

# Assembly and dynamics of an anastral:astral spindle: the meiosis II spindle of *Drosophila* oocytes

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## SUMMARY

The meiosis II spindle of *Drosophila* oocytes is distinctive in structure, consisting of two tandem spindles with anastral distal poles and an aster-associated spindle pole body between the central poles. Assembly of the anastral:astral meiosis II spindle occurs by reorganization of the meiosis I spindle, without breakdown of the meiosis I spindle. The unusual disk- or ring-shaped central spindle pole body forms de novo in the center of the elongated meiosis I spindle, followed by formation of the central spindle poles.  $\gamma$ -Tubulin transiently localizes to the central spindle pole body, implying that the body acts as a microtubule nucleating center for assembly of the central poles. Localization of  $\gamma$ -tubulin to the meiosis II spindle is

dependent on the microtubule motor protein, Nonclaret disjunctional (Ncd). Absence of Ncd results in loss of  $\gamma$ -tubulin localization to the spindle and destabilization of microtubules in the central region of the spindle. Assembly of the anastral:astral meiosis II spindle probably involves rapid reassortment of microtubule plus and minus ends in the center of the meiosis I spindle – this can be accounted for by a model that also accounts for the loss of  $\gamma$ -tubulin localization to the spindle and destabilization of microtubules in the absence of Ncd.

Key words: Anastral:astral spindle, Meiosis, Microtubule motor, Ncd,  $\gamma$ -Tubulin

## INTRODUCTION

Meiotic spindle assembly in oocytes of many species occurs by a pathway that differs from that of mitotic spindles in the same organism. Assembly of the meiosis I spindle has been studied in oocytes of organisms that include *Drosophila*, *Xenopus*, and the mouse. Meiosis I spindles of these oocytes are anastral and lack centrosomes, the microtubule organizing centers associated with most mitotic spindles. Meiosis II spindles of *Xenopus* and mouse oocytes are also anastral, and both meiosis I and II spindles contain  $\gamma$ -tubulin, which has been demonstrated to nucleate microtubules in vitro and is thought to constitute the microtubule nucleating activity of centrosomes (Zheng et al., 1995).  $\gamma$ -Tubulin associates with the chromosomes during the early stages of meiosis I spindle assembly in *Xenopus* oocytes and distributes along the length of the spindle fibers in early bipolar spindles, accumulating at the poles in a crescent after prometaphase I spindle elongation (Gard et al., 1995). In meiotic spindles of mouse oocytes,  $\gamma$ -tubulin is present in several patches near the poles (Gueth-Hallonet et al., 1993). The function of  $\gamma$ -tubulin in *Xenopus* and mouse oocyte spindles is probably the same as in mitotic spindles, to nucleate microtubules for spindle assembly, although the distribution of  $\gamma$ -tubulin in the oocyte and mitotic spindles differs. These anastral oocyte meiotic spindles are therefore similar to mitotic spindles in that they contain the same microtubule nucleating activity, even though they lack

centrosomes, the cellular structure with which  $\gamma$ -tubulin is associated in most cells.

Meiosis I spindles of *Drosophila* oocytes differ from those of *Xenopus* and the mouse in that they lack localized  $\gamma$ -tubulin. Attempts to detect  $\gamma$ -tubulin in mature metaphase I-arrested spindles of *Drosophila* oocytes using  $\gamma$ -tubulin antibodies have resulted in no observable staining (Matthies et al., 1996; Tavosanis et al., 1997). Despite this, meiosis I spindles in oocytes of females mutant for the maternal  $\gamma$ -tubulin isoform are disrupted, leading to the hypothesis that  $\gamma$ -tubulin is required to polymerize microtubules for meiosis I spindle assembly (Tavosanis et al., 1997). Direct evidence in support of this hypothesis has yet to be obtained, however, and the observation of spindle fibers and microtubules in the mutant spindles argues against it. Moreover, meiosis I spindle assembly in *Drosophila* oocytes has been observed to proceed by microtubules first associating with the bivalent chromosomes to form a sphere-like array prior to elongating into a tapered spindle (Hatsumi and Endow, 1992b; Theurkauf and Hawley, 1992), and  $\gamma$ -tubulin has not been detected associated with either the chromosomes or microtubules. Nucleation of microtubules for meiosis I spindle assembly could therefore occur by a different mechanism than proposed by this hypothesis, and the role of  $\gamma$ -tubulin could be to stabilize the bipolar spindles following their assembly, rather than to initially nucleate microtubules.

Organization of the chromosome-associated microtubules of

*Drosophila* oocytes into a bipolar meiosis I spindle requires the minus-end microtubule motor protein, Nonclaret disjunctional (Ncd) (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992b; Matthies et al., 1996). Meiosis I spindles form in the absence of Ncd, but the spindles are multi-polar, or broad or diffuse at the poles (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992b). The abnormal meiosis I spindles of *ncd* mutant oocytes, together with Ncd localization to the spindle fibers (Hatsumi and Endow, 1992a), have led to the proposal that Ncd functions like cytoplasmic dynein (Verde et al., 1991) to organize the minus ends of microtubules associated with the bivalent chromosomes into poles (Hatsumi and Endow, 1992a). Time-lapse imaging of spindle assembly in wild-type and mutant oocytes injected with rhodamine-conjugated tubulin to visualize the spindle microtubules (Matthies et al., 1996) has shown that the Ncd motor is required not only for assembly, but also to stabilize the meiosis I spindle following assembly.

Although the general steps in assembly of the *Drosophila* oocyte meiosis I spindle are now apparent, little is known about assembly of the meiosis II spindle. This is likely to differ from meiosis I spindle assembly, since the meiosis I and II spindles differ strikingly in overall structure. The meiosis II spindle consists of two spindles arrayed in tandem with respect to one another (Sonnenblick, 1950). An unusual disk- or ring-shaped structure (Puro, 1991; Riparbelli and Callaini, 1996), the central spindle pole body, is present in the region between the two central spindle poles. The central spindle pole body has been shown to contain the centrosomal protein DMAP190 (also known as CP190) (Riparbelli and Callaini, 1996), implying a centrosome-like function, and is thought to function in organization of the central poles. Analysis of meiotic divisions in live, in vitro-activated *ncd-gfp* oocytes showed that the meiosis II spindle forms without disassembly of the meiosis I spindle (Endow and Komma, 1997).

Despite previous studies on the meiotic spindle and progression of meiosis I to II in *Drosophila* oocytes, several outstanding questions remain. Not only does the origin of the central spindle pole body and its role in spindle assembly remain to be established, but gaps exist in our understanding of meiosis II spindle dynamics. We now report an analysis of meiotic spindle dynamics in live *ncd-gfp* oocytes that reveals the steps in formation of the meiosis II spindle. The time-lapse images, together with analysis of fixed wild-type and *ncd* null mutant oocytes stained for  $\alpha$ - and  $\gamma$ -tubulin, show that the central spindle pole body of the meiosis II spindle of *Drosophila* oocytes forms de novo in the center of the elongated meiosis I spindle just prior to assembly of the meiosis II spindle. The results demonstrate that assembly of the meiosis II spindle is remarkably different from meiosis I spindle assembly. Based on our cytological observations, together with genetic analysis of *ncd* and  $\gamma$ -tubulin mutant interactions, we propose a model for assembly of the meiosis II spindle.

