A requirement for the Abnormal Spindle protein to organise microtubules of the central spindle for cytokinesis in *Drosophila*

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Summary

***Drosophila abnormal spindle* (*asp*) mutants exhibit a mitotic metaphase checkpoint arrest with abnormal spindle poles, which reflects a requirement for Asp for the integrity of microtubule organising centres (MTOCs). In male meiosis, the absence of a strong spindle integrity checkpoint enables *asp* mutant cells to proceed through anaphase and telophase. However, the central spindle region is not correctly organised and cells frequently fail to complete cytokinesis. This contrasts with meiosis in wild-type males where at late anaphase a dense array of microtubules forms in the central spindle region that has Asp localised at its border. We speculate that Asp is associated with the minus ends of microtubules that have been released from the spindle poles to form the central spindle. A parallel situation arises in female meiosis where Asp not only associates with the minus ends of microtubules at the acentriolar poles but also with the central spindle pole body that forms between the two tandem spindles of meiosis II. Upon fertilisation, Asp is also recruited to the MTOC that nucleates the sperm aster. Asp is required for growth of the microtubules of the sperm aster, which in *asp* mutants remains diminutive and so prevents migration of the pronuclei.**

Key words: Asp, cytokinesis, meiosis, microtubules, contractile-ring, *Drosophila*

Introduction

In metazoans, cytokinesis is accomplished by the contractile ring, a transient structure containing actin and myosin filaments that is anchored to the equatorial cortex. Interactions between filaments lead to the contraction of the ring, which constricts the dividing cell in the middle until cleavage is completed. A large body of data suggests that in animal cells the site of cytokinesis is determined by the position of the spindle. However, it is still unclear which part of the spindle provides the signals for cytokinesis. Experiments to manipulate the position of the spindle in invertebrate eggs suggested that the asters are the source of such signals (Rappaport, 1961). However, other work indicates that the stimulus is provided by the central spindle. For example, when a barrier is placed between the central spindle and the cortex, cytokinesis is blocked (Cao and Wang, 1996).

The central spindle is evident as a dense body of microtubules that forms in the region between the two late anaphase-telophase nuclei. The mechanism and dynamics of its formation are still poorly understood. The establishment of the central spindle in *Drosophila* appears, in part, to depend on at least two kinesin-like proteins: Klp3A (Williams et al., 1995) and Pavarotti (Pav-KLP) (Adams et al., 1998). The counterparts of Pav-KLP in other organisms, the *zen-4* gene product of *C. elegans* (Raich et al., 1998; Severson et al., 2000) or the vertebrate Mklp1 (Vernos et al., 1995), have also been shown to be required for cytokinesis. In addition to the contribution made by the spindle, several proteins, known as chromosomal passengers, dissociate from the chromosomes at the metaphase-anaphase transition and are deposited at the cell equator. The inner centromere protein (INCENP) and the associated Aurora B kinase, for example, transfer to the central spindle and the cell cortex and are necessary for completion of cytokinesis (Adams et al., 2000; Adams et al., 2001; Kaitna et al., 2000; Giet and Glover, 2001). The Polo-like kinases are also required for central spindle formation, and *Drosophila* Polo-kinase and Pav-KLP are mutually dependent for their localisation in the central spindle mid-zone (Adams et al., 1988; Carmena et al., 1998). The analysis of the cytological phenotypes displayed by mutants with disrupted meiotic cytokinesis in *Drosophila* males has provided insight into an intimate relationship between the formation of the central spindle and the contractile ring (Giansanti et al., 1998). Mutations identified as disrupting the central spindle are found in genes that encode a variety of actin-, microtubule- or septin-associated proteins (reviewed by Field et al., 1999; Gatti et al., 2000; Glotzer, 1997; Goldberg et al., 1998).

