The meiotic spindle of the *Drosophila* oocyte: the role of Centrosomin and the central aster

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Summary

We provide here the first evidence that a distinct midzone is present in the *Drosophila melanogaster* female meiosis I spindle. This region has the ability to bind the Pavarotti kinesin-like (PAV-KLP) and Abnormal spindle (Asp) proteins, indicating a correct organization of the central spindle microtubules. We also identified the core component centrosomal protein centrosomin (CNN) at an unexpected site within the anaphase I spindle, indicating a role for CNN during the biogenesis of the female meiotic apparatus. However, there are no apparent defects in the midzone organization of *cnn* oocytes, whereas defects occur later when the central aster forms. The primary mutant phenotype of *cnn* oocytes is the failure to form a developed central microtubule organizing center (MTOC), although twin meiosis II spindles usually do form. Thus the central MTOC may not be essential for the formation of the inner poles of twin meiosis II spindles, as generally proposed, but it might be involved in maintaining their proper spacing. We discuss the proposal that, in the presence of a central MTOC, a chromatin-driven mechanism of spindle assembly like that described during meiosis I may control the morphogenesis of the twin meiosis II spindles.

Key words: *Drosophila*, Female meiosis, Spindle midzone, *centrosomin*, *pavarotti*, *abnormal spindle*

Introduction

Eighty years ago, the pioneering studies of Huettner (Huettner, 1924) on the maturation of the *Drosophila melanogaster* oocyte revealed that chromosome segregation during the first meiosis is supported by a peculiar spindle apparatus that transforms during the second meiosis into twin spindles arranged in tandem and disposed perpendicularly to the longitudinal axis of the egg. These spindles ensure the reductional divisions and the formation of the haploid complements, the innermost of which is the female pronucleus. Huettner (Huettner, 1924) first observed that the meiotic spindles are anastral and lack centrioles at their poles, though they are tapered. Despite the potential of these observations for clarifying acentrosomal pathways of microtubule (MT) organization, the female meiotic apparatus of *Drosophila* has received little attention for many years. During the last decade some researches have concentrated their attention on the morphogenesis and organization of the spindle during metaphase of the first meiotic division. Immunohistochemical analysis failed to reveal centrosomal components, such as CP60, CP190 and γ-tubulin, at the spindle poles (Matthies et al., 1996), confirming, in its essential aspects, the general description of Huettner (Huettner, 1924). The role of γ-tubulin during meiosis is, however, controversial since mutants for this gene have abnormal first meiotic spindles (Tavosanis et al., 1997), but meiosis is not terminally arrested and appears mostly normal (Wilson and Borisy, 1998; Llazamares et al., 1999). Several lines of evidence suggest that meiotic spindle assembly in the *Drosophila* female begins with a chromosome-driven mechanism of MT organization (Theurkauf and Hawley, 1992; McKim and Hawley, 1995). Mutational analysis indicates that, in the absence of centrosomes, MT bundling and bipolarity of anastral meiotic spindles requires kinesin-like proteins with minus-end-directed MT motor activity. Defects in the genes *nonclaret disjunctional* (*ncd*) (Matthies et al., 1996; Endow and Komma, 1997) and *subito* (*sub*) (Giunta et al., 2002), which encode kinesin-like proteins, lead to the formation of abnormal meiotic I spindles with frayed or undefined poles. The cooperative interaction between motor proteins and the products of the genes *mini spindles* (*msps*) and *transforming acidic coiled-coil protein* (*tacc*), localized at the acentrosomal poles of the first meiotic spindle (Cullen and Ohkura, 2001), might be crucial for the bipolarity of the meiotic spindle. The MT minus-end-associated Abnormal spindle (Asp) protein, localized at the extremities of the meiotic spindle, could be also involved in the stabilization of focused poles (Riparbelli et al., 2002).

Oocytes are activated to resume meiosis by passage through the oviduct (Doane, 1960; Mahowald et al., 1983; Page and Orr-Weaver, 1997; Heifetz et al., 2001). The spindle, positioned parallel to the cortex during metaphase arrest (Theurkauf and Hawley, 1992; White-Cooper et al., 1993; Matthies et al., 1996), reorients perpendicular to the oocyte surface (Endow and Komma, 1997) during completion of meiosis I. The spindle elongates during anaphase-telophase of the first meiosis and transforms during meiosis II into two tandemly arranged spindles (Huettner, 1924). Although the meiotic II spindles are anastral, an unusual structure, from which an extensive array of MTs nucleates, forms between the two internal spindle poles (Riparbelli and

Callaini, 1996). It has been shown that this structure, in contrast to the poles of the meiotic spindles, contains several centrosomal proteins usually found at the mitotic spindle poles, such as γ-tubulin (Endow and Komma, 1998), CP60 and CP190 (Riparbelli and Callaini, 1996; Brent et al., 2000) and centrosomin (CNN) (Llamazares et al., 1999). However, although this central MTOC contains centrosomal components and is able to nucleate an astral array of MTs, it lacks centrioles, is unable to duplicate and disappears after the completion of meiosis.

Whereas previous analyses provided insight into the mechanisms of spindle assembly and chromosome segregation during metaphase of the first meiosis, they provided no clear information on the kinetics of spindle morphogenesis during transition from meiosis I to II. This aspect was investigated by time-lapse analysis of *ncd-gfp* oocytes (Endow and Komma, 1998). The main event of the transforming of meiotic I spindle in twin tandem arranged meiotic II spindles is the formation in the elongated meiotic I spindle of lateral puckers, which are correlated with the organization of the central MTOC (Endow and Komma, 1998). However, despite the usefulness of this elegant in vivo approach, some aspects, such as the individualization of the twin spindles and the origin and function of the central MTOC, remain unclear. The findings presented in this report both expand and clarify earlier studies on the structural organization and dynamics of the female meiotic spindle, suggesting an alternative model of meiosis II spindle assembly.

Materials and Methods

Fly stocks

Oregon-R and cnn^{HK}/*CyO* flies were used as wild type. The cnn^{HK} allele has been described previously (Schupbach and Wieschaus, 1989). The cnn^{E2} allele was obtained following ethylmethane sulfonate (EMS) mutagenesis of male flies, and the small deficiency *Df(2R)8-104* was recovered following transposase-based P-element mobilization (Vaizel-Ohayon and Schejter, 1999). All the *cnn* alleles and the deficiency were provided by Eyal Schejter (Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel). Both cnn^{HK}/cnn^{E2} trans-heterozygous and cnn^{HK}/*Df(2R)8-104* hemizygous flies exhibited similar defects in the process of meiotic spindle assembly. Oocytes derived from *cnn* mutant mothers are referred to as *cnn* oocytes.