## MATERIALS AND METHODS

### *Drosophila* stocks

The *ncd* mutants used in this study, *ca<sup>nd</sup>*, *ncd<sup>2</sup>* and *ncd<sup>D</sup>*, are described in Lindsley and Zimm (1992) and are in our stock collection. *ca<sup>nd</sup>* and

*ncd<sup>2</sup>* are null alleles of *ncd* (Yamamoto et al., 1989; Endow and Komma, 1997), and *ncd<sup>D</sup>* is a partial loss-of-function allele that shows weak semi-dominant effects on meiotic nondisjunction and loss (Komma and Endow, 1997).  $\gamma$ Tub37C is encoded by *fs(2)TW1* and uncovered by *Df(2L)VA23* (Tavosanis et al., 1997). The  $\gamma$ Tub37C mutants, *fs(2)TW1<sup>APL10</sup>*, *fs(2)TW1<sup>HL2</sup>* and *fs(2)TW1<sup>RU34</sup>*, are EMS-induced maternal-effect female sterile mutants (Schüpbach and Wieshaus, 1989) and were obtained from T. Schüpbach. *Df(2L)VA23* was obtained from the Bloomington Stock Center. The 4-dose *ncd-gfp\** transgenic *Drosophila* carry 4 copies of *ncd* fused to *S65T* mutant *gfp\** (Heim et al., 1995), regulated by the native *ncd* promoter (Endow and Komma, 1997). The 4-dose *ncd-gfp\** flies are homozygous for *ca<sup>nd</sup>* and 2 independent *ncd-gfp\** insertions, *M3M1* and *M9F1*, on chromosomes 2R and 3L, respectively.

### Time-lapse confocal microscopy

Time-lapse confocal microscopy of live 4-dose *ncd-gfp\** oocytes was carried out as described previously (Endow and Komma, 1997) using a Bio-Rad MRC 600 scanning confocal detector mounted on a Zeiss Axiophot microscope and equipped with a krypton/argon laser and a custom GFP filter block (Endow and Komma, 1996). Oocytes were activated in vitro by brief immersion in *Drosophila* PBS (Robb, 1969). The use of freshly mixed *Drosophila* PBS helped ensure progression of the activated oocytes through the meiotic divisions. Images were collected into stacks of 60 at 20 second intervals using the time-lapse feature of Bio-Rad COMOS software and 3 Kalman-averaged slow scans per image. Image stacks were opened in the public domain program, NIH Image v 1.59, and were analyzed by animating the sequences at different speeds.

### Immunocytochemistry and fluorescence microscopy

Normally fertilized wild-type or mutant eggs were collected at 12-15 minute intervals and dechorionated, vitelline membranes were removed, and eggs were fixed in MeOH/EGTA without taxol. Embryos were rehydrated, stained with antibodies and DAPI, and mounted on slides in anti-fade solution, as described (Hatsumi and Endow, 1992b; Endow and Komma, 1997). Antibodies used in these studies were rhodamine-conjugated  $\alpha$ -tubulin antibody,  $\gamma$ -tubulin antibody, anti-Ncd tail antibody (Hatsumi and Endow, 1992a), and FITC-conjugated anti-rabbit IgG. Eggs were scanned under fluorescence using a Leica Dialux 22 epifluorescence microscope equipped with a 100 W Hg light source. A Princeton Instruments PentaMax-1317-K cooled CCD camera mounted on the microscope was used to collect images of chromosomes and spindles. Images of spindles stained with rhodamine or FITC were collected by laser scanning confocal microscopy using the confocal microscope described above. Digital images of chromosomes or spindles collected at several focal planes were superimposed and merged into a single image using Adobe Photoshop v 4.0.1. Merged CCD images of chromosomes were adjusted to the same size as confocal spindle images and were overlaid on the spindle images to determine the positions of the chromosomes in the spindles.

### Genetic tests for mutant interactions

*ncd* mutants were tested for interactions with  $\gamma$ Tub37C mutants by mating females of the appropriate genotypes to *y<sup>2</sup>w<sup>b</sup>/B<sup>SY</sup>* tester males and monitoring offspring for X chromosome nondisjunction and loss, as described previously (Komma et al., 1991). Egg counts were carried out (Endow et al., 1994) to determine the effects of the *ncd* and  $\gamma$ Tub37C mutants on embryo viability. Meiotic nondisjunction and loss result in X/X/Y and/or X/0 offspring, while loss of the X or chromosome 4 during the first 2-3 cleavage divisions gives rise to gynandromorphs or *Minute* mosaics, respectively (Sturtevant, 1929). Embryo inviability is caused by meiotic or mitotic mis-segregation of chromosome 2 or 3, or mitotic loss of the X or chromosome 4 following the first few cleavage divisions. Other effects of mutants on embryogenesis can also contribute to egg inviability.

