaries as normalised to wild type. (D) Wild-type (wt) and *orbit*^{6} (orbit) egg chambers were stained with anti-lamin (green) antibody and TOTO3 for DNA (blue). The *orbit*^{6} egg chambers contain fewer than 16 germline cells. They show no oocyte differentiation and all cells adopt nurse cell fate. Scale bar: 50 μm.

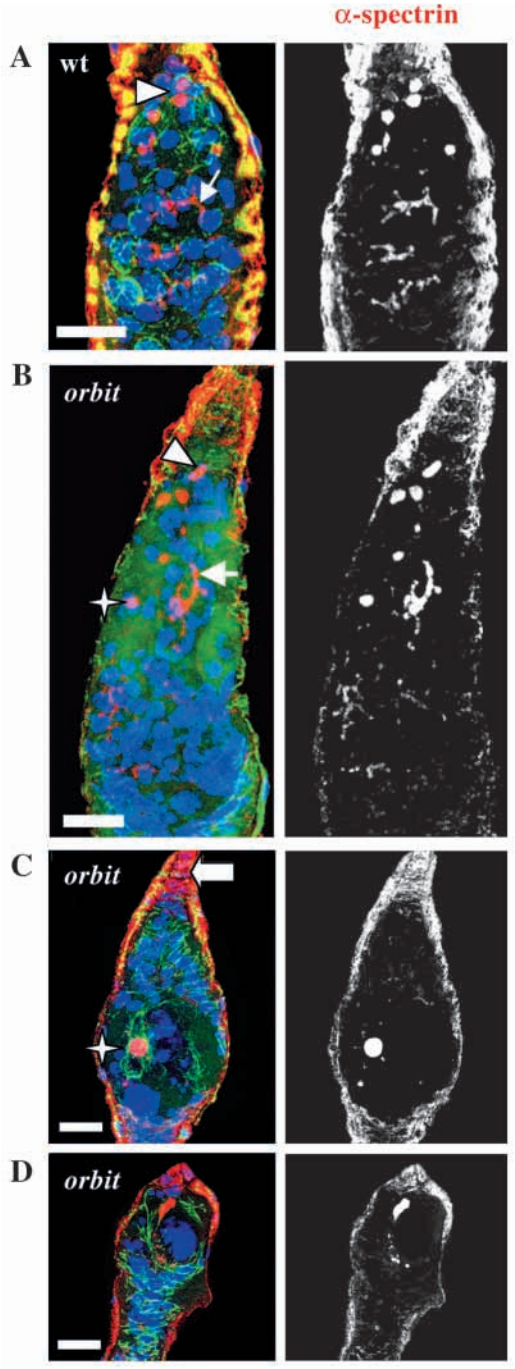

Fig. 3. Disruption of the ovariole niche in germaria of *orbit*^{6} mutants. Germaria are stained with anti-α-spectrin (red), anti-α-tubulin (green) antibodies and TOTO3 (blue) to reveal DNA. (A) The apical region of a wild-type germarium with two stem cells (arrowhead) that each contains a spherical fusome (inferred by α-spectrin staining). Germline cysts have branched fusomes (arrow) linked to microtubule bundles. (B) The germarium of a 1-day-old *orbit*^{6} female showing normal fusome morphology in a stem cell (arrowhead) and a germline cyst (arrow). In other cysts the fusome failed to branch (star). The terminal filament cells are not visible on the picture since they are out-of-focus. (C) The germarium of a 2-day-old *orbit*^{6} female that has no stem cells. Only one germline cell-cyst is present in the germarium containing a spherical fusome (star) and some fusome fragments. Microtubule bundles are cross-linking these fusome elements and the stack of terminal filament cells looks normal (arrow). (D) The germarium of a 3-day-old *orbit*^{6} female that has no stem cells. A single germline cell-cyst is present that contains a single nurse cell-like nucleus, an elongated fusome and some fusome fragments connected through microtubule bundles. A stack of terminal filament cells is not present. Scale bar: 10 μm.

chambers were found in about one fifth of the mutant ovaries, suggesting the potential involvement of a diminished production rate of 16-cell cysts. Oocytes did not differentiate in these mutant *orbit* egg chambers (about 700 were examined), and all the nuclei resembled those of nurse cells but with poor nuclear lamina organisation (Fig. 2D), a condition potentially caused by abnormal germline divisions and/or the failure to establish the correct architecture of the cyst required to support normal oocyte differentiation. In the following, we describe the cytological phenotype of *orbit*^{6} homozygotes, although we have observed similar phenotypes in the above genotypic combinations with *orbit*^{6}.

Stem cell maintenance and fusome growth are compromised in *orbit*^{6} germaria

The finding of Orbit/Mast in the fusome in wild-type ovaries led us to examine whether fusome integrity might be affected in *orbit* mutants. The fusome is first evident in the ovariolar niche of wild-type germaria in the asymmetric divisions of the stem cells. The proliferative activity and identity of ovarian stem cells is regulated in this microenvironment, situated at the tip of the germarium, where two stem cells interact with the terminal filament and cap cells, both of somatic origin. We found defects in the structure of ovariole niches in *orbit*^{6} females from their emergence through three consecutive days of development (Fig. 3) that increased in severity as the amount of Orbit/Mast protein decreased (Fig. 2C). In the wild-type ovariolar niche, the position of two stem cells could be inferred from the α-spectrin positive spherical fusome situated close to the terminal filament and cap cell (Fig. 3A, arrowhead). More distally, the fusome branched, reflecting its growth in successive germline division cycles (Fig. 3A, arrow). In about 50% of germaria of newly emerged and 1-day-old *orbit*^{6} females, fusome-containing stem cells could be recognised that were associated with terminal filament and cap cells in the ovariolar niche (Fig. 3B, arrowhead). Some cysts at later stages had developed branched fusomes of normal appearance (Fig. 3B, arrow). In many other cysts, the fusome appeared as though it had failed to branch suggesting its growth was compromised (Fig. 3B, star), although this structure could be a spherical fusome often seen after ring canals have coalesced at the four-cell stage. The remaining 50% of germaria of 0- to 1-day-old females, showed no fusome material in the ovariolar niche and thus would appear not to contain any stem cells, despite the presence of both terminal filament and cap cells (Fig. 3C). Such ovaries would have appeared at one time to have had stem cells since they frequently contained either aggregates or fragments of fusome material often contacting irregular microtubule bundles located in more distal cysts (Fig. 3C, star). Such abnormal germaria predominated in 2- to 3-day-old *orbit*^{6} females (Fig. 3D). Thus, neither the fusome organisation or cycling ability of stem cells appeared to be maintained in the ovariolar niche of *orbit*^{6} germaria.