Reagents

Mouse monoclonal anti-β-tubulin (Boehringer-Mannheim, UK) was used at a 1:200 dilution; rat monoclonal YL1/2 directed against tyrosinated α-tubulin (Harlan Sera-Lab, England) at a dilution of 1:20; a mouse anti-γ-tubulin monoclonal antibody (Sigma, St Louis, MO) at 1:100; a rabbit polyclonal anti-CP190 serum Rb188 (Whitfield et al., 1988) at 1:400 dilution; a rabbit polyclonal anti-Asp serum Rb3133 (Saunders et al., 1997) at 1:50 dilution; a rabbit polyclonal anti-centrosomin antibody (Vaizel-Ohayon and Schejter, 1999) at 1:400 dilution; a rabbit polyclonal anti-PAV-KLP serum Rb3301 (Adams et al., 1998) at 1:100. Goat anti-mouse, anti-rat or anti-rabbit secondary antibodies coupled to fluorescein or rhodamine (Cappel, West Chester, PA, USA) were used at 1:600 dilution. DNA was stained with propidium iodide, Hoechst 33258 (Sigma, St Louis, MO, USA) or TOTO-3 iodide (Molecular Probes, Eugene, OR). Bovine serum albumin (BSA) and ribonuclease A (RNAse) were obtained from Sigma (St Louis, MO).

Fluorescence preparations

Oocytes were obtained using the method of Riparbelli and Callaini (Riparbelli and Callaini, 1996) with minor modifications. Flies were raised in groups of 80 males and 50 females on standard cornmeal, agar and yeast medium in 200 ml plastic containers. Eggs from 4- to 5-day-old flies were collected at 24°C on small agar plates supplemented with acetic acid and yeast three times for 10 minutes each. Egg precollection needs to have many fertilized oocytes at the earliest stages of meiosis. After discarding the eggs from the first collections, fertilized eggs were collected again fifteen times for 5 minutes. This collection led us to obtain an average of about 190 wild-type and 80 *cnn* oocytes. Each antibody was tested on the oocytes obtained from three separate sets of collections. Eggs dechorionated in a 50% bleach solution were fixed for 10 minutes in cold methanol, washed in PBS and incubated for 1 hour in PBS containing 0.1% BSA. For double staining of microtubules and Asp, CNN or PAV-KLP oocytes were incubated overnight at 4°C with the specific antisera and then with anti-β-tubulin antibody for 4-5 hours at room temperature. For simultaneous identification of microtubules and γ-tubulin the embryos were incubated overnight at 4°C with the anti-γ-tubulin antibody, then the YL1/2 antibody was added and the incubation continued for 2 hours at room temperature. After washing in PBS-BSA the embryos were incubated for 1 hour with the appropriate secondary antibodies. Controls, of the secondary antibodies alone, were done for all staining. For simultaneous tubulin and DNA staining, the eggs were incubated for 4-5 hours at room temperature, or overnight, in the anti-β-tubulin antibody. After washing in PBS-BSA, the eggs were incubated in the goat anti-mouse antibody to which 1 mg/ml RNAse was added. After washing in PBS the eggs were incubated for 30 minutes in 1 μg/ml propidium iodide. Eggs were mounted in small drops of 90% glycerol containing 2.5% *n*-propyl gallate.

Confocal microscopy

Digital optical sections of whole-mount oocytes were examined using a Leica TCS 4D laser scanning confocal microscope equipped with an argon-krypton laser and coupled to a Leica DMRBE microscope equipped with a 63× PL Apo 1.4 objective (Leica Lasertechnik, Heidelberg). For double staining, the images of the two fluorochrome distributions were recorded separately by averaging 8-16 scans of a single optical section to improve the signal-to-noise ratio. Images collected at several focal planes were superimposed, merged into a single file and imported into Adobe Photoshop to adjust size and contrast.

Results

The female meiotic spindle

After oocyte activation, meiosis resumes and the metaphase-arrested spindle elongates and undergoes a pivoting movement to reorient perpendicularly to the surface (Endow and Komma, 1997). Two distinct populations of interpolar MTs, interior and peripheral, form a top-like anaphase spindle (Fig. 1A: early anaphase, 8.2%; n=33/401). The interior MTs run parallel to the main axis of the spindle to form longitudinal thick bundles, whereas the peripheral ones spread out from the poles to form opposite cone-shaped half spindles (Fig. 1A). The peripheral MTs, which form a cage around the interior bundles, intersect at the equatorial plane of the spindle and extend both deeply into and above this plane until they reach the cell cortex. Thus, the spindle appears to be anchored to the oocyte surface through the peripheral MT set that forms the inner half spindle. Some clusters of MTs appear through the equatorial region of the spindle where opposite MTs overlapped and/or interacted

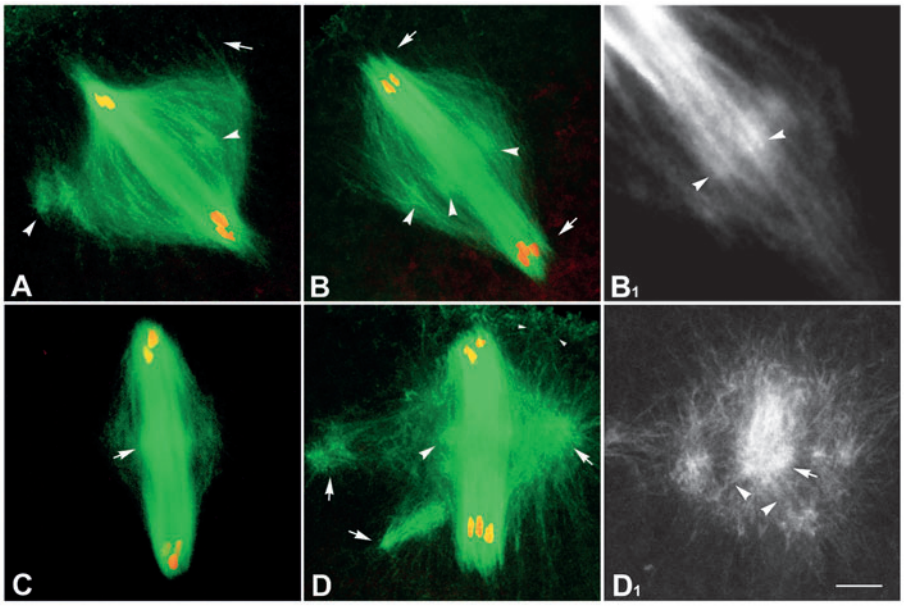

Fig. 1. Spindle organization during meiosis I-early meiosis II. Projected series of optical sections of oocytes stained with propidium iodide (orange) and an anti-β-tubulin (green) antibody. (A) Early anaphase I: peripheral MTs intersect at the spindle equator to form discrete clusters (arrowheads) or extend toward the cell cortex (arrow). (B) Late anaphase: the spindle has elongated and the poles appear broad and frayed (arrows), MT clustering at the spindle equator is more pronounced (arrowheads). (B_1) A single optical section of the spindle in B at the level of the equator showing the overlapping of some antiparallel MTs (arrowheads). (C) Telophase: a distinct tubulin accumulation is present midway along the spindle where the opposite MT bundles overlap (arrow). (D) Early prophase II: the peripheral MT network expands and some MTs contact the oocyte surface (small arrowheads); several astral arrays of MTs appear within the equator of the spindle and outside its boundaries (arrows) and a distinct tubulin cluster bulges out the spindle midzone (large arrowhead). (D_1): single optical section of the spindle in D showing that the equatorial tubulin cluster (arrow) is connected by thin bundles to the peripheral MT network (arrowheads). Bars, 2 μm in A-D,D_1 and 0.7 μm in B_1.