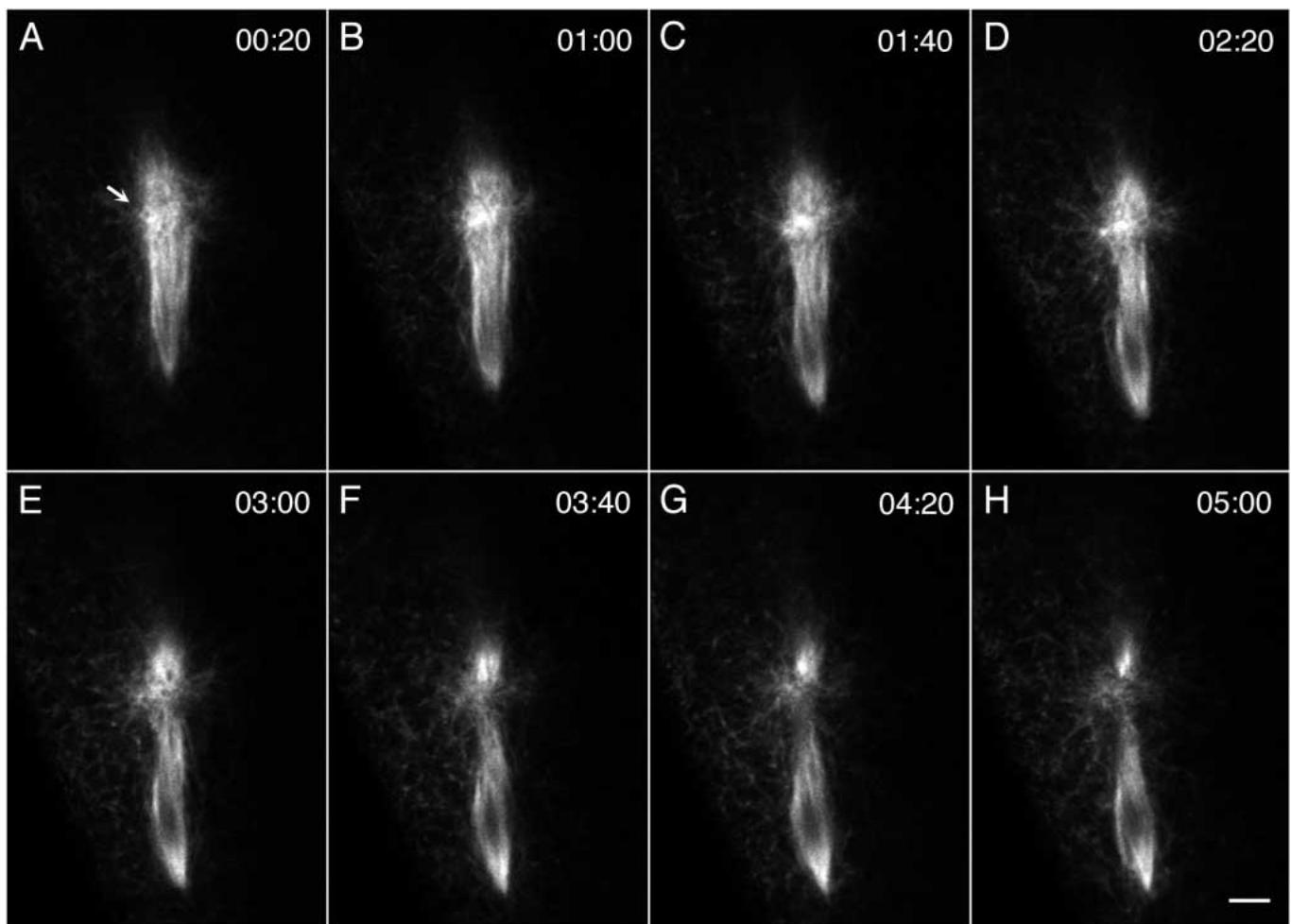
## RESULTS

**Assembly of the *Drosophila* oocyte meiosis II spindle**

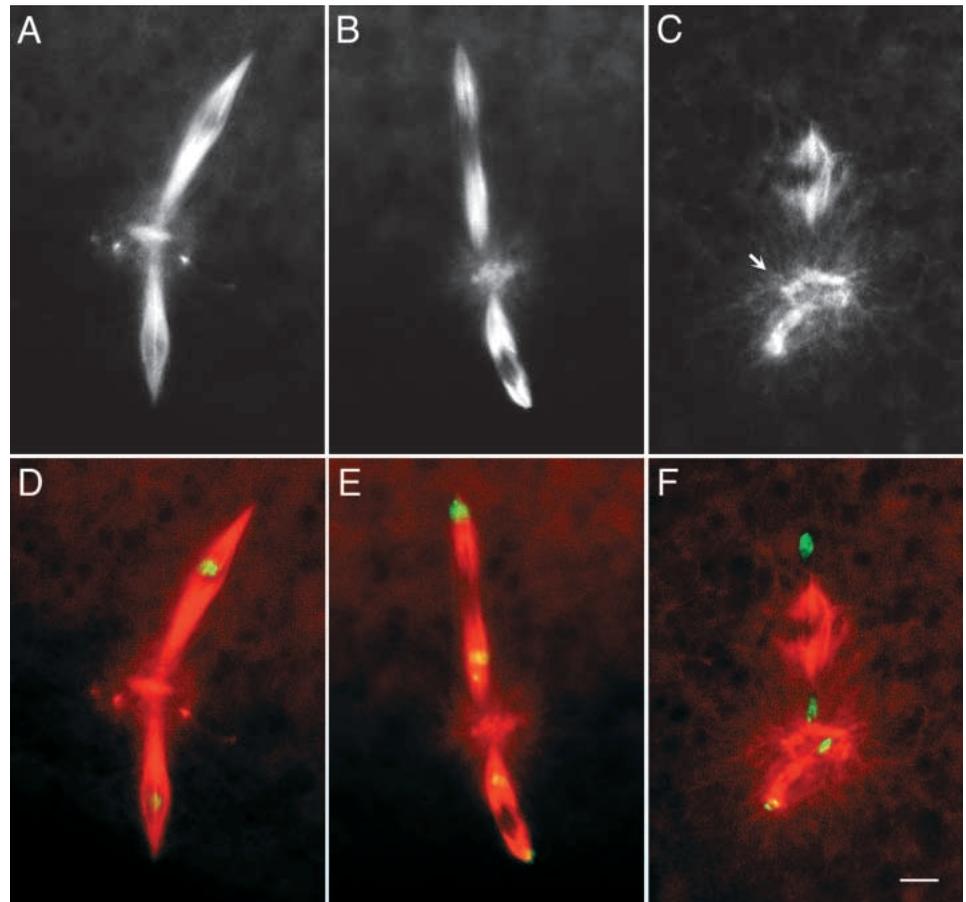
Formation of the meiosis II spindle in live, in vitro-activated *ncd-gfp* oocytes was analyzed using time-lapse laser-scanning confocal microscopy. Mature oocytes were dissected from ovaries of 3-5 day old females and activated by brief immersion in Robb's *Drosophila* PBS (Robb, 1969; Endow and Komma, 1997). Oocytes were mounted under light halocarbon oil onto microscope slides and time-lapse confocal images of Ncd-GFP-decorated meiotic spindles were collected at 20 second intervals. Spindles in oocytes that were favorably oriented for observation clearly showed the transition from meiosis I to II, with assembly of the meiosis II spindle and formation of the central spindle pole body (Fig. 1). Small puckers were first observed in the center of the elongated meiosis I spindle (Fig. 1A, arrow), followed by the pinching out of the puckered

regions from the center of the spindle to form the central spindle pole body (Fig. 1B-D). Assembly of the meiosis II spindle from the meiosis I spindle was rapid, occurring within 1-2 minutes after the puckers in the center of the meiosis I spindle were first observed. An aster of microtubules emanating from the disk- or ring-shaped structure could be observed during assembly of the meiosis II spindle (Fig. 1D). After definition of the central spindle pole body, the spindle microtubules to either side narrowed into poles (Fig. 1D-E) and one of the two tandem spindles of the meiosis II spindle became apparent. The second spindle, deeper in the oocyte, was imaged by quickly focusing down into the oocyte during the time-lapse sequence, then returning to the original plane of focus.