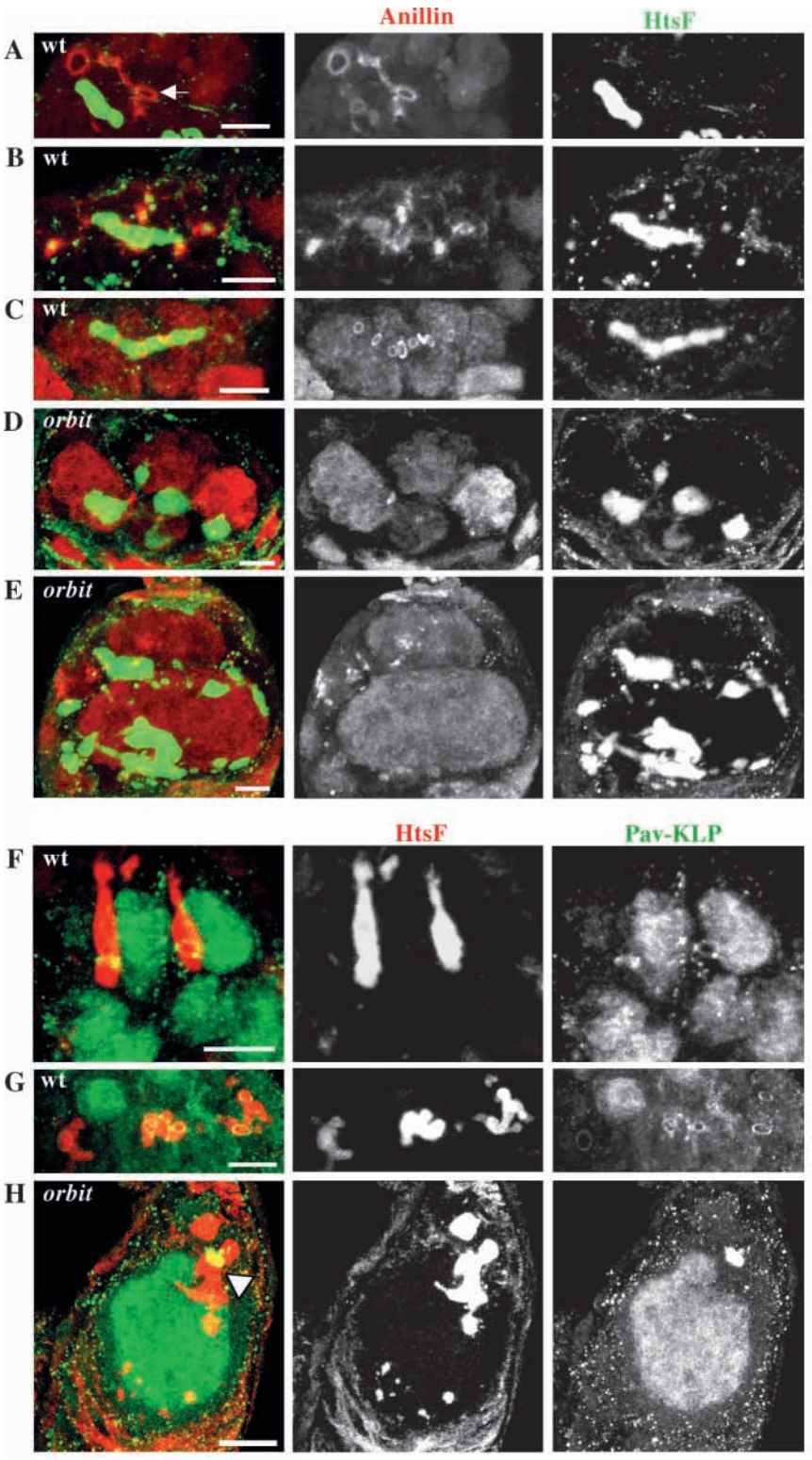

Fig. 4. Anillin and Pav-KLP fail to be recruited to ring canals in *orbit*^{6} mutants. (A-E) Germaria stained with anti-Anillin (red) and anti-HtsF (green) antibodies. (A) Wild-type cyst showing Anillin recruited to the newly formed contractile rings (arrow) before the initiation of fusome plug formation. (B) Wild-type cyst with Anillin present in the constricting and migrating ring canals. (C) Wild-type 16-cell cyst that has completed the four rounds of divisions. Anillin is present in the ring canals situated along the fusome. Only eight ring canals and the corresponding fusome part are visible; the others are out of focus. (D,E) *orbit*^{6} germaria that contain one and two germline cysts respectively, each with several fusome pieces. No Anillin-containing ring canal-like structures are visible. However, as with wild-type, the interphase nuclei of mutant cysts contain Anillin. Scale bar: 5 μm. (F-H) Germaria were stained with anti-Pav-KLP (green) and anti-HtsF (red) antibodies. (F) Wild-type stem cell and cystoblast interconnected through a Pav-KLP-containing ring canal at the time of fusome partition. The nuclei of these cells contain elevated levels of Pav-KLP, suggesting that these cells are in interphase. (G) Pav-KLP recruited to the ring canals surrounding the pre-existing fusome in wild-type germline cysts. (H) *orbit*^{6} germline cyst with an abnormal fusome and Pav-KLP accumulated in a single ring canal-like structure (broad arrow). Fusome pieces seen at the posterior of this cyst are of different sizes and show little or no Pav-KLP staining. Scale bars: 5 μm in F,G; 10 μm in H.

We further assessed the morphology and growth of the fusome in *orbit*^{6} germaria by focusing on its interactions with ring canals. In the wild-type ovary, the fusome plug first forms in each newly assembled ring canal following mitosis of stem cells, cystoblasts and cystocytes. These plugs then migrate and fuse with the pre-existing fusome. The development of the spherical fusome of the stem cell and cystoblast into a large branched structure that extends through the ring canals into every cell of the cyst was revealed by HtsF or α-spectrin staining (Fig. 4; Fig. 3A). We monitored the progressive growth of the fusome in wild-type and *orbit*^{6} mutant females revealed by immunostaining HtsF with respect to Anillin and Pav-KLP, two components of the ring canals that form from the cytokinetic cleavage ring that in the germline does not undergo complete closure (Fig. 4). Anillin accumulates at the contractile ring and binds to actin filaments during cytokinesis in many cell types including stem cells, cytoblasts and cystocytes (de Cuevas and Spradling, 1998). The protein was recruited to the contractile ring in cystoblasts and cystocytes before fusome plug formation (Fig. 4A, arrow), and was present in the constricting and migrating ring canals (Fig. 4B). Moreover, it persisted in the ring canals that had reached the fusome until after the cyst had left the germarium (Fig. 4C). In contrast to wild type, we found no Anillin stained ring canals in *orbit*^{6} cysts although there were HtsF containing fusome-like bodies (Fig. 4D,E). Similar findings were obtained by monitoring Pav-KLP (Fig. 4F-H). Pav-KLP acts in the early stages of cytokinesis to organise the central spindle and persists in the ring canals of germline cysts in both oogenesis and

spermatogenesis (Adams et al., 1998; Carmena et al., 1999; Minestrini et al., 2002). In wild-type germaria, we found higher levels of Pav-KLP in the nuclei of stem cells and cystoblasts than in cystocytes. In mitotically dividing stem cells, cystoblasts and cystocytes, Pav-KLP was present in the mid-zone of the central spindle (data not shown). It was then to be incorporated into the cleavage furrows and so the ring canals through which the fusome was seen to pass (Fig. 4F,G). In *orbit*^{6} cysts we could not assess the recruitment of Pav-KLP to the central spindle as we were unable to find a single anaphase or telophase spindle. However, in some mutant cysts, we were able to see occasional ring-like, Pav-KLP-containing structures associated with fusome-like bodies, while the other fusome-like pieces could be scattered within a cyst and showed no Pav-KLP staining (Fig. 4H). These multiple fusome pieces appeared to have failed to fuse and form a properly branched fusome. Thus, we conclude that not only is there a reduction in the formation of fusomes, but those that do form fail to grow in part due to defects in ring canal formation and migration.

orbit^{6} mutants disrupts the asymmetric orientation of mitotic spindles

As aspects of the *orbit* phenotype described above suggested defects in cell division of the germarium stem cells or cystocytes, we searched for cells in the process of division in wild-type and *orbit*^{6} ovaries, staining them to reveal DNA, γ-tubulin and Orbit/Mast protein. In wild-type stem cells at prophase, γ-tubulin was recruited to the centrosomes and the Orbit/Mast protein accumulated on both centrosomes and the centrosome nucleated microtubules, its staining increasing in intensity at metaphase (Fig. 5A). Although the levels of Orbit/Mast protein were dramatically reduced in total ovaries from 1- to 3-day-old *orbit*^{6} females, we were still able to detect faint staining of spindle MTs by the anti-Orbit antibody within a small number of cystoblasts and cytocytes of newly eclosed females. In such germaria the structure of the cystoblast and/or cytocyte spindles was severely affected (Fig. 5B,C). Spindles were much shorter (average length 3 μm; arrowheads in Fig. 5B,C) than the metaphase spindles (average length 7 μm) observed in wild-type two- or four-cell cysts, and could even be monopolar (Fig. 5B, arrow). This suggests that bipolar spindles might first form and then collapse to monopolar structures as recently described in *orbit/mast*-derived embryos and following *orbit/mast* RNAi in cultured cells (Maiato et al., 2002). We analysed over 2000 wild-type germaria and identified 48 that contained mitotic stem cells, and 54 that had cystoblasts or cystocytes at various stages of mitosis. The stem cell specific mitotic index (MI) of 2.4 was slightly higher, but still consistent with the observations of Deng and Lin (Deng and Lin, 1997). In about 2000 germaria of *orbit*^{6}/*orbit*^{6} females, we found no stem cells in mitosis and only seven cystoblast and cytocyte divisions. The mitotic index of *orbit*^{6} cystoblasts-cystocytes was thus dramatically reduced compared with wild type (0.35 versus 2.7). Thus, unlike larval neuroblasts, cystocytes appear not to arrest in mitosis, suggesting that if they have a mitotic checkpoint it is over-ridden by the developmental process of oogenesis. However, reduced proliferation ability of stem cells or their failure to be maintained in *orbit*^{6} germaria as noted above would further account for the reduction in mitotic index.