Mutations in *abnormal spindle* (*asp*) have not previously been thought to affect cytokinesis. Male meiotic spindles in *asp* mutants are bipolar, with particularly long and wavy microtubules (Ripoll et al., 1985; Casal et al., 1990). Mitotic cells accumulate at metaphase and show spindles with disorganized broad poles at which γ-tubulin has an abnormal distribution (Avides and Glover, 1999). *asp* encodes a 220 kDa microtubule-associated protein found at the spindle poles and centrosomes from prophase to early telophase. The protein has

consensus phosphorylation sites for CDK1 and MAP kinases, an actinin-type actin-binding domain and multiple calmodulin-IQ-binding motifs (Saunders et al., 1997). Asp and γ-tubulin are present in partially purified centrosomes and are both required for the organization of microtubules into asters (Avides and Glover, 1999). This activity is dependent on the phosphorylation of Asp by the kinase Polo (Avides et al., 2001).

Here, we now show that *asp* function is also required for cytokinesis in male meiosis and that the protein becomes localised at late anaphase in a manner consistent with a function in organising the spindle mid-zone. We have also examined the distribution of Asp protein in female meiosis. These divisions are unusual because the first meiotic spindle is acentriolar and appears not to contain any centrosomal proteins, such as γ-tubulin and CP190 (Riparbelli and Callaini, 1996; Tavosanis et al., 1997). The female meiotic spindle microtubules are initially nucleated from chromatin and require the minus-end motor Ncd to focus the poles of the spindle for meiosis I (Endow and Komma, 1996). At telophase of meiosis I, a central pole body is formed. Microtubules in the central part of the spindle dissociate and reorganise with reverse polarity such that their minus ends are associated with this central pole body. This results in the tandemly linked second meiotic spindles (Endow and Komma, 1997; Endow and Komma, 1998; Riparbelli and Callaini, 1996). Asp is a component of this central spindle pole body. We suggest that Asp has a dual role not only organising microtubule-nucleating centres at the poles at the onset of M-phase but also participating in organising the central spindle region at telophase.

Materials and Methods

Stocks

Drosophila mutant stocks used in this work were $asp^{dd4}/TM6B$, $asp^{1}/TM6B$ and $Df(3R)H\delta^{71}/TM6B$ (Flybase (Gelbart et al., 1999; White-Cooper et al., 1996)).

Reagents

A mouse monoclonal anti-β-tubulin antibody (Boehringer, Mannheim UK) was used at a 1:200 dilution; a rabbit polyclonal anti-Asp serum Rb3133 (Saunders et al., 1997) at 1:50 dilution; a rabbit anti-Pav-KLP polyclonal Rb3301 (Adams et al., 1998) at 1:100; a rabbit anti-γ-tubulin polyclonal Rbcs1 at 1:100; a mouse monoclonal anti-Peanut 4C9H4 at 1:4; a rabbit HsCen1p polyclonal antibody (Paoletti et al., 1996) at 1:400 dilution; a rabbit polyclonal anti-centrosomin antibody (Li and Kaufman, 1996) at 1:400 dilution. Goat anti-mouse or anti-rabbit secondary antibodies coupled to fluorescein or rhodamine (Cappel, West Chester, PA) were used at 1:600 dilution. DNA was stained with propidium iodide (Sigma, St. Louis, MO), Hoechst 33258 (Sigma) or TOTO-3 (Molecular Probes, Europe, BV). The actin cytoskeleton was stained with Rhodamin-phalloidin (Molecular Probes). Bovine serum albumin (BSA) and Ribonuclease A (RNAse) were obtained from Sigma.

Indirect immunofluorescence and confocal images

Immunostaining was performed either by the methanol/acetone fixation method described by Gonzalez and Glover (Gonzalez and Glover, 1993) or by the ethanol/formaldehyde fixation method as described by Hime et al. (Hime et al., 1996). Briefly, testes from pupae or newly eclosed adults were dissected in phosphate-buffered saline (PBS) and placed in a small drop of 5% glycerol in PBS on a glass slide. Testes were squashed under small cover glasses and frozen on a copper bar precooled in liquid nitrogen.