tangentially (Fig. 1A). As anaphase progresses the clustering of MTs is more evident through the spindle equator (Fig. 1B: late anaphase, 4.7%; *n*=19/401). Most of the interior MT bundles terminate in a small non-fluorescing area at the opposite poles. Co-labeling with propidium iodide indicated that these regions of lower MT density correspond to the chromosomes and that the bundles might therefore be kinetochore fibers. The kinetochore MTs of different chromosomes form separated bundles and do not focus on a common pole. Thus the spindle poles appear broad and frayed (Fig. 1B). Although the density of the MTs made it difficult to resolve the structure of the central region of the spindle, suitable optical sections showed that some MT bundles overlap in this region (Fig. $1B_1$). During telophase a diffuse tubulin accumulation was observed at the middle of the spindle at the sites where the interior MTs overlap (Fig. 1C: telophase, 5.7%; *n*=23/401).

Transition from meiosis I to meiosis II is marked by the expansion of the cage of MTs that surround the interior interpolar MTs to form an extensive network. Some of the peripheral MTs also contact the oocyte cortex (Fig. 1D: early prophase II, 7.2%; *n*=29/401). Astral arrays of MTs of various size become apparent at this time within the equator of the spindle and outside its boundaries (Fig. 1D). Intermediate to the formation of these structures might be the clusters of overlapping MTs observed during the first meiosis. These clusters become increasingly compact at the onset of second meiosis and could become centers from which MTs spread. A distinct tubulin aggregate was seen to

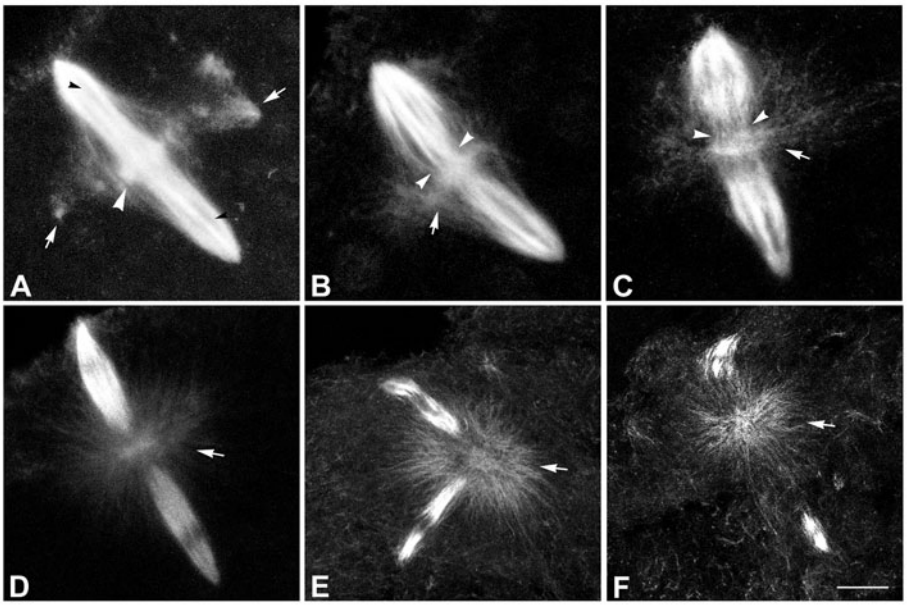

Fig. 2. Organization of the meiosis II spindle. Projected series of optical sections of oocytes stained with anti-β-tubulin antibody. (A) Late prophase: the peripheral MT network is dismantled, but small asters are still observed outside the spindle (arrows), the belt-shaped tubulin cluster is prominent and protruding at the spindle equator (large arrowhead); 'minispindles' (small arrowheads) are focused at the outer poles. (B) Prometaphase: the interior MTs become focused on separate bundles of different size at the spindle equator (arrowheads), where short MTs spread out to form a loose cloud (arrow). (C) Late prometaphase: the interior MT bundles partially coalesce in three to four distinct bundles (arrowheads) that are focused at the spindle equator where MTs form an extensive astral array (arrow), the central aster. (D) Metaphase: the twin spindles have separated and the central aster (arrows) becomes clearly evident and enlarges during (E) anaphase and (F) telophase. Bar, 2 μm.

bulge out at the middle of the interior MT bundles. Kinetochore MTs run almost parallel in each half spindle both outward and inwards to each chromosome set they hold. Thus, kinetochore fibers form distinct parallel 'minispindles' and the outer poles appear frayed (Fig. 1D). The inner poles cannot be distinguished at this time. Optical sections midway along the spindle show an irregular belt-shaped equatorial tubulin aggregate (Fig. $1D_1$) that could be derived from the tubulin accumulation observed during telophase of the first meiosis. Several MTs that span from this aggregate end at the cytoplasmic asters or mingle within the peripheral network (Fig. $1D_1$).

As meiosis progresses the peripheral MT array gradually disassembles and the MT asters become free in the cytoplasm or remain connected to the remnant of the peripheral MT framework or to the spindle by thin threads (Fig. 2A: late prophase II, 6.0%; *n*=24/401) and the equatorial tubulin cluster is more evident and protruding. Free asters are transient structures that disappear over time and are no longer visible from anaphase II on. The outward extremities of the minispindles formed by the kinetochore fibers focus on common outer poles, whereas the inner kinetochore fibers still run almost parallel (Fig. 2A). Thus, the individualization of the meiosis II twin spindles occurs gradually and requires the formation of the internal poles that start to be organized during late prophase/early prometaphase when the inwards extremities of the minispindles have partially coalesced midway along the spindle (Fig. 2B: prometaphase, 7.1%; *n*=28/401). Short MTs form a loose cloud at the spindle equator. During prometaphase/early metaphase, the twin spindles begin to be distinguishable, though their inner poles are formed by three to four distinct bundles and appear broad (Fig. 2C: late prometaphase, 4.2%; 17/401). The MTs at the spindle equator are more prominent and form an extensive astral array (Fig. 2C), the 'central aster', which increases in size during subsequent metaphase (Fig. 2D: metaphase, 22.2%; *n*=89/401) and anaphase (Fig. 2E: anaphase, 18.0%; *n*=72/401). At telophase (Fig. 2F: telophase, 16.7%; *n*=67/401) the MTs of the central aster reach their maximum length, although they have decreased in density.