Further differentiation of the central pole region into somewhat narrower poles and transition of the central spindle pole body from a ring into an asterlike structure followed in the later stages of meiosis II (Fig. 1F-H). Disassembly of the



**Fig. 1.** Assembly of the *Drosophila* oocyte meiosis II spindle. Images collected over time following activation of a live *ncd-gfp*\* oocyte show steps in the formation of the meiosis II spindle. Only the spindle closer to the oocyte surface is visible in the plane of focus shown. The more internal spindle was imaged by quickly changing the focus during the time-lapse sequence. The images show small puckers in the center of the elongated meiosis I spindle (A, arrow), the pinching out of microtubules surrounding the puckers to form the disk- or ring-shaped central spindle pole body (B-D), narrowing of the spindle to either side of the body to form the central poles of the two tandem spindles (D-E), further narrowing of the poles and transition of the central spindle pole body into an asterlike array in the final stages of meiosis II (F-H). Astral microtubules associated with the central spindle pole body are especially clear in D. The puckered appearance of the central spindle pole body is still apparent in E. Time in minutes:seconds is shown on each image. Bar, 5  $\mu$ m.



**Fig. 2.** Changes in the meiosis II spindle during the cell cycle. Meiosis II spindles in wild-type eggs, fixed and stained with DAPI and rhodamine-conjugated  $\alpha$ -tubulin antibody. The rhodamine fluorescence is shown at the top (A-C) and the merged DAPI (green) and rhodamine (red) fluorescence at the bottom (D-F). The central spindle pole body appears in metaphase II (A,D) as a bright disk associated with foci of tubulin, and as a ringlike structure in anaphase II (B,E). In early telophase II (C,F), the central spindle pole body (C, arrow) has enlarged into a ring that lies between the two central nuclei. Bar, 5  $\mu$ m.

spindle upon completion of meiosis II occurred by a splintering fragmentation of the spindle microtubules (not shown).

Changes in the central spindle pole body over time were observed in the time-lapse images and correlated with stages of the meiotic cell cycle by staining fixed early eggs for DNA and tubulin to visualize the chromosomes and spindles. After its formation in late meiosis I, the central spindle pole body of the metaphase II spindle appears as a thick disk between the two central spindle poles, associated with bright foci of tubulin and a faint microtubule aster (Fig. 2A,D). During anaphase II, the central spindle pole body is present as a disk or ring of bright tubulin foci with an array of astral microtubules (Fig. 2B,E). In early telophase II, the ring (Fig. 2C, arrow) has enlarged in size in the plane perpendicular to the spindle midbodies, and is associated with emanating microtubules (Fig. 2C,F).

#### $\gamma$ -Tubulin localization to the meiosis II spindle

Fixed wild-type early eggs were stained with rhodamine-conjugated anti- $\alpha$ -tubulin antibody and an antibody specific for the maternal  $\gamma$ -tubulin at 37C on chromosome 2 to determine the distribution of  $\gamma$ -tubulin in the meiosis II spindle. Spindles in different stages of meiosis II are shown in Fig. 3 stained for  $\alpha$ -tubulin (Fig. 3A-C) and  $\gamma$ -tubulin (Fig. 3D-F). The overlaid images show the  $\alpha$ -tubulin staining in red,  $\gamma$ -tubulin in green, and DAPI-stained chromosomes in blue (Fig. 3G-I). The superimposed  $\alpha$ - and  $\gamma$ -tubulin staining appears yellow.

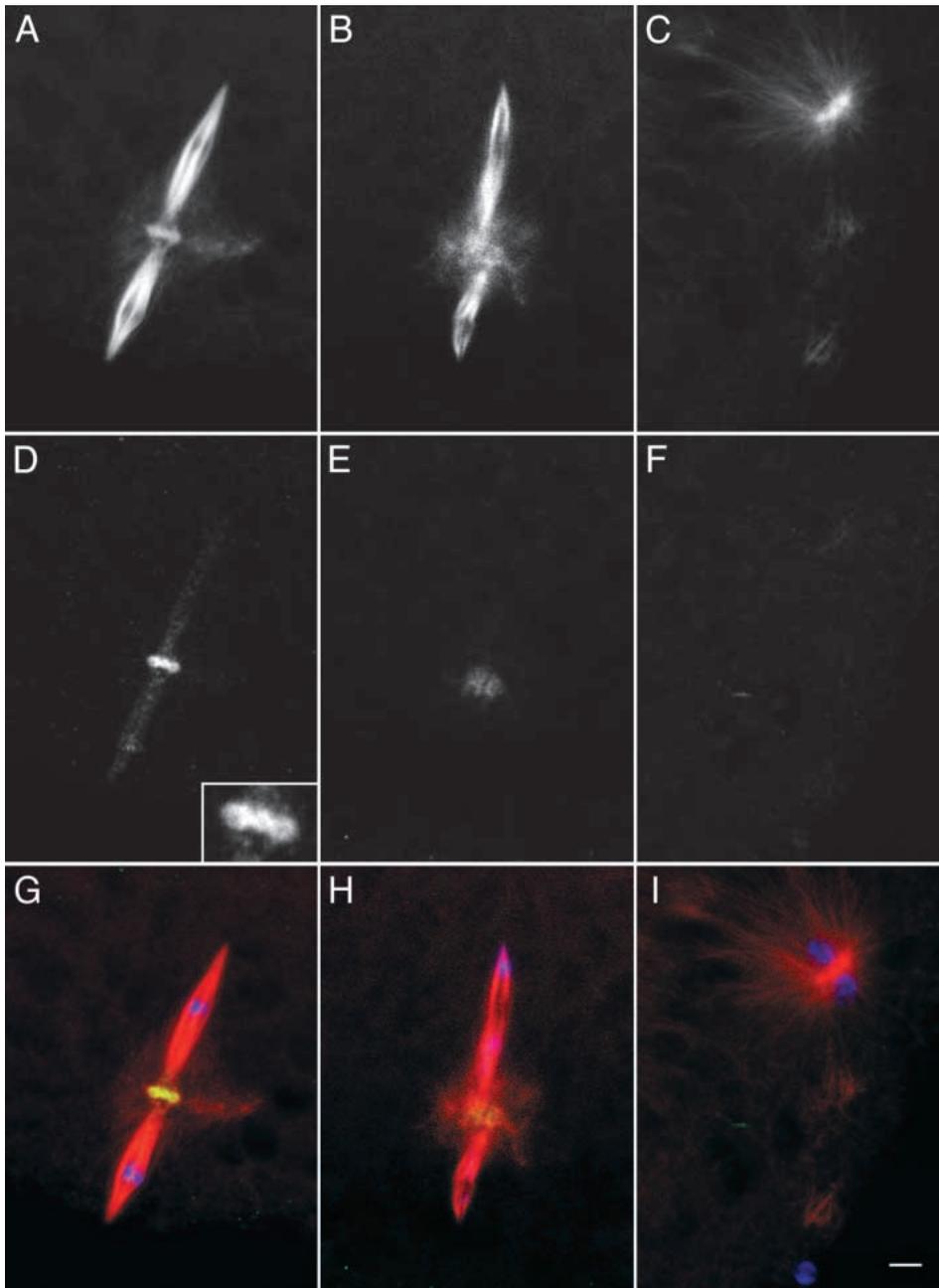
The maternal  $\gamma$ -tubulin is localized to the central spindle pole body of the metaphase II spindle (Fig. 3D,G), where it is present in a puckered disk or ring (Fig. 3D, inset). The  $\gamma$ -