It is a characteristic of stem cell, cystoblast and cystocyte divisions that one of the poles of the mitotic spindles associates with the fusome leading to the asymmetry of division proposed to play a determinative role in generating and maintaining cyst asymmetry and to be a precondition for oocyte formation. In wild-type germaria we found the pole of each spindle most proximal to the terminal filament cells was attached to a spherical fusome (Fig. 5D). In mitotic cystoblasts, the fusome kept its spherical shape and was often located in the posterior half of the cell with the posterior spindle pole anchored to it (data not shown). In two-cell, four-cell and eight-cell mitotic cysts, we found spindles arranged in compact clusters with one pole of each spindle in close contact with a single branch of the fusome (Fig. 5E). To find examples of spindles in the *orbit*^{6} mutant, we examined about 2000 mutant germaria. In the most affected germaria, Orbit/Mast staining was very weak, and no spindles were visible. Whenever there was sufficient residual Orbit/Mast protein to allow some spindles to form, such spindles never made contact with the fusome. This can be seen in the cyst shown in Fig. 5F, where two bipolar and one monopolar (arrow) spindles lie in the vicinity of a fusome but show no association with it. In general, fusomes looked fragmented in most cysts (Fig. 5G, arrowhead). Taken together, our observations suggest that the *orbit*^{6} mutation affects the bipolar organisation of the spindles and also prevents the asymmetric interaction between the mitotic spindle and fusome.

orbit^{6} egg chambers have ring canals of abnormal structure and number

The 16 cystocytes of each germline cyst are connected by 15 ring canals representing the arrested cleavage furrows of the preceding four rounds of germline cyst divisions. Once these divisions are completed and concomitant to formation of the polarised microtubule network, the ring canals acquire outer and inner rims by recruiting different actin binding proteins. Filamin is recruited to both inner and outer rims of the ring canals (Li et al., 1999; Sokol and Cooley, 1999), whereas HtsRC (the ring canal specific product of the *hts* gene) (Yue and Spradling, 1992), further F-actin (Theurkauf et al., 1993) and Kelch (Xue and Cooley, 1993) accumulate in the inner rims. The Orbit/Mast protein did not associate extensively with the ring canals of wild-type egg chambers, although some punctate accumulation may be inferred based on co-immunolocalisation studies with other ring canal proteins (Fig. 6E).

In *orbit*^{6} egg chambers, we observed a gradient of ring canals defects, the severity of which varied as a function of the age of the female (Fig. 6). Egg chambers from younger females (Fig. 6B,C,F) showed milder defects than older ones (Fig. 6D,G). Whereas F-actin and Filamin formed the expected overlapping rings in wild-type ring canals, in the egg chambers of younger *orbit*^{6} females, these proteins appeared disorganised, extending into and obstructing partially if not totally the lumen of the canals (Fig. 6B,C). The inner rim proteins Filamin and HtsRC extended to the very centre of the canal (insets to Fig. 6C,F). We also noted these *orbit*^{6} egg chambers showed varying loss of actin at the nurse cell boundaries indicating a disruption to the cortical cytoskeleton within these cells (Fig. 6B-D). In older *orbit*^{6} females, the egg chambers often had no ring canals, although ring canal specific proteins such as Pav-KLP (not shown), phosphotyrosine

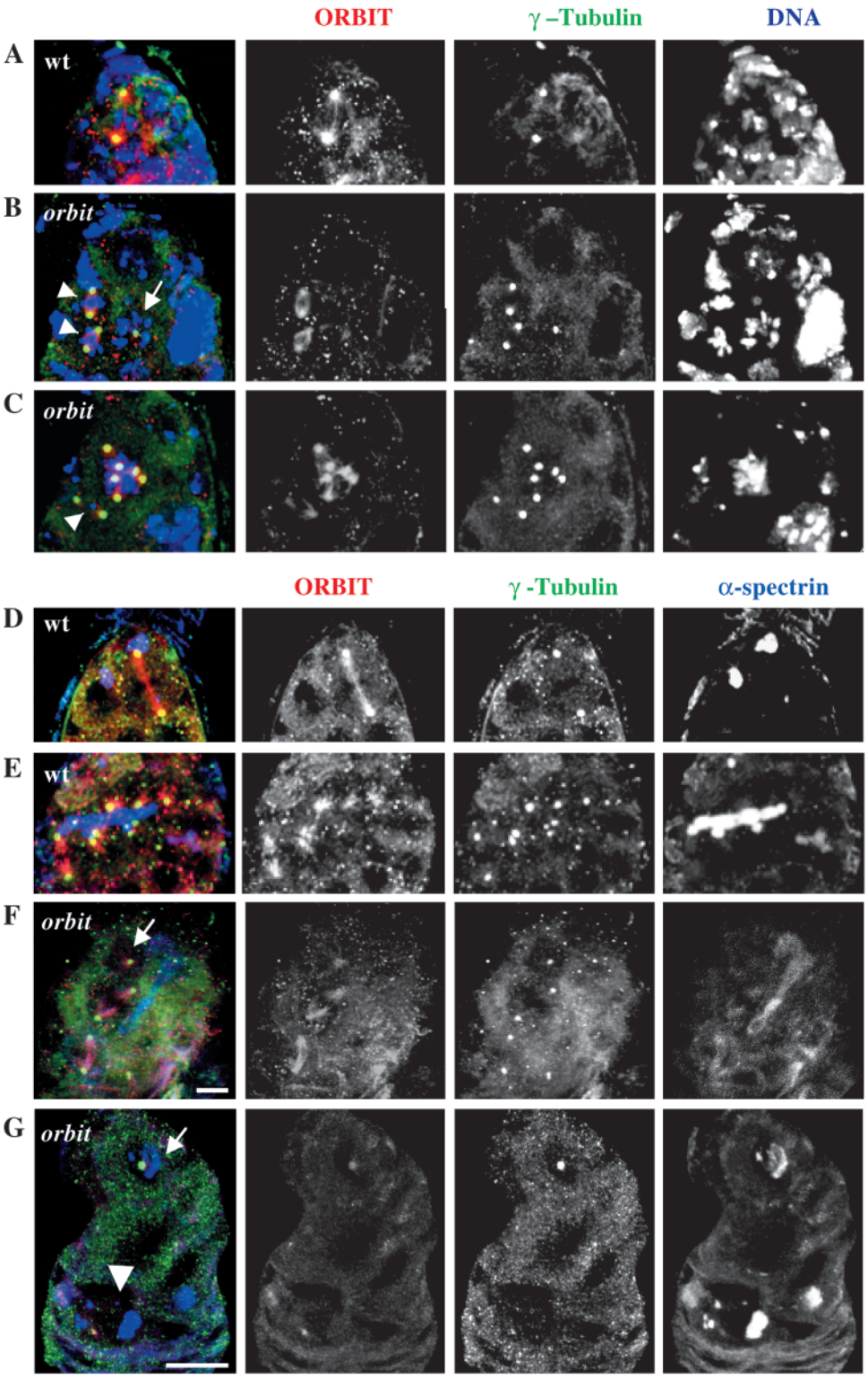

Fig. 5. Defective spindles fail to associate with fusome in *orbit*^{6} mutants. (A-C) Germaria stained with anti-Orbit/Mast (red), anti-γ-tubulin (green) antibodies and TOTO3 to reveal DNA (blue). (A) At metaphase, Orbit/Mast concentrates at spindle poles and localises along spindle MTs in wild-type stem cells. (B) Short bipolar (arrowheads) and monopolar (arrow) spindles seen at the tip of an *orbit*^{6} germarium. (C) Multiple short spindles at the tip of an *orbit*^{6} germarium. Arrowhead indicates a short bipolar spindle. (D-G) Germaria stained with anti-Orbit/Mast (red), anti-γ-tubulin (green) and anti-α-spectrin (blue). (D) Wild-type stem cell at metaphase, showing the anterior spindle pole attached to the fusome. (E) Wild-type eight-cell germline cyst at metaphase with the spindles being attached to fusome through one of their poles. (F) Monopolar (arrow) and bipolar spindles that fail to associate with the fusome in an *orbit*^{6} germarium. (G) *orbit*^{6} germarium showing a monopolar spindle (arrow) close to the very apical region of the germarium, and a poorly organised germline cyst (arrowhead) situated more posteriorly. Both contain fusome pieces of different sizes. Scale bars: 5 μm A-E; 10 μm in F.

protein(s) (data not shown), actin, Filamin (Fig. 6D) and HtsRC (Fig. 6G) were detected in the form of aggregates. Surprisingly, we were unable to detect any Kelch protein in the ring canals of either younger or older *orbit*^{6} females (data not shown). Together this suggests defects in the differentiation of ring canals from the cleavage furrows of the cystocytes.