A distinct spindle midzone is present in the female meiotic apparatus

The late anaphase meiosis I spindle shows a striking concentration of MTs corresponding to the mid-region of the spindle, whereas the density of the MTs appears lower at the opposite pole regions. This MT distribution is similar to that observed during anaphase/telophase of mitotic cells and spermatocytes, when the release of internal MTs from the poles and their concentration at the equator leads to the formation of a structured mid-zone and ultimately to a mid-body. Although female meiosis is a peculiar kind of division that occurs without cytokinesis, we wondered if the central region of the first meiotic spindle might show common features with male meiosis or mitosis. As the product of the gene *abnormal spindle* (*asp*) appears to perform a major role in the organization and dynamic of the central spindle MTs in both mitotic cells and spermatocytes, we asked if this protein was also present within the central region of the post-anaphase meiosis I female spindle. The MT minus-end binding protein Asp localizes to the polar regions of mitotic and meiotic spindles (Saunders et al., 1997) and exhibits a striking enrichment at the terminal regions of their mid-zone MTs (Wakefield et al., 2001; Riparbelli et al., 2002).

In oocytes stained with the Asp antibody during anaphase of

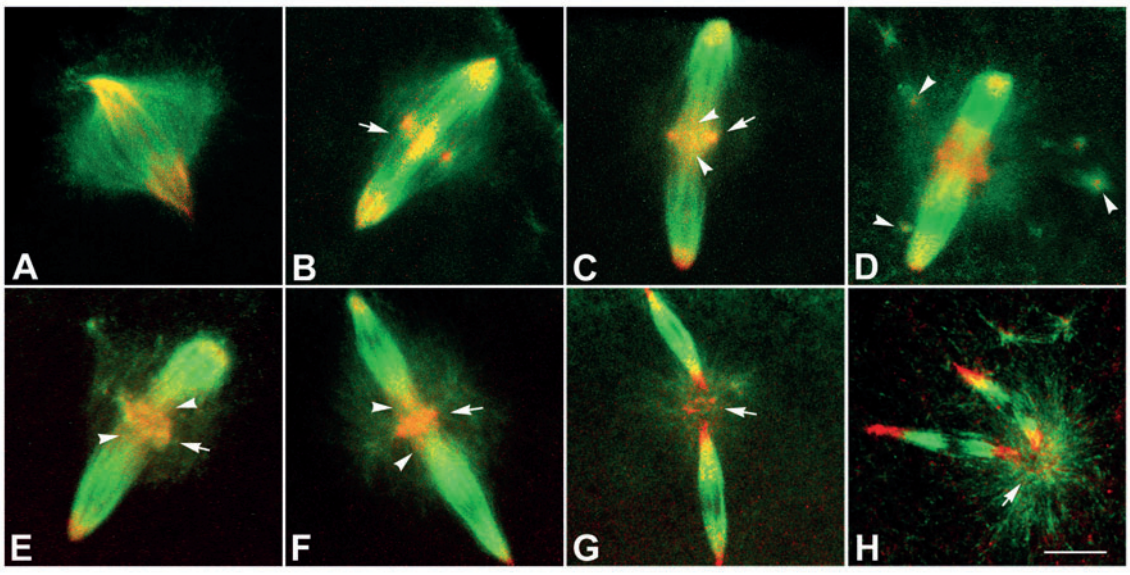

Fig. 3. Localization of Asp protein during female meiosis. In all panels MTs are stained green and Asp, orange. (A) Anaphase of meiosis I: Asp is present at the spindle poles. (B) Late anaphase of meiosis I: Asp is localized at the poles and at the spindle equator (arrow). (C) Telophase of meiosis I: Asp localizes in a ring-like structure (arrow) around the accumulation at the central spindle (arrowheads). (D) Early prophase: Asp staining is evident at the spindle poles, at the central spindle, and it is also found at the foci of the sparse asters (arrowheads). (E) Prometaphase and (F) metaphase: Asp remains localized at the outer spindle poles and within the central region of the spindle at the site where the inner poles are forming (arrowheads); a strong labeling is also observed within the individualizing spindles where the central aster is developing (arrow). (G) Anaphase II: Asp accumulates at the spindle poles and at the minus ends of the MTs that organize the central aster (arrow), where a feeble ring-like staining remains. (H) Telophase II: the spindle poles are strongly stained by the anti-Asp antibody, whereas a weak labeling is found within the central aster (arrow). Bar, 3 μm.

the first meiosis, the opposite regions of the spindle, corresponding to the minus ends of the MTs at the focused poles, show strong Asp labeling (Fig. 3A). Asp staining is undetectable above the background levels in the central region of the spindle, suggesting that MT minus ends are found only at the spindle poles. During late anaphase-early telophase there is an additional class of Asp distribution within the equator of the spindle, where the MT density seems to be higher (Fig. 3B). During late telophase I the Asp protein also concentrates in a thin equatorial band surrounding the former accumulation at the central spindle (Fig. 3C). There is also Asp labeling at the foci of the MT asters that appeared outside the spindle boundaries at the beginning of meiosis II (Fig. 3D).

The twin meiosis II spindles become apparent during transition from prophase to metaphase of the second meiosis. However, only their outer poles are focused, whereas the inner ones have frayed MT bundles. Asp accumulates at the outer poles and within the central region of the spindle at the presumptive inner poles where the antibody detects a diffuse staining (Fig. 3E). A distinct ring-like accumulation of the protein is also evident at the spindle equator. During metaphase, Asp is still diffusely localized within the central region of the spindle where the inner poles formed. A strong ring-like staining was present in the region between the inner extremities of the twin spindles (Fig. 3F). Twin meiotic spindles become well separated, with focused inner and outer poles at anaphase (Fig. 3G) and telophase (Fig. 3H). The region corresponding to the MT minus ends at the spindle poles was strongly labeled by the anti-Asp antibody, whereas the minus ends of the MTs that organized the central aster and the remnant of the equatorial ring-like structure were weakly recognized.

The striking dynamic of the MTs in the central region of the meiotic spindle led us to examine the distribution of the kinesin-like protein (PAV-KLP) encoded by *pavarotti*. This motor protein, required for central spindle formation and cytokinesis, associates with putative plus ends of the MTs that emanate from the opposite poles and interdigitate with one another to form antiparallel arrays in the overlap region at the spindle midzone of anaphase/telophase mitotic and meiotic cells (Adams et al., 1998; Minestrini et al., 2003). During anaphase of the first female meiosis PAV-KLP is found along the interior MTs in the predicted spindle midzone (Fig. 4A). The staining at the central spindle is more evident in late anaphase (Fig. 4B) and becomes prominent at the spindle midzone during telophase (Fig. 4C). During prophase of the second meiosis, the accumulation of PAV-KLP at the central spindle MTs is no longer visible (Fig. 4D), and the protein forms a loose circular band midway along the spindle (Fig. 4D). The PAV-KLP–localization remains prominent at the developing central aster where it forms a distinct ring at the beginning of metaphase (Fig. 4E) and compacts throughout subsequent stages of meiosis (Fig. 4F). During late anaphase of the second meiosis PAV-KLP is also clearly bound to the midzone of the twin tandem spindles (Fig. 4F) at the putative plus ends of the antiparallel overlapping MTs.