tubulin staining is restricted to the central spindle pole body – it is not present, or is present in greatly reduced amounts on the spindle microtubules and distal poles of the meiosis II spindle. The  $\gamma$ -tubulin staining remains in the form of puckers in the central pole region in anaphase II (Fig. 3E,H), but it is less tightly localized and diminished in amount. It is barely detectable in late telophase II (Fig. 3F,I), when the central spindle pole body appears as a series of bright foci of tubulin between the central pair of nuclei that are formed upon completion of the meiosis II division (Fig. 3C,I).

The specificity of the  $\gamma$ -tubulin antibody used in these studies was confirmed by the staining of mitotic spindles from early embryos on the same slide as the meiosis II eggs. The spindle fibers, centrosomes, and asters of the mitotic spindles were brightly stained with  $\alpha$ -tubulin antibody, but the staining of mitotic spindles by the  $\gamma$ -tubulin antibody was restricted to the centrosomes. This is consistent with the staining of centrosomes by  $\gamma$ Tub37C antibody reported previously (Wilson et al., 1997) and the enrichment of  $\gamma$ -tubulin in the *Drosophila* centrosome determined biochemically (Moritz et al., 1995).

#### $\gamma$ -Tubulin distribution in *ncd* mutant eggs

A previous study showed that Ncd is associated with the meiosis I and II spindles in spindle/chromosome preparations (Hatsumi and Endow, 1992a). This has been confirmed by visualization of Ncd-GFP fluorescence associated with the meiosis I and II spindles, including the developing central spindle pole body, in live *ncd-gfp* oocytes (Endow and Komma, 1997) (Fig. 1). Antibody staining experiments for the present study showed



**Fig. 3.** Localization of  $\gamma$ -tubulin to the meiosis II spindle. Meiosis II spindles in wild-type eggs, fixed and stained with  $\alpha$ - and  $\gamma$ -tubulin antibodies and DAPI. The  $\alpha$ -tubulin staining is shown at the top (A-C), the  $\gamma$ -tubulin staining in the middle (D-F), and the merged  $\alpha$ -tubulin (red),  $\gamma$ -tubulin (green) and DAPI (blue) images at the bottom (G-I). The superimposed  $\alpha$ - and  $\gamma$ -tubulin staining appears yellow. The  $\gamma$ -tubulin is present in the central spindle pole body in metaphase II (A,D,G). The inset in D is enlarged by  $\times 2.3$  to show the puckered appearance of the staining. The faint outline of the spindle is probably due to bleedthrough from the rhodamine channel, but could be due to dispersed  $\gamma$ -tubulin distribution. The  $\gamma$ -tubulin staining is diminished in anaphase II (B,E,H) and barely detectable in late telophase II (C,F,I). Bar, 5  $\mu$ m.

that Ncd localization to the central spindle pole body in fixed wild-type eggs is detectable in anaphase II, but greatly diminished by telophase II (not shown). Despite the diminished central spindle pole body staining by Ncd in telophase II, Ncd staining of the meiosis II spindle fibers and midbody remained bright in telophase II. Bright fluorescence of telophase II spindle microtubules and the midbody was also observed in live activated *ncd-gfp* oocytes. These results indicate that localization of Ncd to the central spindle pole body is dependent on the meiotic cell cycle, paralleling that of  $\gamma$ Tub37C.

*ncd* mutant eggs were stained for  $\gamma$ Tub37C to determine the effect of absence of Ncd on  $\gamma$ -tubulin localization. The *ncd* mutant used in these experiments was *ca<sup>nd</sup>* (Lewis and Gencarella, 1952), a null allele caused by deletion of the promoter and 5' end of the *ncd* gene which does not produce

detectable *ncd* RNA (Yamamoto et al., 1989). The meiosis II spindles of *ca<sup>nd</sup>* eggs were highly abnormal and did not show detectable labeling by  $\gamma$ Tub37C antibody. The inability to detect  $\gamma$ Tub37C staining could be due to mis-localization of  $\gamma$ Tub37C or to the disruption of spindle structure in the abnormal meiotic spindles that are present in *ca<sup>nd</sup>* mutant eggs. However, centrosomes associated with early (cycle 1-8) mitotic spindles of *ca<sup>nd</sup>* embryos on the same slides also showed faint or barely detectable staining by  $\gamma$ -tubulin antibody, indicating a correlation between diminished  $\gamma$ Tub37C localization to the centrosome and absence of Ncd in the *ca<sup>nd</sup>* embryos.

#### Destabilization of microtubules in *ncd* mutant spindles

The fixed, antibody-stained *ca<sup>nd</sup>* eggs that were observed

**Table 1. Dominant enhancement of *ncd<sup>D</sup>* by a mutant of  $\gamma$ Tub37C**

Female parent	Total <i>M</i> <sup>+</sup> gametes	Gametic		Zygotic X loss	Total adults*	Total embryos	Embryo viability	Total mis-seg
		X nd	X loss					
1. <i>ncd<sup>D</sup>/+</i>	2,511	0.004	<0.001	<0.001	2,506	2,874	0.872	0.004
2. <i>ncd<sup>D</sup>/ncd<sup>D</sup></i>	3,550	0.028	0.002	0.002	3,724	5,123	0.727	0.031
3. <i>fs(2)TW1<sup>HL2</sup>/+</i>	2,556	0.003	0.002	<0.001	2,552	3,337	0.765	0.005
4. <i>fs(2)TW1<sup>HL2</sup>/+;</i> <i>ncd<sup>D</sup>/+</i>	1,949	0.002	0.001	<0.001	1,947	2,437	0.799	0.003
5. <i>fs(2)TW1<sup>HL2</sup>/+;</i> <i>ncd<sup>D</sup>/ncd<sup>D</sup></i>	1,649	0.181	<0.001	0.007	1,667	6,588	0.253	0.187
6. <i>Df(2L)VA23/+</i>	1,859	<0.001	<0.001	<0.001	1,859	2,229	0.834	<0.001
7. <i>Df(2L)VA23/+;</i> <i>ncd<sup>D</sup>/+</i>	1,272	0.006	<0.001	<0.001	1,271	1,715	0.741	0.006
8. <i>Df(2L)VA23/+;</i> <i>ncd<sup>D</sup>/ncd<sup>D</sup></i>	386	0.026	<0.001	<0.001	393	808	0.486	0.026
9. <i>+/+</i>	2,086	<0.001	<0.001	<0.001	2,086	2,324	0.898	<0.001