In wild-type cysts, the number of ring canals is one fewer than the cell number providing a record of each incomplete cytokinesis involved in the formation of the 16-cell cyst. The egg chambers of younger *orbit*^{6} females frequently had seven ring canals and eight nurse cell nuclei, suggesting that they might have had developed from eight-cell germline cysts that fail to execute the fourth round of mitotic division (Fig. 6B). However, in other egg chambers the number of ring canals and nurse cell nuclei did not seem to correlate and it was often impossible to determine their exact number, suggesting a more extensive failure of cell division. Many egg chambers contained between one and six nurse nuclei and no ring canals, which suggested that germline cyst mitoses had occurred but that cytokinesis had not been completed correctly. Thus, defects in the structural integrity of eggs chambers in the absence of Orbit/Mast protein appeared to be due in part to cytokinesis defects. Moreover, the defects seen in ring canal differentiation in *orbit*^{6} egg chambers further suggest that during cytokinesis ring canal – fusome interactions were affected.

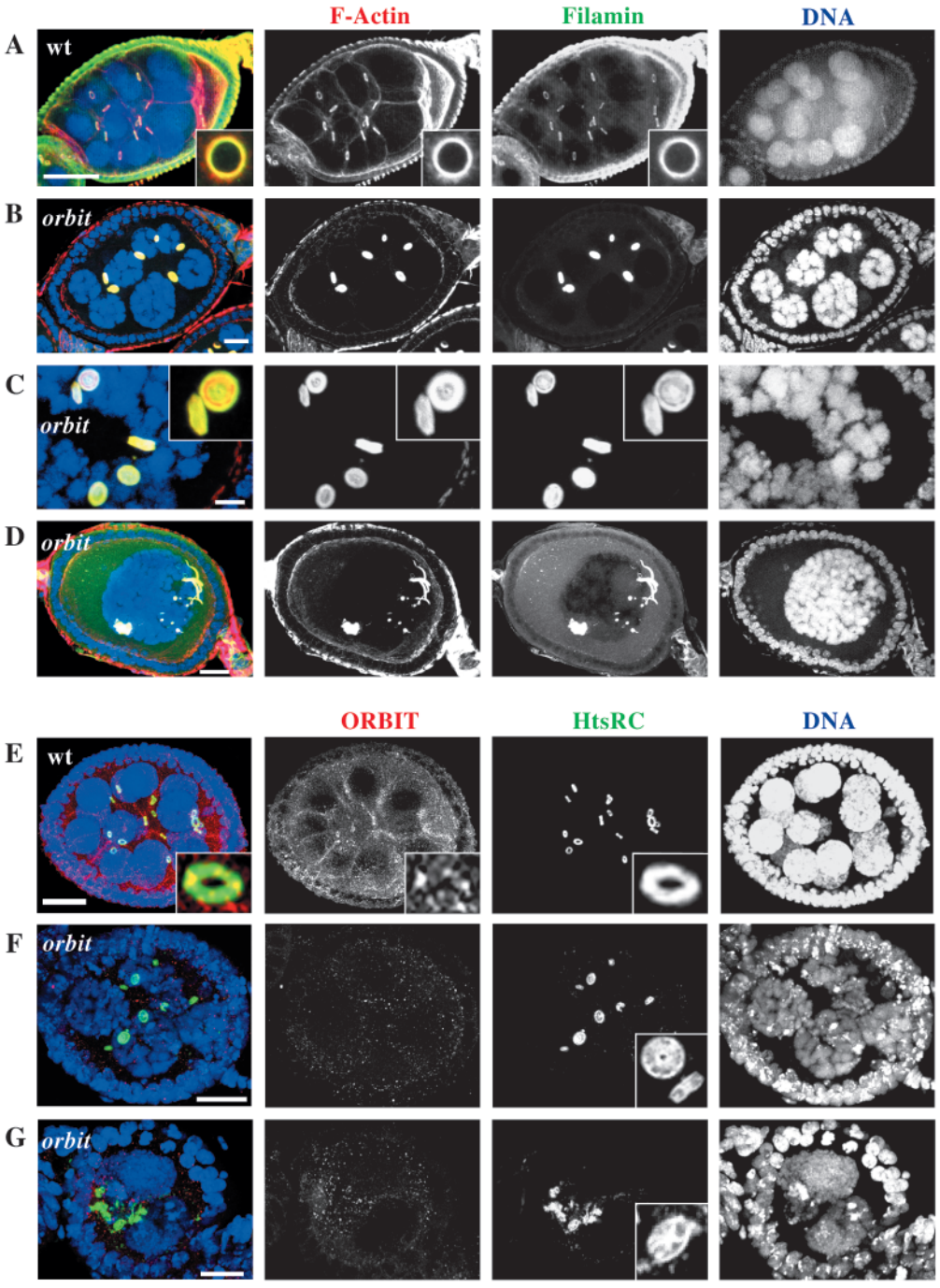

Fig. 6. Ring canal organisation is affected in egg chambers of *orbit*^{6} mutants. (A-D) Germaria were stained with rhodamine-phalloidine (red), anti-Filamin (green) antibody and TOTO3 to reveal DNA (blue). (A) Wild-type egg chamber in which Filamin and F-actin co-localise in the ring canals. Scale bar: 50 μm. (B) Egg chamber of 1-day old *orbit*^{6} female containing seven nurse cell nuclei and six ring canals. Filamin and F-actin accumulate not only in the rims but also in the lumen of the ring canals. Note the weak F-actin staining in the cortex of the nurse cells. Scale bar: 10 μm. (C) Filamin and F-actin are not restricted to the rims of ring canals in *orbit*^{6} egg chambers. Scale bar: 5 μm. (D) Severely affected egg chambers from 3-day old *orbit*^{6} female with a single large nurse cell nucleus; ring canals fail to form although filamentous structures containing Filamin and F-actin are visible. Scale bar: 10 μm. (E-G) Germaria were stained with anti-Orbit (red), anti-HtsRC (green) antibodies and TOTO3 to reveal DNA (blue). (E) Wild-type egg chamber showing the HtsRC and Orbit/Mast (inset) proteins in the ring canals. Scale bar: 25 μm. (F) Egg chamber from 1-day old *orbit*^{6} female in which HtsRC accumulates in the rims and obstructs the lumen of the ring canals. Orbit/Mast staining is weak in the cortex of the nurse cells. Scale bar: 10 μm. (G) Abnormal egg chamber of 3-day old *orbit*^{6} female showing irregular structures containing HtsRC protein, some of which resemble ring canals that have completely occluded lumens. Scale bar: 10 μm.

The posterior MTOC fails to develop correctly in *orbit*^{6} egg chambers and the microtubule network is perturbed

In wild-type ovaries, egg chambers form and mature along the length of the ovariole as the germline cysts complete their divisions and are encapsulated by follicle cells of somatic origin. One of the 16 cystocytes will acquire oocyte identity as the ‘stage 1’ egg chamber buds off the germarium and enters the vitellarium. Here, the oocyte differentiates at the expense of nurse cells and surrounding follicle cells. After completion of cyst divisions, the Orbit/Mast protein showed punctate localisation pattern within the 16-cell cyst, and co-localised with the regressing fusome and microtubules but disappeared from the differentiating ring canals (data not shown). As oogenesis progressed, the Orbit/Mast protein became concentrated in the oocyte cytoplasm of stage 1 egg chambers and persisted until stage 6-7 of oogenesis, when it translocated into the oocyte nucleus (Fig. 7A,D). The localisation of the Orbit/Mast protein in the oocyte cytoplasm during stages 1 to 7 of oogenesis is largely coincident with the single MTOC located behind the oocyte nucleus. Microtubule bundles nucleated at this centre extend through the ring canals to form a network that interconnects the oocyte-nurse cell complex within each egg chamber. The punctate accumulations of