CNN loss does not affect meiotic spindle assembly

Because the central aster that develops at the onset of second meiosis seems to be the result of a local MT nucleation process caused by a discrete MTOC that was organized during the first meiosis around the spindle midzone, we wished to know whether centrosomal components might be already present within this region during meiosis I. The localization of major *Drosophila* centrosomal components within the meiosis I spindle would be unexpected, because labeling with antibodies against γ-tubulin, CP60, CP190 and CNN was undetectable above background levels, although these proteins have been found in the central aster of meiosis II (Megraw and Kaufman, 2000). Accordingly, we did not find distinct immunostaining within the anaphase I spindle with antibodies against γ-tubulin and CP190, whereas we identified these proteins throughout meiosis II at the focus of the central aster (our unpublished data). However, when we stained meiosis I oocytes with antibodies against CNN, a core component of the *Drosophila* centrosome (Megraw et al., 1999) required for proper targeting of γ-tubulin and other centrosomal components to the centrosome itself (Terada et al., 2003), an unexpected localization of the protein was readily found. Although the poles of the anaphase I spindle lacked CNN staining, as previously reported, the protein accumulates in small aggregates at the spindle mid-zone (Fig. 5A). At the onset of

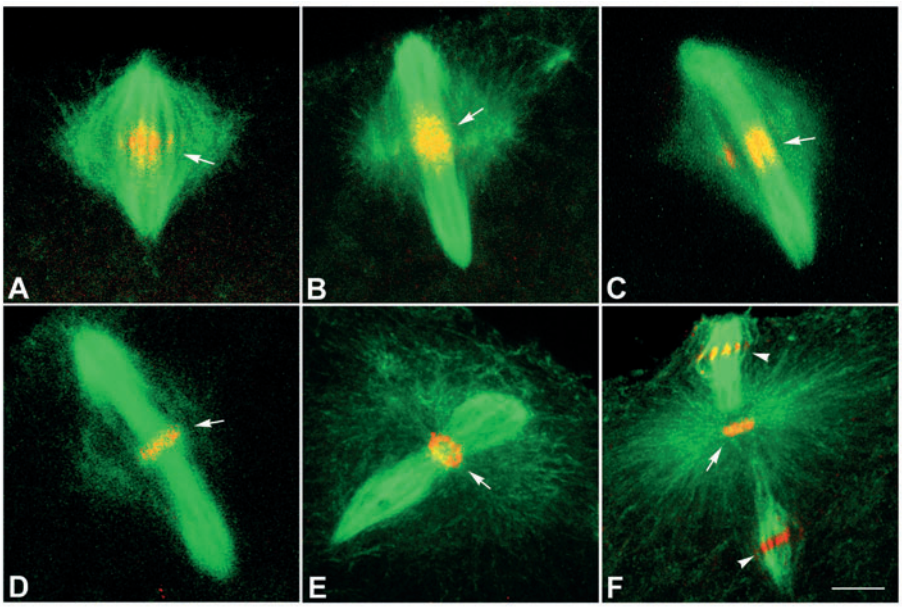

Fig. 4. Localization of PAV-KLP in the female meiotic spindle. Oocytes were stained with anti-β-tubulin (green) and anti- PAV-KLP (orange) antibodies. First meiosis: (A) anaphase, (B) late anaphase, (C) telophase: PAV-KLP accumulates at the middle of the spindle (arrow), at the presumptive midzone. Second meiosis: (D) prophase, (E) metaphase: the staining at the spindle midzone is no longer visible, but PAV-KLP localizes on a discrete ring-like structure at the spindle equator (arrows). (F) Anaphase: PAV-KLP concentrates at the focus of the central aster (arrow) and at the midzone of the twin spindles (arrowheads). Bar, 2 μm.

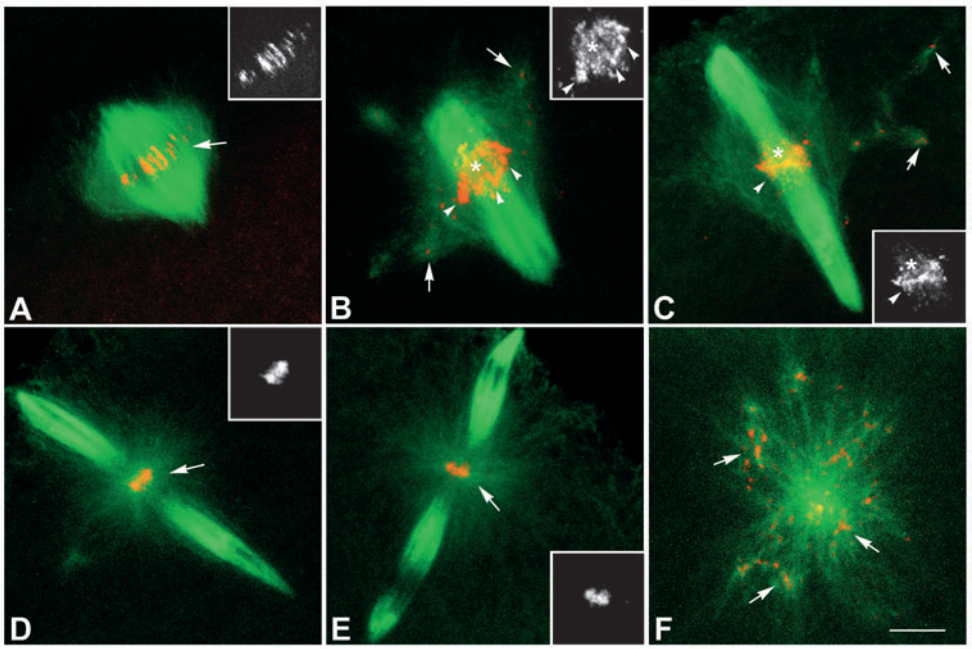

Fig. 5. Localization of CNN at the female meiotic spindle. Projected series of optical sections of oocytes stained with antibodies against β-tubulin (green) and CNN (orange). In each panel the inset shows, in monochrome, the localization of CNN protein. (A) Anaphase I: CNN accumulates on distinct bundles at the central spindle (arrow). (B) Prophase II: the protein accumulates at the middle of the spindle in a punctate ring-like structure (arrowheads) that surrounds a faint localization (asterisk) on the interior MT bundles; punctate aggregates of the protein are also found at the center of the MT asters (arrows). (C) Prometaphase II: the CNN localization at the middle of the spindle decreases in intensity (asterisk), although a thin ring-like fluorescent structure is still visible (arrowheads); the protein has also accumulated at the focus of the cytoplasmic asters (arrows). (D) Metaphase II, (E) anaphase II: CNN staining is no longer found within the spindles, but a distinct accumulation is visible at the focus of the central aster (arrows). (F) CNN aggregates are also observed within the sperm aster, at the sites of MT clustering (arrows). Bar, 2 μm.

prophase II, CNN is localized around the central spindle to form a discontinuous equatorial belt composed of dot-like aggregates (Fig. 5B). This protein is also found as small faint dots occupying the area of the central spindle (Fig. 5B) and small aggregates at the focus of the MT asters lying at the spindle periphery (Fig. 5B). During prometaphase, CNN remains confined to the middle of the spindle where the protein forms a thin ring-like structure that encircles a faint punctate distribution and is still found at the foci of the MT asters outside the spindle apparatus (Fig. 5C). As meiosis progresses, and the central aster increases in dimension, the distribution of CNN is reduced (Fig. 5D,E). The staining persists until the end of meiosis when the central spindle disappears. Dot-like aggregates were also observed in association with the MT clusters that form throughout the sperm aster (Fig. 5F).