\*Including *M* offspring

Females of the indicated genotypes were tested for embryo viability and X chromosome segregation by mating to tester  $\gamma^2w^{bf}/B^S Y$  males. Wild-type (*+/+*) females are Oregon R. Total mis-segregation (mis-seg) refers to gametic + zygotic mis-segregation of the X chromosome. nd, nondisjunction; *M*, Minute.

contained depolymerizing microtubules in the region of the meiosis II spindle where the central spindle pole body forms in wild-type eggs. A haze of fragmented microtubules is present in the center of the metaphase II spindle shown in Fig. 4. The distal ends of the spindle are split into two or three poles, and spindle spurs are associated with a chromosome that has partially detached from the spindle at the top. Fragmentation of meiotic spindle microtubules was observed in previous studies of live, activated *ncd<sup>2</sup>-gfp* oocytes and fixed *ncd<sup>2</sup>-gfp* embryos (Endow and Komma, 1997), but it was not certain whether destabilization of spindle fibers was due to loss of *ncd* function or to the slight semi-dominant gain of function of *ncd<sup>2</sup>*. The present results show that depolymerization of meiosis II spindle fibers is caused by loss of *ncd* function. This implies that wild-type Ncd is needed to stabilize spindle microtubules for completion of the meiotic divisions.

#### Genetic interactions between *ncd* and $\gamma$ Tub37C

Three EMS-induced  $\gamma$ Tub37C mutants, *fs(2)TW1<sup>APL10</sup>*, *fs(2)TW1<sup>HL2</sup>* and *fs(2)TW1<sup>RU34</sup>*, and a deficiency that uncovers  $\gamma$ Tub37C, *Df(2L)VA23*, were tested for genetic interactions with the *ncd* mutants, *ca<sup>nd</sup>*, *ncd<sup>2</sup>* and *ncd<sup>D</sup>*. *ca<sup>nd</sup>*, described above, is an *ncd* null mutant caused by partial deletion of *ncd*, *ncd<sup>2</sup>* is a missense mutant that shows complete loss of function (Endow and Komma, 1997), and *ncd<sup>D</sup>* is a partial loss-of-function mutant that causes meiotic chromosome nondisjunction and loss, but is near wild type with respect to mitotic chromosome distribution (Komma et al., 1991).

Females homozygous for *ca<sup>nd</sup>* or *ncd<sup>2</sup>* produce the same or almost the same number of eggs as wild-type females, but ~90% of the mutant eggs fail to hatch (Endow and Komma, 1997). *ca<sup>nd</sup>* or *ncd<sup>2</sup>* females heterozygous for the  $\gamma$ Tub37C mutant alleles, *fs(2)TW1<sup>HL2</sup>* and *fs(2)TW1<sup>RU34</sup>*, were sterile – the females laid eggs but >99% of the eggs did not hatch. Tests of *ca<sup>nd</sup>* or *ncd<sup>2</sup>* females heterozygous for the  $\gamma$ Tub37C deficiency, *Df(2L)VA23*, showed embryo inviability of ~90%, indicating that the enhanced inviability caused by *fs(2)TW1<sup>HL2</sup>* and *fs(2)TW1<sup>RU34</sup>* requires the mutant  $\gamma$ Tub37C protein. The

enhanced inviability does not require Ncd, however, since it is observed with *ca<sup>nd</sup>*, a deficiency allele of *ncd*.

Results of tests of *ncd<sup>D</sup>* with a  $\gamma$ Tub37C mutant are shown in Table 1. *ncd<sup>D</sup>* females heterozygous for *fs(2)TW1<sup>HL2</sup>* (cross 5) showed a strong dominant enhancement of the *ncd<sup>D</sup>* effect on meiotic nondisjunction (cross 2), resulting in a ~6-fold increased frequency of nondisjunctional offspring. Embryo viability was also greatly reduced compared to *ncd<sup>D</sup>* alone. Tests of *ncd<sup>D</sup>* females heterozygous for *Df(2L)VA23* (cross 8) showed reduced embryo viability, but no significant difference in meiotic or mitotic chromosome distribution compared to *ncd<sup>D</sup>* females (cross 2). These results indicate that the enhancement of *ncd<sup>D</sup>* meiotic nondisjunction by *fs(2)TW1<sup>HL2</sup>* requires the mutant  $\gamma$ Tub37C protein. The interaction between *ncd<sup>D</sup>* and *fs(2)TW1<sup>HL2</sup>* is allele-specific with respect to  $\gamma$ Tub37C – *fs(2)TW1<sup>RU34</sup>/+*; *ncd<sup>D</sup>/ncd<sup>D</sup>* caused only a small increase (0.050) in meiotic nondisjunction compared to *ncd<sup>D</sup>* (0.028), while tests of *fs(2)TW1<sup>APL10</sup>/+*; *ncd<sup>D</sup>/ncd<sup>D</sup>* resulted in a ~5-fold increase in nondisjunctional offspring compared to *ncd<sup>D</sup>*, similar to that observed for *fs(2)TW1<sup>HL2</sup>/+*; *ncd<sup>D</sup>/ncd<sup>D</sup>*.

*fs(2)TW1<sup>APL10</sup>/+*; *ncd<sup>D</sup>/+* females further produced a frequency of 0.038 nondisjunctional offspring (not shown) compared to 0.004 for *ncd<sup>D</sup>/+* females (cross 1). This effect, known as extragenic noncomplementation (Regan and Fuller, 1988; Stearns and Botstein, 1988), was not observed for *fs(2)TW1<sup>RU34</sup>/+*; *ncd<sup>D</sup>/+* (not shown) or *fs(2)TW1<sup>HL2</sup>/+*; *ncd<sup>D</sup>/+* (cross 4). It was also not observed for *Df(2L)VA23/+*; *ncd<sup>D</sup>/+* (cross 7) or *fs(2)TW1<sup>APL10</sup>/+*; *ca<sup>nd</sup>/+* (not shown), indicating that the effect requires both the mutant  $\gamma$ Tub37C and Ncd proteins.

These results can be summarized as follows. The  $\gamma$ Tub37C mutants, *fs(2)TW1<sup>HL2</sup>* and *fs(2)TW1<sup>APL10</sup>*, cause a strong dominant enhancement of the effect of *ncd<sup>D</sup>* on meiotic nondisjunction, while *fs(2)TW1<sup>RU34</sup>* shows only a weak effect. These allele-specific genetic interactions are not observed with a deficiency of  $\gamma$ Tub37C, *Df(2L)VA23*, indicating that the enhancement of *ncd<sup>D</sup>* by  $\gamma$ Tub37C requires the mutant  $\gamma$ Tub37C protein. *fs(2)TW1<sup>APL10</sup>* also shows extragenic

noncomplementation of *ncd<sup>D</sup>*, an effect that is not observed when one or the other mutant is replaced by a deficiency allele, indicating a requirement for both mutant proteins.