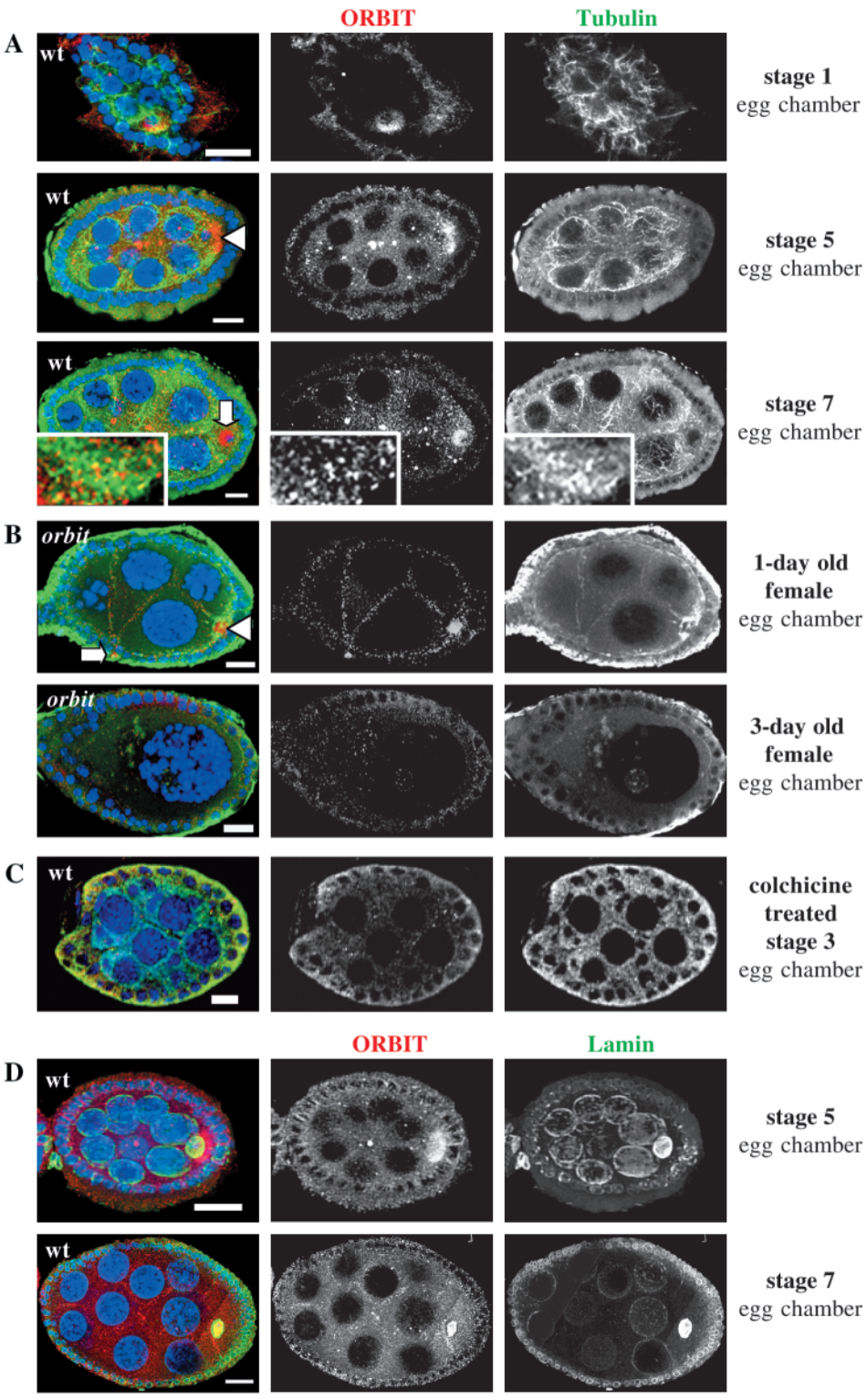

Fig. 7. The polarised MT network fails to develop correctly in egg chambers of *orbit*^{6} mutants. (A-C) Egg chambers were stained to reveal Orbit/Mast (red), α-tubulin (green) and DNA (blue). (A) Wild-type egg chambers at stages 1-, 5 and 7 showing the Orbit/Mast protein accumulates at the MTOC of the oocyte (arrowhead) and is present in the oocyte nucleus from stage 7 onwards (arrow). The punctate pattern of Orbit/Mast seems to show some correlation with the microtubule network (inset) of the egg chambers. (B) Egg chambers of 1- and 3-day old *orbit*^{6} females showing the age-dependent severity of the mutant phenotype. In egg chambers of younger females, the residual Orbit/Mast protein decorates a diminished number of microtubule bundles, which seem to contact local accumulations of Orbit/Mast (arrow). There is also reduced staining of a putative MTOC (arrowhead). (C) Colchicine-treated, wild-type stage 3 egg chamber showing no MTOC-specific localisation of Orbit/Mast. The microtubule network is destroyed and the Orbit/Mast staining is diffuse in the cytoplasm of cells. Scale bar: 10μm. (D) Wild-type egg chambers stained to reveal Orbit/Mast (red), lamin (green) and DNA (blue). Note the MTOC and nuclear localisation of Orbit and Mast, respectively. Scale bar: 20 μm.

Orbit/Mast appeared only in part connected to microtubule bundles with a substantial amount of protein remaining apparently free in the cytoplasm. To demonstrate that localisation of Orbit/Mast protein in egg chambers was dependent upon microtubules, we used colchicine treatment to disassemble microtubules after the completion of cyst divisions, as described by Theurkauf and colleagues (Theurkauf et al., 1993). When wild-type flies were fed with 20-50 μg/ml colchicine for 16-48 hours, the newly produced egg chambers had 16 nurse cell nuclei indicating that oocyte fate was not properly established and/or maintained. Moreover, in such egg chambers the Orbit/Mast protein failed to accumulate at any site equivalent to a MTOC and the punctate nature of its localisation was diminished (Fig. 7C). Together, these observations indicate that the microtubule network is essential for recruitment of Orbit/Mast protein to the posterior of the stage 1-6 egg chamber and for maintaining the identity of the oocyte.

In contrast to wild-type egg chambers, the microtubule network of *orbit*^{6} mutants was severely disrupted or completely abolished (Fig. 7B). The severity of the observed defects reflected the age of the female and paralleled the levels of Orbit/Mast protein in ovaries. The egg chambers of younger females had reduced numbers of microtubules in irregular bundles that were associated with some residual Orbit/Mast protein. Weak Orbit staining of a body apparently equivalent to the MTOC could be seen in such egg chambers of younger