The characteristic co-localization of CNN with the central spindle during anaphase of the first meiosis suggested that this protein could play some role in spindle assembly. We then asked whether loss of CNN could affect the spindle structure. We carried out a functional analysis of the protein by studying the effects on female meiosis of mutations in the *cnn* gene. As expected, no accumulation of CNN protein is observed in any of the mutant oocytes (n=304) stained from anaphase I to telophase II. Progression throughout meiosis I, however, occurs normally, and the late anaphase I spindle is indistinguishable from the corresponding wild-type spindle (Fig. 6A). The prometaphase II spindle has a wild-type shape and is composed of interior MT bundles surrounded by an extensive MT network (Fig. 6B). The outer poles are focused, whereas the MT bundles in the central region of the spindle coalesce to form the inner poles. There are small aster-like structures at the periphery of the MT network (Fig. 6B). As the peripheral MT array dismantles during transition to metaphase, short MTs appear to spread throughout the spindle equator to form a faint central aster (Fig. 6C). As twin spindles individualize, the MTs of the central aster, which are very prominent in wild-type oocytes, diminish in density and the central aster becomes hardly detectable (Fig. 6D,E). This indicates that *cnn* mutant oocytes are unable to maintain a normal-looking central aster. However, the sperm-associated aster is present throughout all meiotic stages (Fig. 6F), although it is much smaller than the wild-type sperm asters. 31% (n=62/193) of the mutant oocytes examined during anaphase/telophase of meiosis II displayed spindles that were not properly spaced or rather abnormal in shape (Fig. 6G). 41% (n=79/193) of the twin meiotic spindles also appeared misaligned during late telophase (Fig. 6F).

To investigate whether mutations in *cnn* could affect γ-tubulin localization during female meiosis, as reported for centrosomes of *Drosophila* embryos (Vaizel-Ohayan and Schejter, 1999) and neuroblasts (Megraw et al., 2001), we stained mutant oocytes at various stages of meiosis I and II with an antibody raised against this protein. However, all our attempts to identify γ-tubulin in mutant oocytes failed (our unpublished data). Perhaps this protein does not accumulate at the meiotic spindle in the absence of CNN or its amount is too low to be detected with our conventional immunofluorescence methods.

The elongation of the central spindle during anaphase of the first meiosis and the formation of four haploid complements suggests that the spindle MTs behave in the same way in mutant and wild-type oocytes. This observation led us to ask if mutant *cnn* oocytes were able to assemble a normal-looking mid-zone within the meiotic spindle. Therefore, we examined whether the correct segregation of the *pav* gene product occurred during anaphase/telophase transition of the first meiosis. The protein is found at the equator of the late anaphase spindle during meiosis I, in association with the interior MT bundles (Fig. 7A) and is concentrated at the putative spindle

midzone during telophase (Fig. 7B). During early metaphase II, PAV-KLP accumulation is dramatically diminished in comparison with the same stage wild-type oocytes and the protein is barely detectable at the focus of the faint central aster (Fig. 7C). During anaphase of the second meiosis, the central aster becomes hardly detectable and the PAV-KLP staining between the inner poles of the twin spindles is not longer visible (Fig. 7D). However, there is a distinct accumulation of the protein at the midzone of the spindles, in the region where antiparallel MTs overlap (Fig. 7D).

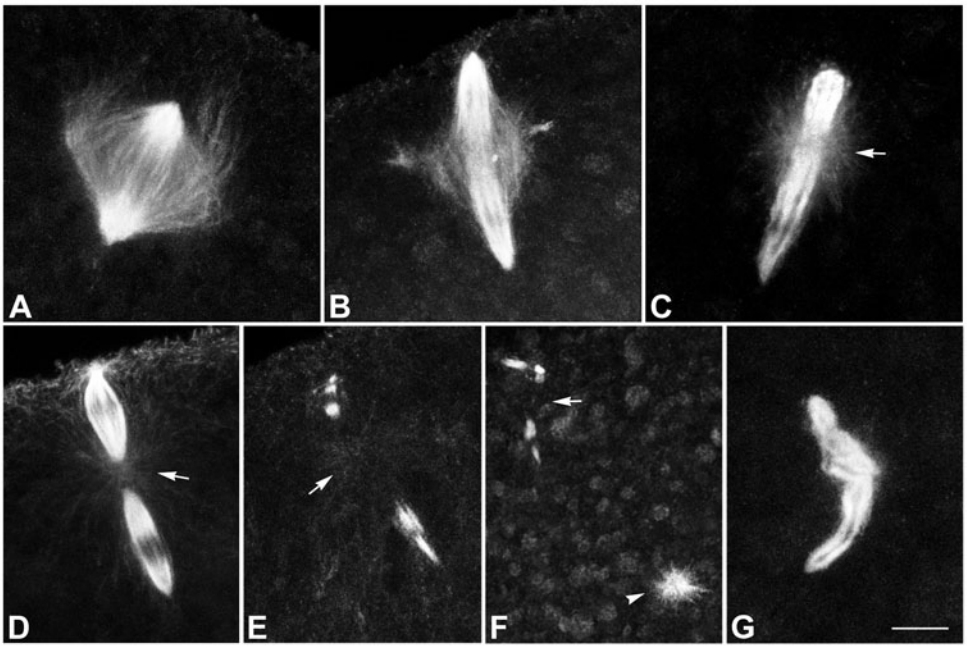

Fig. 6. Female meiosis in *cnn* mutants. Oocytes derived from *cnn* mothers were fixed and stained for tubulin. (A) Anaphase I: the meiotic spindle is organized as in wild type. (B) Prometaphase II: the peripheral MT network has dismantled and the inner poles of the twin spindles start to organize. (C) Early metaphase II: a faint central aster (arrow) is visible between the twin spindles. (D) Early anaphase II and (E,F) telophase II: the central aster becomes hardly detectable (arrows), however, a distinct sperm aster is present (arrowhead, F), although diminutive compared to that in the wild-type. (G) Example of twin anaphase II spindles not properly spaced and rather abnormal in shape. Bars, 2.5 μm in A-E,G and 3.5 μm in F.