## DISCUSSION

### A hybrid anastral:astral spindle

Several differences exist between the meiosis I and II spindles of *Drosophila* oocytes. The meiosis I spindle is anastral with narrow, tapered spindle poles (e.g. see Figs 1 and 2 of Endow and Komma, 1997), while the meiosis II spindle consists of two tandem spindles that lack asters at the distal poles but contain an unusual aster-associated structure, the central spindle pole body, between the two central poles. The central spindle pole body is disk- or ring-shaped and associated with the centrosomal proteins DMAP190 (CP190) (Riparbelli and Callaini, 1996) and  $\gamma$ -tubulin. The finding of  $\gamma$ -tubulin localized to the central spindle pole body implies that the body probably functions as a microtubule nucleating and organizing center. The *Drosophila* oocyte meiosis II spindle is therefore an unusual hybrid anastral:astral spindle consisting of distal anastral spindle poles and central astral poles that are associated with a novel microtubule organizing center.

### Assembly of the meiosis II spindle

The differences in meiosis I and II spindle structure probably reflect differences in spindle assembly. The meiosis I spindle assembles from short microtubules associated with the meiotic chromosomes. Spindle assembly requires the Ncd microtubule motor protein, which is required to form and maintain spindle poles. Ncd has been proposed to crosslink the microtubules associated with the bivalent chromosomes and focus the microtubules into poles (Hatsumi and Endow, 1992a). Multipolar spindles are observed in *ncd* null mutant oocytes and oocytes mutant for the partial loss-of-function motor protein, *Ncd<sup>D</sup>*, which has a slower velocity of movement than wild-type Ncd (Moore et al., 1996). Thus, both the minus-end motility of Ncd and its crosslinking activity are probably needed to focus microtubules into spindle poles for the meiosis I spindle.

The meiosis II spindle undergoes assembly by reorganization of the meiosis I spindle fibers without disassembly of the meiosis I spindle, and the meiosis I spindle poles become the distal poles of the new meiosis II spindle (Endow and Komma, 1997). The disk- or ring-shaped central spindle pole body of the meiosis II spindle forms de novo in the center of the meiosis I spindle just prior to assembly of the meiosis II spindle. The central spindle pole body has been observed by other workers (Puro, 1991; Riparbelli and Callaini, 1996), but its origin and role in spindle assembly have not been reported previously. The first step in formation of the central spindle pole body is the appearance of puckers in the center of the meiosis I spindle, followed by the pinching out from the spindle of a disk or ring of microtubules that becomes the central spindle pole body. The manner in which the central spindle pole body forms suggests the involvement of a microtubule motor. If so, the motor involved is likely to be different than Ncd, since loss of Ncd function does not seem to prevent its formation (Endow and Komma, 1997). Following the formation of the central spindle pole body, the microtubules

to either side of the body narrow into poles, forming the mature meiosis II spindle. The central poles become more tapered during progression through meiosis II, and the central spindle pole body also changes in morphology – the disk or ring becomes asterlike, then enlarges into a ring that lies between the two central telophase II nuclei.

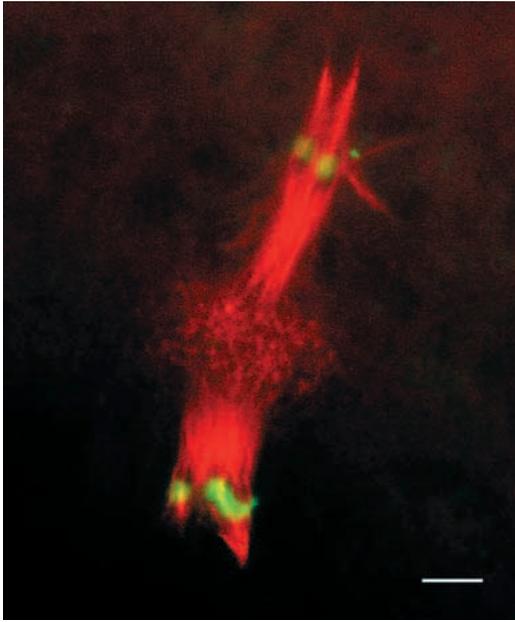
### $\gamma$ -Tubulin localization to the meiosis II spindle

Failure to detect  $\gamma$ -tubulin in the metaphase I-arrested meiotic spindle of *Drosophila* oocytes has been reported by others (Matthies et al., 1996; Tavosanis et al., 1997), an indication that  $\gamma$ -tubulin is either not present in the spindle or present in low amounts. The latter possibility is supported by the abnormal meiotic spindles observed in oocytes mutant for  *$\gamma$ Tub37C* (Tavosanis et al., 1997). The maternal  $\gamma$ -tubulin encoded at 37C can, however, be detected in the meiosis II spindle where it is localized to the central spindle pole body, as demonstrated by antibody staining of fixed eggs. Localization of  $\gamma$ -tubulin to the meiosis II spindle has also been observed by others (Riparbelli and Callaini, 1998) (A. MacQueen, A. Brent and T. Hazelrigg, personal communication). Staining by  $\gamma$ -tubulin of the metaphase II, but not the metaphase I spindle implies that  $\gamma$ -tubulin is redistributed in the spindle or recruited to the spindle at some time between metaphase I and II, probably in late anaphase I, based on changes in the spindle observed in live oocytes. The puckers that form in the center of the elongated meiosis I spindle of live oocytes resemble the puckered  $\gamma$ -tubulin localization to the central spindle pole body of the metaphase II spindle, and probably reflect redistribution of  $\gamma$ -tubulin in the spindle or recruitment to the spindle.  $\gamma$ -Tubulin does not localize to the distal poles of the metaphase II spindle which are retained from the meiosis I spindle, consistent with the failure by others to localize  $\gamma$ -tubulin in the meiosis I spindle.

$\gamma$ -Tubulin shows a transient, cell cycle-dependent association with the central spindle pole body of the meiosis II spindle – it is present in metaphase II but absent by late telophase II. The cell cycle dependence of  $\gamma$ -tubulin localization to the central spindle pole body is paralleled by Ncd, which is present in metaphase II but greatly diminished by telophase II. Localization of  $\gamma$ -tubulin to the central spindle pole body appears to be dependent on Ncd, since no  $\gamma$ -tubulin staining is observed in meiosis II spindles of the *ncd* null mutant, *ca<sup>nd</sup>*. This could be due to the extremely disrupted spindles observed in *ca<sup>nd</sup>* eggs, however, the staining by  $\gamma$ -tubulin of centrosomes of mitotic spindles is also greatly reduced in *ca<sup>nd</sup>* early embryos compared to wild type. These findings suggest that Ncd is needed to recruit or anchor  $\gamma$ -tubulin to the central spindle pole body, as well as to the centrosomes of early mitotic spindles. The function of  $\gamma$ -tubulin in the central spindle pole body is probably to nucleate microtubules for central spindle pole formation.  $\gamma$ -Tubulin may also play a role in spindle pole maintenance by continuing to nucleate microtubule growth until anaphase is completed.