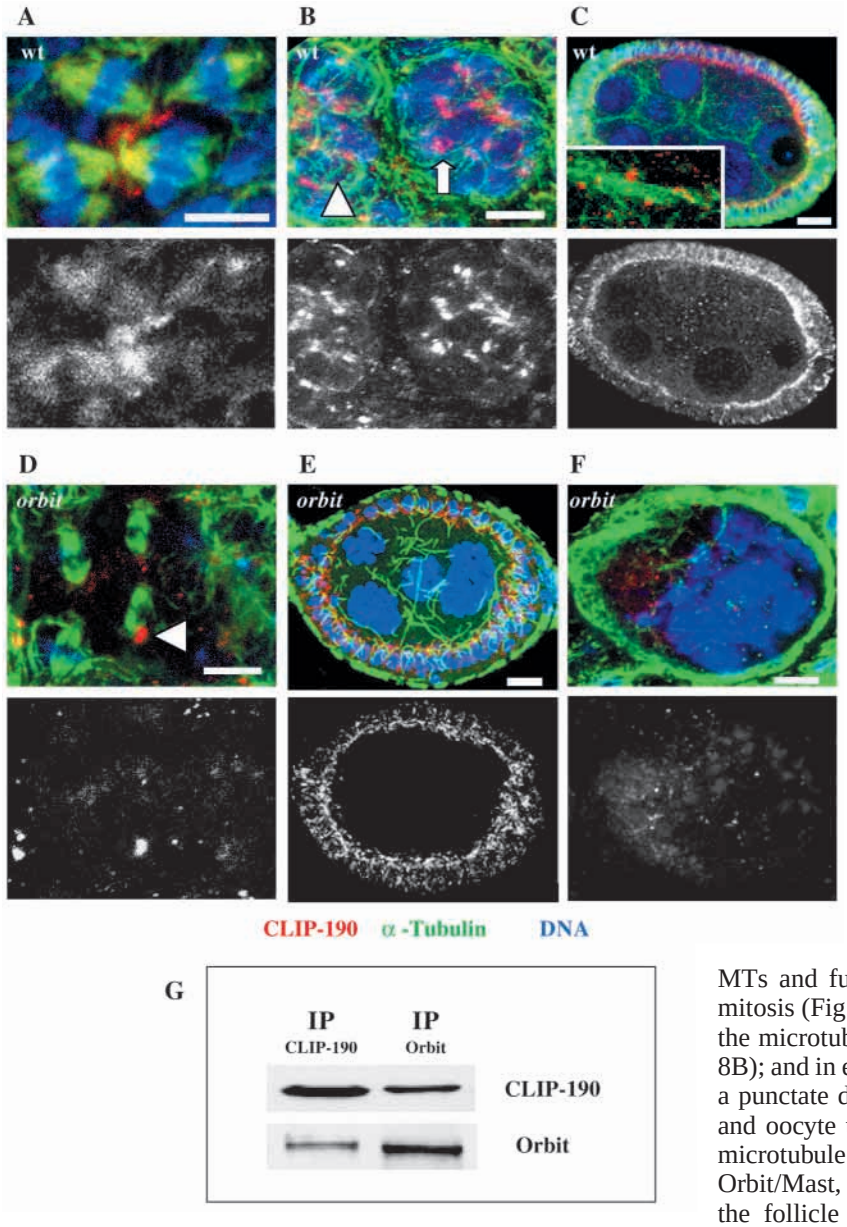

Fig. 8. Localisation of CLIP-190 is disrupted in *orbit*^{6} mutants. (A-F) Germaria and egg chambers stained to reveal CLIP-190 (red), α-Tubulin (green) and DNA (blue). The monochrome panels correspond to the staining pattern of CLIP-190. (A) Wild-type four-cell cyst at metaphase with CLIP-190 localising on the spindle and fusome. (B) Wild-type 16-cell stretching (arrowhead) and lens-shaped (arrow) cysts showing specific accumulation of D-CLIP-190. (C) Wild-type stage 6-7 egg chamber; CLIP-190 displays a punctate localisation along MT bundles (inset) and accumulates in the apical cortical region of the follicle cells. (D) Mitotic cyst in *orbit*^{6} germarium with no CLIP-190 on the spindles and a fusome-like body containing CLIP-190 (arrow) that contacts a spindle pole. (E) Egg chamber of a young *orbit*^{6} female with irregular microtubule bundles that show no accumulation of CLIP-190. (F) Egg chamber of an older *orbit*^{6} female with a few irregular microtubule bundles. Scale bars: 10 μm. (G) Western blots of total ovarian extracts immunoprecipitated with anti-CLIP-190 or anti-Orbit/Mast specific antibodies, subjected to western blotting and probed with the indicated antibodies.

females, even though they had reduced numbers of nurse cells and had not specified an oocyte nucleus. In such cases, the MTOC varied in its position. In older females, Orbit/Mast protein, microtubule bundles and MTOC were not detected. Thus, the Orbit/Mast protein appears to play a role in maintaining microtubules in the post-mitotic egg chamber and in establishing the MTOC that is usually formed adjacent to the oocyte nucleus.

CLIP-190 interacts with Orbit/Mast during oogenesis

In mammalian cells, CLASP, the counterpart of Orbit/Mast, has been shown to interact with CLIP-170, a protein implicated in the interphase functions of microtubules (Akhmanova et al., 2001). We therefore wished to know whether Orbit/Mast protein interacted with CLIP-190 (the fly orthologue of CLIP-170) (Lanz and Miller, 1998) and whether the localisation of CLIP-190 in ovaries was in any way dependent upon Orbit/Mast. To this end, we carried out immunoprecipitation experiments on total protein extracts made from wild-type ovaries with antibodies to the Orbit/Mast protein and found that CLIP-190 co-precipitated. Similarly, immunoprecipitations with anti-CLIP-190 antibodies also demonstrated an interaction with Orbit/Mast (Fig. 8G). Moreover, in wild-type germaria, CLIP-190 followed a similar localisation pattern to Orbit/Mast protein: it associated with spindle MTs and fusomes during stem cell and cystoblast-cystocyte mitosis (Fig. 8A): it was present in punctate masses contacting the microtubule network of newly formed egg chambers (Fig. 8B); and in egg chambers that had left the germaria, it followed a punctate distribution along the cytoplasm of the nurse cells and oocyte that seemed to associate with the interconnecting microtubule bundles (Fig. 8C and inset). In contrast to Orbit/Mast, CLIP-190 also accumulated in the apical region of the follicle cells and did not show any localisation to the oocyte-associated MTOC. In *orbit*^{6} germaria, CLIP-190 was not observed on the spindles, although occasionally in younger females local accumulations of it were seen in close contact with some spindle poles (Fig. 8D, arrowhead). CLIP-190 was also absent from the irregular microtubule bundles surrounding nurse cells of younger *orbit*^{6} females (Fig. 8E). However, the follicle cells of such egg chambers contained normal levels of CLIP-190, indicating that its localisation in these cells was independent of Orbit/Mast. In older *orbit*^{6} females, few germline cysts had completed their divisions and these rarely showed microtubule bundles. In such cysts there was very weak accumulation of CLIP-190 in the cytoplasm apparently not associated with microtubules (Fig. 8F). Thus, CLIP-190 and Orbit/Mast show considerable overlap in their subcellular distribution. Moreover, the dependence of CLIP-190 localisation on Orbit/Mast function suggests that colocalisation seen in these regions is likely to be mediated through the interaction between the two proteins. The localisation of the two proteins to both the fusome and the polarised MT network,

structures that are aberrant in *orbit* mutants, suggests a role for the complex in maintaining these structures.

DISCUSSION

The Orbit/Mast protein facilitates a wide array of cytoskeletal events in both mitotic and interphase cells during *Drosophila* oogenesis. Failure to provide these functions affects organisation of the stem cell niche, the polarised growth of the fusome, the division of germline cells, specification and maintenance of oocyte fate, and the establishment of the microtubule mediated system for polarised nurse cell – oocyte transport.

As *orbit*^{6} females age they ultimately show a complete failure of egg chamber production, reflecting an eventual loss of stem cells from the ovariolar niche. The wild-type ovariolar niche consists of two germline stem cells and the somatic terminal filament and cap cells thought to cooperate in establishing and maintaining stem cell identity (reviewed by Spradling et al., 2001). This process requires that stem cells divide asymmetrically along the anteroposterior axis of the germarium such that only their anterior daughter cells inherit cap cell contact and a larger piece of fusome. The posteriorly situated daughter cells that inherit a smaller piece of fusome loose contact with cap cells and differentiate into cytoblasts. The *orbit*^{6} phenotype indicates that the gene product is required for stem cell maintenance. One possible explanation may be offered for this that the absence of Orbit/Mast protein leads to stem cell division defects that cause stem cells to lose contact with the cap cells and the terminal filament cells.

The ovaries of younger females do have stem cells that are able to produce egg chambers, although these have reduced numbers of nurse cells. This reflects directly the requirement of Orbit/Mast protein for cell division and/or fusome growth. During mitosis of the germline cells, we found that the Orbit/Mast protein associated with the spindle microtubules and spindle poles, and that towards the completion of cell division it became concentrated in the spindle remnants, as previously reported in other cell types (Inoue et al., 2000; Lemos et al., 2000). Indeed, mitotically dividing *orbit*^{6} cystoblasts showed a variety of abnormalities consistent with defects seen in dividing somatic cells of other *orbit* alleles (Inoue et al., 2000; Lemos et al., 2000). These included short bipolar and monopolar spindles, supporting recent observations that the mitotic requirement for Orbit/Mast is to maintain spindle bipolarity (Maiato et al., 2002). Orbit/Mast protein is also found in the fusome that, in wild-type cells, is always associated with one pole of each mitotic spindle in the cyst of dividing cells. By contrast, the *orbit*^{6} spindles never made contact with fusome fragments. Loss of connections between spindles and fusome have been previously described in mutants for the heavy chain of the minus end directed motor dynein (Dhc64C) (McGrail and Hays, 1997) and in germline clones of null alleles of *Lis1*, which encodes the *Drosophila* homologue of the *lissencephaly* disease gene, the product of which interacts with dynein (Liu et al., 1999; Swan et al., 1999). The phenotypes in each case have some similarities to *orbit*^{6}: the spindles often fail to attach to fusomes, cysts are produced with fewer than 16 cells, and the oocyte is not specified. It has been suggested that these defects were due to interactions between fusome and spindles or interphase microtubules. Although the defects seen in *orbit*^{6} could arise in this way, the mitotic defects seen in the mutant give greater emphasis to the necessity to maintain correct interactions between the metaphase spindle and the fusome and suggest that Orbit/Mast protein may have a direct role in ensuring such connections.