Discussion

Although female meiosis in *Drosophila* differs from male meiosis and mitosis in that spindle poles lack centrosomes and cytokinesis does not occur, we demonstrated that the central region of the spindle has a common organization in these systems and that a structured midzone exists in the female meiotic spindle. At least two main events are involved in the formation of the spindle midzone in *Drosophila* cells (Inoue et al., 2004): the overlay of antiparallel MTs and the release of a subpopulation of MTs from the spindle poles. We showed that the central region of the female meiosis spindle during late anaphase I has the ability to bind PAV-KLP, the *Drosophila* ortholog of MKLP (Adams et al., 1998). This protein has been found in association with the putative MT plus ends at the midzone of anaphase spindles of mitotic cells (Minestrini et al., 2003) and spermatocytes (Carmena et al., 1998) where it might function in regulating the dynamics and organization of the overlapping MTs. Thus the female meiotic spindle could have a structured midzone formed by overlapping antiparallel MTs. This is consistent with the finding, from sagittal optical sections, that opposite MT bundles overlap at the middle of the central spindle. The increased density of MTs in the central region of the spindle at telophase of the first meiosis might be consistent with the release of a subset of MTs from the opposite spindle poles. When this process occurs in mitotic cells (Wakefield et al., 2001) and spermatocytes (Riparbelli et al., 2002), the Asp protein accumulates at the minus ends of the central spindle MTs. The Asp staining observed in the central region of the spindle during telophase of the first meiosis could be such a protein localization, at the predicted MT minus ends, suggesting that MTs of the central spindle undergo similar dynamics during female meiosis and in both mitosis and male meiosis.

Morphological evidence indicates that several differences exist between the meiosis I and II spindles: the meiosis I spindle is anastral, whereas the twin meiosis II spindles have outer anastral poles and an unusual central aster between the inner poles (Riparbelli and Callaini, 1996; Endow and Komma, 1997). These observations suggest that spindle assembly could require different mechanisms during meiosis I and II. Whereas the organization of the meiosis I spindle involves a chromosomal-dependent pathway of MT organization (Theurkauf and Hawley, 1992; Matthies et al., 1996), the individualization of the twin spindles during meiosis II requires the formation of new poles in the center of the prophase II spindle. However, the mechanism by which the inner poles form is unclear. This issue has been difficult to address because the formation of the inner spindle poles occurs in a region of very high MT density, and thus any stage of this process is obscured from view. It has been proposed that the formation of the inner poles might require the reorganization of the central spindle in which the polarity of MT ends reverse (Endow and Komma, 1998). This hypothesis is supported by the presence of an unusual MTOC that has been postulated to play a major role in the process of MT nucleation for the formation of the inner half spindles during meiosis II (reviewed by Megraw and Kaufman, 2000). However, normal-looking twin bipolar spindles can form during meiosis II, both when the central aster is defective as in *polo* mutants (Riparbelli et al., 2000) and when it is very reduced as in *wispy* (Brent et al., 2000), *KLP3A* (Williams et al., 1997) and *cnn* (this paper) mutants.

We suggest, here, a new model of spindle assembly during meiosis II. Spindle elongation during anaphase/telophase of the first meiosis moves the homologous chromosomes to opposite

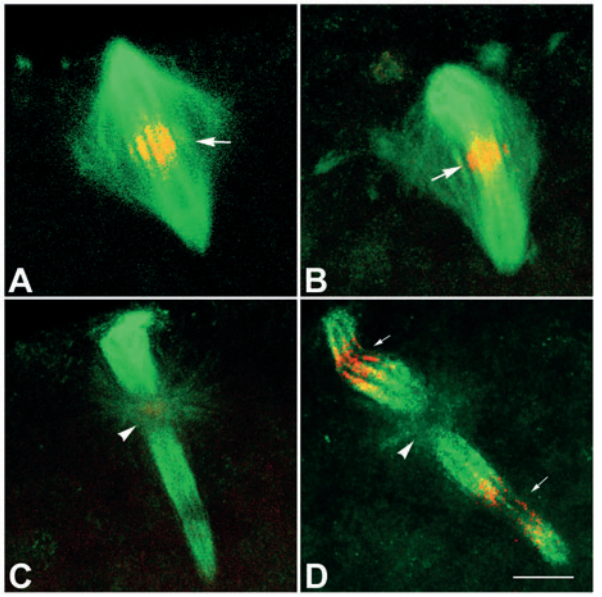

Fig. 7. PAV-KLP localization in *cnn* mutants. The merged images show PAV-KLP (orange) and MT (green) staining. (A) Anaphase I, (B) telophase of the first meiosis, (C) metaphase, (D) late anaphase of the second meiosis. PAV-KLP strongly accumulates at the central spindle during first meiosis (arrows), but the protein is barely detectable or absent within the focus of the faint central aster during the second meiosis (arrowheads); however, a discrete PAV-KLP labeling is found at the midzone of the twin meiotic spindles (small arrows). Bar, 2 μm.

poles that appear broad and frayed because the kinetochore fibers of different chromosomes do not focus on a common pole during later meiosis I stages. Starting from late prophase II, two opposite half spindles become evident, with outer focused poles where Asp accumulates. By contrast, inner poles are not evident at this point. However, the accumulation of Asp midway along the spindle suggests that the minus ends of opposite interpolar MTs are facing into the central region of the spindle. These observations are consistent with the finding in each prophase II half spindle of two opposite MT populations, with their plus ends near the chromatin and their minus ends outwards. The outward minus ends are focused on distinct poles, whereas the minus ends facing the spindle equator remain unbound. Control of spindle assembly could be achieved during meiosis II by the same chromosome-driven mechanisms of MT organization working in meiosis I. MTs in the vicinity of the condensed chromatin could be sorted into bundles and focused in stable poles by the cooperative interaction of molecular motors and cross-linking proteins. Loss of *ncd* function results, indeed, in the destabilization and fragmentation of both inner and outer spindle poles (Endow and Komma, 1998). Accordingly, the inner half spindles are formed during late prophase by parallel MTs that are first bundled at the spindle equator in distinct foci, and that then coalesce into a single pole. This model of meiotic spindle assembly is consistent with the formation of twin spindles in oocytes lacking normal-shaped central asters. We propose, therefore, that the acentrosomal pathway makes an essential contribution to spindle formation during meiosis II, even when a functionally active MTOC is present in the oocyte. An alternative mechanism might be that the inner half meiosis II spindles could be formed by a mixture of two MT populations: MTs nucleated in the vicinity of the chromosomes and MTs of the central aster. The gradual tethering together of these two MT populations could give rise to the tapered inner spindle poles, as proposed during the assembly of the mitotic spindle in cultured vertebrate cells (Gruss et al., 2002; Wadsworth and Kodjakov, 2004). To what extent inner half spindles might be formed by two MT populations is difficult to determine, since individual MTs and their origin cannot be resolved within the assembling spindle. However, the Asp staining observed during transition from prophase to metaphase of the second meiosis suggests that the MTs radiating from the central aster contribute minimally, if at all, to the formation of the internal half spindles. The Asp staining is strong in the central region of the spindle, whereas it is weaker in the central aster, pointing to a lower MT density. This suggests that the contribution of the central MTOC is not essential for the higher MT density within the region where the inner poles will organize. Consistently, optical sections at the level of the spindle equator during prophase of the second