### Destabilization of microtubules by *ncd* mutants

Microtubules of *ca<sup>nd</sup>* meiotic spindles undergo depolymerization in the center of the meiosis II spindle, where the central spindle poles form in wild-type eggs. Fragmentation of meiotic spindle fibers was observed previously in live *ncd<sup>2-</sup>*



**Fig. 4.** Destabilization of meiosis II spindle microtubules in an *ncd* null mutant egg. Metaphase II spindle in a *ca<sup>nd</sup>* egg, fixed and stained with rhodamine-conjugated  $\alpha$ -tubulin antibody (red) and DAPI (green). A cloudlike haze of microtubule fragments is present in the central region of the spindle. The mutant spindle is multipolar and microtubule spurs are associated with a partially detached chromosome (top). Bar, 5  $\mu$ m.

*gfp* mutant oocytes activated by brief immersion in *Drosophila* PBS and analyzed by time-lapse imaging, and in fixed *ncd<sup>2</sup>-gfp* mutant embryos (Endow and Komma, 1997). Depolymerization of microtubules in the central region of the spindle of some live *ncd<sup>2</sup>-gfp* mutant oocytes caused the spindle poles to move far away from one another and to become widely separated as the meiotic divisions proceeded (Endow and Komma, 1997). The present results indicate that microtubule destabilization is caused by loss of *ncd* function, and is not specific to *ncd<sup>2</sup>* or *ncd<sup>2</sup>-gfp*. These findings imply that wild-type Ncd acts to stabilize the microtubules in the center of the spindle for formation of the central poles.

### Interactions between Ncd and $\gamma$ -Tubulin

The proposed recruitment or anchoring of  $\gamma$ -tubulin to the spindle by Ncd could be direct or indirect, i.e. mediated by other proteins. However, the genetic interactions of *ncd<sup>D</sup>* with  $\gamma$ Tub37C mutants reported here imply that Ncd interacts directly with  $\gamma$ Tub37C. *Ncd<sup>D</sup>* contains a missense mutation that causes the motor to bind to microtubules more weakly than wild type (Moore et al., 1996). The three EMS-induced  $\gamma$ Tub37C mutants show allele-specific interactions with *ncd<sup>D</sup>* – *fs(2)TW1<sup>HL2</sup>* and *fs(2)TW1<sup>APL10</sup>* cause a strong dominant enhancement of the effect of *ncd<sup>D</sup>* on meiotic nondisjunction, while *fs(2)TW1<sup>RU34</sup>* shows only a weak effect. The allele-specificity of the interaction is an indication of protein-specific interactions. The genetic interactions observed require the mutant  $\gamma$ Tub37C protein, since a deficiency that uncovers  $\gamma$ Tub37C, *Df(2L)VA23*, shows little or no effect on meiotic nondisjunction of *ncd<sup>D</sup>*. *fs(2)TW1<sup>APL10</sup>* further shows extragenic noncomplementation of *ncd<sup>D</sup>*, a mutant effect that



**Fig. 5.** Reassortment of microtubules in the center of the meiosis I spindle for assembly of the meiosis II spindle. The meiosis I and II spindle microtubules are assumed to be oriented with minus ends at the poles and plus ends near the chromosomes. Formation of meiosis II spindle poles in the center of the meiosis I spindle would require a ‘sorting out’ of microtubule ends so that minus ends replace plus ends in the center of the spindle. Only the polarity of microtubule ends in the center of the meiosis I spindle is indicated. +, plus; –, minus.

is not observed with the other two  $\gamma$ Tub37C mutants, that requires the mutant  $\gamma$ Tub37C and Ncd proteins, and one that also indicates protein:protein interactions.

### A model for assembly of the meiosis II spindle

The orientation of microtubules in meiotic spindles has not been carefully investigated, but is assumed to be with minus ends at the poles and plus ends near the chromosomes, the same as mitotic spindles that have been examined (Euteneuer and McIntosh, 1981; Telzer and Haimo, 1981). If so, formation of new spindle poles in the center of the meiosis I spindle must involve a ‘sorting out’ of the microtubules to position microtubule minus ends in the center of the spindle where the plus ends formerly resided (Fig. 5). The reassembly of microtubules must also be rapid to permit assembly of meiosis II spindles within the brief time observed. How does this occur?

A model for assembly of the *Drosophila* oocyte meiosis II spindle is the following.  $\gamma$ -Tubulin is first recruited or relocalized, possibly as  $\gamma$ -TuRC (Zheng et al., 1995), to the midbody of the meiosis I spindle, where it functions to nucleate microtubules for formation of the meiosis II central spindle poles. The loss of  $\gamma$ -tubulin localization to the spindle in the absence of Ncd suggests that the Ncd motor serves to recruit or anchor  $\gamma$ -tubulin to the center of the spindle. The Ncd motor would then stabilize newly nucleated microtubule minus ends and focus the microtubules into poles. The unstabilized plus ends of microtubules in the center of the spindle would undergo rapid depolymerization as a consequence of dynamic instability (Belmont et al., 1990). Stabilization of the newly nucleated microtubule minus ends and depolymerization of the plus ends would cause a rapid ‘sorting out’ of the microtubules in the center of the meiosis I spindle, replacing microtubule plus ends with minus ends. The distal poles of the meiosis II spindle would be retained from the meiosis I spindle and maintained by the same forces that originally formed them, the crosslinking activity and minus-end movement of Ncd along spindle microtubules.

This model accounts for the loss of  $\gamma$ -tubulin localization to the spindle and destabilization of microtubules in the central region of meiotic spindles in the absence of Ncd. It is also consistent with the genetic interactions that we observe between *ncd<sup>D</sup>* and  $\gamma$ Tub37C mutants. An important feature of the model is that it explains how rapid reassembly of microtubules in the center of the meiosis I spindle could occur during assembly of the meiosis II spindle. Some aspects of the

model are supported by observations in other systems, e.g.  $\gamma$ -tubulin has been reported to localize to the midbody of mitotic spindles in cultured mammalian cells (Julian et al., 1993) and microtubule-associated proteins are known to modulate microtubule dynamic instability in vitro (Pryer et al., 1992). Further studies are needed to explain how  $\gamma$ -tubulin is recruited or relocalized to the central spindle pole body, and to identify other proteins involved in meiotic spindle assembly. These studies will provide important information not only about spindle assembly in *Drosophila* oocytes, but also about the assembly of centrosomes and other microtubule nucleating and organizing centers.

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