The fusome provides a physical basis to ensure asymmetry of the developing cyst that is a precondition for cyst polarisation and oocyte specification (Lin et al., 1994; Theurkauf, 1994; deCuevas and Spradling, 1998; Grieder et al., 2000). Fusome material does develop in the cysts of younger *orbit*^{6} females, but tends to remain as separate fragments. The failure of correct fusome development is most likely to be the underlying cause behind the failure to specify the oocyte in *orbit*^{6} mutants. Our data suggest that not only is Orbit/Mast required for interactions of the fusome with spindles, but it is also needed for its subsequent interactions with spindle remnants and the ring canals. We confirm that fusome material accumulates in the vicinity of the spindle remnants in wild-type cysts. These spindle remnants have been suggested to help restrict constriction of the cleavage furrows (Mahajan-Miklos and Cooley, 1994) and so guide fusome growth by supporting the migration of fusome plugs and their ring canals towards the pre-existing fusome (Storto and King, 1989). The absence of spindle remnants in the mutant *orbit*^{6} cysts may therefore also contribute to the lack of fusome growth. One possibility is that the transfer of Orbit/Mast protein from the degenerating spindle remnants to the fusome plugs inside the ring canals themselves may facilitate migration of fusome plugs along interphase microtubule bundles to fuse with pre-existing fusome. This would ensure the regular and polarised growth of the fusome.

The defects we observe in ring canal formation in *orbit*^{6} mutants are almost certainly secondary to the failure of the fusome to develop correctly. The central spindle (spindle remnant) late in mitosis is important to arrest closure of the cleavage furrows so they may be transformed into ring canals. However, the ring canals continue to differentiate even after completion of cystocyte divisions (Robinson et al., 1994). Anillin (de Cuevas and Spradling, 1998), glycoprotein D-mucin (Sokol and Cooley, 1999), Orbit/Mast and Pav-KLP (this study) are the earliest known proteins to associate with the ring canals during germline cell divisions. Upon completion of these divisions, Anillin and Orbit/Mast disappear from the ring canals, while glycoprotein D-mucin and Pav-KLP will be joined by Filamin, HtsRC and Kelch. The ring canals that did form in younger *orbit*^{6} females were occluded by Filamin, F-actin and HtsRC. A failure to recruit Kelch may be a secondary consequence of the formation of such aberrant structures. Ring canal occlusion could be the result of the irregular structure of the spindle remnant in dividing *orbit*^{6} germline cysts and the failure to plug the newly formed ring canal with fusome components. Mutant egg chambers from older females showed stronger defects; often they contained irregular structures that contained HtsRC, Filamin and F-actin but did not resemble ring canals. This gradient of phenotype along the ovariole may reflect once again the exhaustion of a diminished pool of Orbit/Mast protein as development proceeds.

Molecules found in the fusome are normally found in the

cortical cytoskeleton of most other cell types. Thus, the involvement of Orbit/Mast in the interactions of microtubules with the fusome may be akin to the interphase functions ascribed to CLASP, its counterpart in mammalian cells (Akhmanova et al., 2001). Interactions between the CLASP, CLIP-170, EB1 and APC proteins have been proposed to mediate the crosstalk between cellular structural elements, particularly actin filaments, microtubules and the plasma membrane (Sisson et al., 2000; Akhmanova et al., 2001; Rosin-Arbesfeld et al., 2001; Schuyler and Pellman, 2001). The notion of Orbit/Mast having an equivalent role in the *Drosophila* egg chamber is supported by our finding that the Orbit/Mast protein exists in a complex with CLIP-190, the counterpart of CLIP-170. The two proteins can be co-immunoprecipitated and overlap considerably in their pattern of subcellular localisation throughout oogenesis.

A further interphase role for the Orbit/Mast protein in oogenesis would seem to be in facilitating the organisation of the polarised network of microtubules essential for transport of mRNAs and proteins from the nurse cells to the growing oocyte at stages 1 to 7 (for a review, see Johnstone and Lasko, 2001). The minus ends of microtubules in this network are nucleated by a unique MTOC situated at the posterior of the oocyte. The Orbit/Mast protein is present in punctate bodies along these microtubules and accumulates at this MTOC. Its accumulation at the MTOC requires functional microtubules, as it fails to accumulate in females treated with a microtubule depolymerising drug. This is one aspect of its localisation in which it forsakes its partner CLIP-190, which (although present in punctate bodies associated with the microtubule network) is absent from the MTOC. The mechanisms that regulate the association of these two proteins with respect to the polarity of microtubules are likely to be significant in the full understanding of their biological roles. The polarised microtubule network is disrupted and the MTOC is reduced or absent from egg chambers of *orbit*^{6} females. Once again this phenotype is age dependent: younger mutant females feature only irregular MT bundles and diminutive irregularly placed MTOCs whereas the bundles and MTOC are totally absent in egg chambers of older females. The localisation of CLIP-190 to the fusome and to the polarised microtubule array is also prevented in the *orbit*^{6} mutant, suggesting that it requires complexing to the Orbit/Mast protein to achieve this distribution.

Taken together, our observations strongly argue that the Orbit/Mast protein is required for major polarisation events in wild-type egg chambers: the establishment of the polarised fusome that ultimately enables a single cell within the 16-cell cyst to be specified as the oocyte and in the formation of the polarised MT network in mid-oogenesis. Further studies are needed to determine the precise mechanism whereby the Orbit/Mast protein participates in the organisation of the mitotic spindle, fusome and ring canals, on the one hand, and in the organisation of polarised arrays of interphase microtubules, on the other; and to determine whether the other *Drosophila* orthologues that are members of the CLASP complex are also involved in these processes.

We are grateful to Alfonso Martinez-Arias, Matthew Savoian, Joseph Laycock and Marisa Segal for stimulating discussions. We would like to thank Kathryn Miller for the anti-CLIP-190; Lynn Cooley for the anti-Filamin, anti-HtsRC and anti-Kelch; and Christine Field for the anti-anillin. The monoclonal antibody 1B1 developed by Howard Lipshitz was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa. This work was supported by a grant from Cancer Research UK to D. M. G.

REFERENCES

Adams, R. R., Tavares, A. A., Salzberg, A., Bellen, H. J. and Glover, D. M. (1998). *Pavarotti* encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev.* **12**, 1483-1494.

Akhmanova, A., Hoogenraad, C. C., Drabek, K., Stepanova, T., Dortland, B., Verkerk, T., Vermeulen, W., Burgering, B. M., de Zeeuw, C. I., Grosveld, F. and Galjart, N. (2001). CLASPs are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. *Cell* **104**, 923-935.

Allan, V. and Näthke, I. S. (2001). Catch and pull a microtubule: getting a grasp on the cortex. *Nat. Cell Biol.* **3**, E226-E228.

Büning, J. (1994). The insect ovary: ultrastructure, previtellogenic growth and evolution. New York: Chapman & Hall.

Carmena, M., Riparbelli, M. G., Minestrini, G., Tavares, A. M., Adams, R., Callaini, G. and Glover, D. M. (1999). *Drosophila* polo kinase is required for cytokinesis. *J. Cell Biol.* **143**, 659-671.

de Cuevas, M. and Spradling, A. C. (1998). Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification. *Development* **125**, 2781-2789.

de Cuevas, M., Lee, J. K. and Spradling, A. C. (1996). α-spectrin is required for germline cell division and differentiation the *Drosophila* ovary. *Development* **122**, 3959-3968.

de Cuevas, M., Lilly, M. A. and Spradling, A. C. (1997). Germline cyst formation in *Drosophila*. *Annu. Rev. Genet.* **31**, 405-428.

Deák, P., Omar, M. M., Saunders, R. D. C., Pál, M., Komonyi, O., Szidonya, J., Maróy, P., Zhang, Y., Ashburner, M., Benos, P. et al. (1997). P-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: correlation of physical and cytogenetic maps in chromosomal region 86E-87F. *Genetics* **147**, 1697-1722.

Deng, W. and Lin, H. (1997). Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarised microtubule array for oocyte specification in *Drosophila*. *Dev. Biol.* **189**, 79-94.

Field, C. D. and Alberts, B. M. (1995). Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex. *J. Cell Biol.* **131**, 165-178.