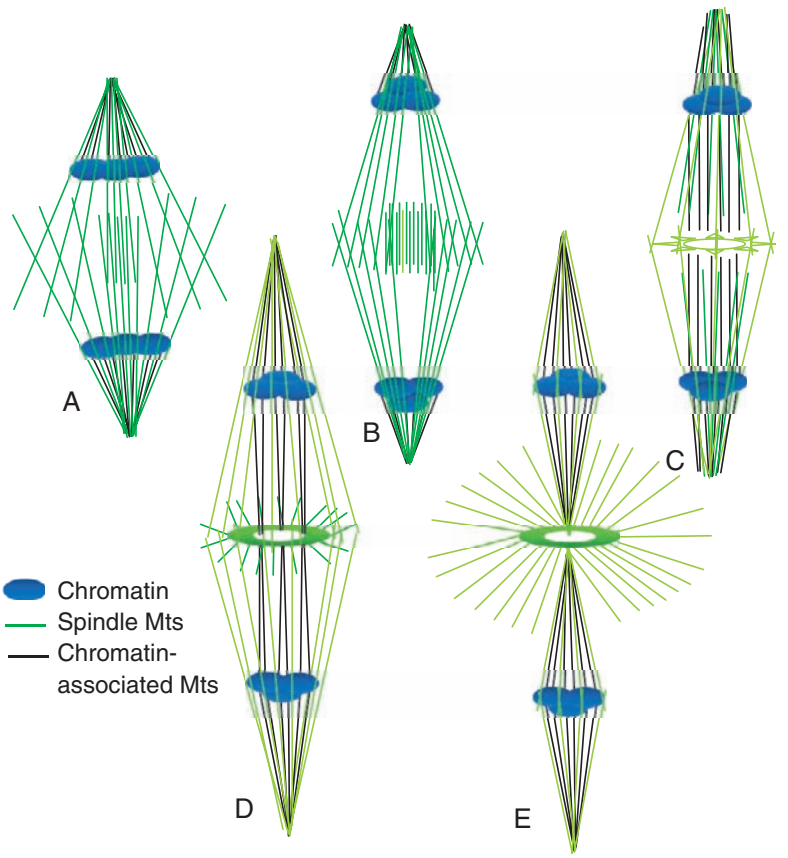

Fig. 8. Diagram of the main features of female spindle morphogenesis during transition from meiosis I to meiosis II. (A) Anaphase I. (B) Telophase I. (C) Prophase II. (D) Prometaphase II. (E) Metaphase II.

meiosis reveal a continuity between the peripheral MT network and the ring-like tubulin aggregate, but no link is observed between this structure and the MTs of the central spindle. Finally, staining of Asp indicates that the minus ends of the inner half meiosis II spindle MTs start to coalesce into a single pole and, therefore, are completed before a distinct central aster is apparent. On the basis of these considerations we favor the first hypothesis in which the function of the central aster is redundant for the meiosis spindle organization.

However, if the meiosis II spindles are assembled by an acentrosomal pathway, what is the role of the central aster? In the absence of a developed central aster, the twin spindles can be improperly spaced or oriented with respect to the long axis of the oocyte, resulting in the failure to correctly position the female pronucleus. The central aster could, therefore, be needed to keep the correct spacing between neighboring twin spindles. Accordingly, twin spindles are misaligned and at variable distances from each other when the central aster is defective as in *polo* mutants (Riparbelli et al., 2000). Mutation in *cnn* has been shown to impair the spatial organization of mitotic spindles in the early *Drosophila* embryo (Vaizel-Ohayan and Schejter, 1999).

In vivo observations revealed that the precursor of the central spindle could be identified by a tubulin pucker midway along the central spindle at the end of meiosis I (Endow and Komma, 1998). We showed that this structure first appears as a tubulin aggregate to the spindle midzone, where antiparallel MTs overlap and recruit the PAV-KLP motor and CNN proteins. There are two possible mechanisms in the formation of the central aster. One possibility is that the MTs are nucleated by a discrete MTOC. The other possibility is that motor proteins may cross-link and organize randomly nucleated MTs into aster-like structures. The finding of several centrosomal proteins at the focus of the central aster during meiosis II (Megraw and Kaufman, 2000), strongly points to the first possibility. Since γ-tubulin at the centrosome seems to be dependent on *cnn* function (Terada et al., 2003), the finding of CNN at the spindle midzone during anaphase I could indicate the prerequisite for the recruitment of γ-tubulin at the central spindle and, therefore, for the nucleation of the central aster MTs. However, in *cnn* mutants that lack this protein at the spindle midzone in anaphase I and fail to accumulate γ-tubulin, a faint astral array of MTs forms at the middle of the spindle during prometaphase/early metaphase II. In the absence of γ-tubulin the central aster becomes poorly organized during subsequent meiotic stages and then disappears. We therefore propose (see Fig. 8) that the assembly of this structure might first rely on the cooperative interaction of motor proteins and acentrosomal MTs to form a polarized array of MTs that spread out from the equatorial region of the spindle. This process does not require *ncd* function, since mutants for the Ncd motor protein assemble an astral array of MTs at the beginning of meiosis II (Endow and Komma, 1998). Recruitment of material along these MTs might then contribute to the accumulation of centrosomal proteins, thus leading to the formation of a true MTOC that in turn could lead to further growth of the central aster. According to this model the presence of centrosomal material and other components, such as PAV-KLP, at its focus might be due to a fortuitous recruitment along its MTs. This is consistent with the finding that when the integrity of the central aster is affected in *cnn* mutant oocytes, there is little or no accumulation of PAV-KLP between twin spindles. By contrast, the motor protein is always found at the midzone of mutant spindles during both anaphase I and anaphase II, where it is needed for spindle dynamics. This is consistent with the observation that the accumulation of pericentrin and γ-tubulin at the vertebrate centrosome is inhibited in the absence of tubulin or by microinjection of antibodies against cytoplasmic dynein (Young et al., 2000). This two step mechanism could explain why γTub37CD mutants show an astral array of MTs at the beginning of metaphase II in the absence of detectable γ-tubulin (Wilson and Borisy, 1998). The sperm aster is usually retained to be assembled by a true centrosome derived from both paternal and maternal sources. In particular the male gamete provides the centriole around which maternal components accumulate to form a mature centrosome able to nucleate MTs and to reproduce. Although rigorous conclusions are difficult to draw from negative results, the observation that *cnn* mutant oocytes have a developed sperm aster lacking CNN and γ-tubulin, points to alternative mechanisms of sperm aster assembly. We cannot, however, exclude the possibility that the amount of these proteins could be so much lower in mutant oocytes that they have escaped our immunofluorescence analysis.

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Note added in proof

Since submission of this manuscript, Sköld et al. have published a paper on the assembly of the meiosis I spindle in the *Drosophila* oocyte (Sköld et al., 2005). These authors reported new findings regarding nucleation of microtubules and the role of the Ncd motor in the formation of the meiosis I spindle. These findings compel modification for model of metaphase spindle assembly during the first female meiosis based on previous observations.

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