Grieder, N. C., de Cuevas, M. and Spradling, A. C. (2000). The fusome organizes the microtubule network during oocyte differentiation in *Drosophila*. *Development* **127**, 4253-4264.

Huynh, J. R. and St Johnston, D. (2000). The role of BicD, Egl, Orb and the microtubules in the restriction of meiosis to the *Drosophila* oocyte. *Development* **127**, 2785-2794.

Inoue, Y. H., Avides, M. C., Shiraki, M., Deák, P., Yamaguchi, M., Nishimoto, Y., Matsukage, A. and Glover, D. M. (2000). Orbit, a novel microtubule-associated protein essential for mitosis in *Drosophila melanogaster*. *J. Cell Biol.* **149**, 153-165.

Johnstone, O. and Lasko, P. (2001). Translational regulation and RNA localization in *Drosophila* oocytes and embryos. *Annu. Rev. Genet.* **35**, 365-406.

King, R. C., Bahns, M., Horowitz, R. and Larremendi, P. (1978). A mutation that affects female and male cells differentially in *Drosophila* melanogaster. *Int. J. Insect Morphol. Embryol.* **7**, 359-375.

Knowles, B. A. and Cooley, L. (1994). The specialized cytoskeleton of *Drosophila* egg chamber. *Trends Genet.* **10**, 235-241.

Komitopoulou, K., Gans, M., Margaritis, L., Kafatos, F. C. and Masson, M. (1983). Isolation and characterization of sex-linked female sterile mutations in *Drosophila melanogaster* with special attention to eggshell mutants. *Genetics* **105**, 897-920.

Lantz, V. A. and Miller, K. G. (1998). A class VI unconventional Myosin is associated with a homologue of a microtubule-binding protein, Cytoplasmic Linker Protein-170, in neurons and at the posterior pole of *Drosophila* embryos. *J. Cell Biol.* **140**, 897-910.

Lemos, C. L., Sampaio, P., Maiato, H., Costa, M., Omel'yanchuk, L. V., Liberal, V. and Sunkel, C. E. (2000). Mast, a conserved microtubule-associated protein required for bipolar mitotic spindle organization. *EMBO. J.* **19**, 3668-3682.

León, A. and McKearin, D. (1999). Identification of TER94, an AAA ATPase protein, as a bam-dependent component of the *Drosophila* fusome. *Mol. Biol. Cell* **11**, 3825-3834.

Li, M. G., Serr, M., Edwards, K., Ludmann, S., Yamamoto, D., Tilney, L. G. and Field, C. M. (1999). Filamin is required for ring canal assembly and actin organization during *Drosophila* oogenesis. *J. Cell Biol.* **146**, 1061-1073.

Lin, H., Yue, L. and Spradling, A. C. (1994). The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* **120**, 947-956.

Lin, H. and Spradling, A. C. (1995). Fusome asymmetry and oocyte determination in *Drosophila*. *Dev. Genet.* **16**, 6-12.

Liu, Z., Xie, T. and Steward, R. (1999). Lis1, the *Drosophila* homolog of a human *lissencephaly disease* gene, is required for germline cell division and oocyte differentiation. *Development* **126**, 4477-4488.

Mahjan-Miklos, S. and Cooley, L. (1994). Intercellular cytoplasm transport during *Drosophila* oogenesis. *Dev. Biol.* **165**, 336-351.

Maiato, H., Sampaio, P., Lemos, C. L., Findlay, J., Carmena, M., Earnshaw, W. C. and Sunkel, C. E. (2002). Mast/Orbit has a role in microtubule – kinetochore attachment and is essential for chromosome alignment and maintenance of spindle bipolarity. *J. Cell Biol.* **157**, 749-760.

McKearin, D. and Ohlstein, B. (1995). A role for the *Drosophila* Bag-of-marbles protein in the differentiation of cystoblasts from germ line stem-cells. *Development* **121**, 2937-2947.

McGrail, M. and Hays, T. S. (1997). The microtubule motor cytoplasmic Dynein is required for spindle orientation during germline cell divisions and oocyte differentiation in *Drosophila*. *Development* **124**, 2409-2419.

McGrail, M., Gepner, J., Silvanovich, A., Ludmann, S., Serr, M. and Hays, T. S. (1995). Regulation of cytoplasmic dynein function in vivo by the *Drosophila* Glued complex. *J. Cell Biol.* **131**, 411-425.

McKearin, D. (1997). The *Drosophila* fusome, organelle biogenesis and germ cell differentiation: if you bild it… *BioEssays* **19**, 147-152.

McNally, F. J. (2001). Cytoskeleton: CLASPing the end to the edge. *Curr. Biol.* **11**, R477-R480.

Minestrini, G., Máthé, E. and Glover, D. M. (2002). Domains of the Pavarotti kinesin-like protein that direct its subcellular distribution: effects of mislocalisation on the tubulin and actin cytoskeleton during *Drosophila* oogenesis. *J. Cell Sci.* **115**, 725-736.

Navarro, C., Lehmann, R. and Morris, J. (2001). Oogenesis: setting one sister above the rest. *Curr. Biol.* **11**, R162-R165.

Paddy, M. R., Belmont, A. S., Saumweber, H., Agard, D. A. and Sedat, J. W. (1990). Interphase nuclear envelope lamins form a discontinuous network that interacts with only a function of the heterochromatin in the nuclear periphery. *Cell* **62**, 89-106.

Riechmann, V. and Ephrussi, A. (2001). Axis formation during *Drosophila* oogenesis. *Curr. Opin. Genet. Dev.* **11**, 374-383.

Robinson, D. N., Cant, K. and Cooley, L. (1994). Morphogenesis of *Drosophila* ovarian ring canals. *Development* **120**, 2015-2025.

Rosin-Arbesfeld, R., Ihrke, G. and Bienz, M. (2001). Actin-dependent membrane association of APC tumour suppressor in polarized mammalian epithelial cells. *EMBO J.* **20**, 5929-5939.

Schuyler, S. C. and Pellman, D. (2001). Microtubule 'plus-end-tracking proteins': the end is just the beginning. *Cell* **105**, 421-424.

Shulman, J. M., Benton, R. and St Johnstone, D. (2000). The *Drosophila* homolog of *C. elegans* Par-1 organizes the oocyte cytoskeleton and directs *oskar* mRNA localization to the posterior pole. *Cell* **101**, 377-388.

Sisson, J. C., Field, C., Ventura, R., Royou, A. and Sullivan, W. (2000). Lava lamp, a novel peripheral Golgi protein, is required for *Drosophila melanogaster* cellularization. *J. Cell Biol.* **151**, 905-917.

Sokol, N. S. and Cooley, L. (1999). *Drosophila* Filamin encoded by the *cheerio* locus is a component of ovarian ring canals. *Curr. Biol.* **9**, 1221-1230.

Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001). Stem cells find their niche. *Nature* **414**, 98-104.

Storto, P. D. and King, R. C. (1989). The role of polyfusomes in generating branched chains of cystocytes during *Drosophila* oogenesis. *Dev. Genetics* **10**, 70-86.

Swan, A., Nguyen, T. and Suter, B. (1999). *Drosophila* Lissencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. *Nat. Cell Biol.* **1**, 444-449.

Telfer, W. H. (1975). Development and physiology of the oocyte-nurse cell syncitium. *Adv. Insect Physiol.* **11**, 223-319.

Theurkauf, W. E. (1994). Microtubules and cytoplasm organization during *Drosophila* oogenesis. *Dev. Biol.* **165**, 352-360.

Theurkauf, W. E., Alberts, B. M., Jan, Y. N. and Jogens, T. A. (1993). A central role for microtubules in the differentiation of *Drosophila* oocytes. *Development* **118**, 1169-1180.

Tomancak, P., Piano, F., Reichmann, V., Gunsalus, K. C., Kemphues, K. J. and Ephrussi, A. (2000). A *Drosophila melanogaster* homologue of *Caenorhabditis elegans* par-1 acts at an early step in embryonic axix formation. *Nat. Cell Biol.* **2**, 458-460.

Xue, F. and Cooley, L. (1993). *kelch* encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell* **72**, 681-693.

Yue, L. and Spradling, A. (1992). *Hu-li tai shao*, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. *Genes Dev.* **6**, 2443-2454.

Zaccai, M. and Lipshitz, H. D. (1996). Differential distribution of two adducin-like protein isoforms in the *Drosophila* ovary and early embryo. *Zygote* **4**, 159-166.