To localise microtubules, Asp, Pav-KLP, γ-tubulin, Centrosomin, Peanut and centrin, the frozen samples were fixed in methanol at –20°C, washed for 15 minutes in PBS and incubated for 1 hour in PBS containing 0.1% BSA (PBS-BSA) to block non-specific staining. For double labelling experiments the samples were incubated overnight at 4°C with the specific antisera against Asp, Pav-KLP, γ-tubulin, Centrosomin, Peanut or centrin antigens and then with anti-β-tubulin antibody for 4-5 hours at room temperature. After washing in PBS-BSA the samples were incubated for 1 hour at room temperature with the appropriate secondary antibodies.

For simultaneous localisation of actin and tubulin, the frozen testes were immersed for 7 minutes in cold ethanol and 10 minutes in 4% paraformaldehyde. After washing in 0.2% Triton in PBS, the samples were incubated with the anti-β-tubulin antibody for 1 hour at room temperature. Samples were then washed in PBS-BSA and incubated for 1 hour in the appropriate secondary antibody to which rhodamine-labeled phalloidin was added. In all cases DNA was stained with TOTO-3, Hoechst or propidium iodide. The samples were rinsed in PBS and mounted in 90% glycerol containing 2.5% n-propyl-gallate.

Metaphase I oocytes and oocytes at subsequent meiotic stages were obtained either by dissection of the ovaries or by collection from 5-7 day old females. Eggs were dechorionated in a 50% bleach solution for 2-3 minutes, rinsed in distilled water, dried on filter paper and transferred to a cold 1:1 mixture of heptane and methanol to remove the vitelline envelope. Eggs were then fixed for 10 minutes in cold methanol, washed in PBS and incubated for 1 hour in PBS containing 0.1% BSA. The eggs were incubated in the rabbit polyclonal anti-Asp serum Rb3133 overnight at 4°C and then with an anti-β-tubulin antibody for 4-5 hours at room temperature. After washing in PBS-BSA the eggs were incubated for one hour with the appropriate secondary antibodies. For simultaneous DNA staining, the eggs were incubated in propidium iodide or TOTO-3 iodide. Eggs were mounted in small drops of 90% glycerol containing 2.5% n-propyl-gallate.

Confocal images were obtained using a Leica TCS4D confocal microscope equipped with a Krypton/Argon laser (Leica Microsystems, Eidelberg). Images were collected using low laser emission to attenuate photobleaching and 8 frame-averaged scans made per image to improve the signal/noise ratio. Images collected at several focal planes were superimposed and merged into a single file and imported into Adobe Photoshop to adjust the size and contrast.

Results

Asp is found at the spindle poles and midzone during mitosis and meiosis in *Drosophila* testis

The role of Asp in nucleating microtubules at centrosomes is consistent with its localisation at the spindle poles during progression throughout mitosis in the embryonic and larval stages of *Drosophila* development. However, a functional rationale of the observation that Asp localises to the spindle midzone at late telophase in embryos has never been offered (Saunders et al., 1997). We decided to study this aspect of Asp localisation in male meiosis as the larger meiocytes allow greater resolution of subcellular localisation. The progenitors of spermatocytes are small cells that undergo four rounds of mitosis within cysts before undertaking a prolonged period of cell growth. Asp is associated with the spindle poles during these mitotic stages (Fig. 1A; only metaphases are shown) and relocates to the mid-zone at late telophase, where it appears to form a ring-like structure (Fig1B, arrow). The protein shows a similar localisation pattern during the two meiotic divisions and is localised at the spindle poles from prophase (Fig. 1C)

through metaphase (Fig. 1D) to early anaphase (not shown). A diffuse faint punctuate staining is also shown by the anti-Asp antibody in the central part of the spindle as early as metaphase (arrowheads in Fig. 1D). By telophase, this central accumulation becomes concentrated at the ends of microtubules that form the mid-zone of the spindle (Fig. 1E,F; arrowheads). Thus, at this point there are two prominent regions of Asp localisation: at the spindle poles (already duplicated in anticipation of meiosis II, Fig. 1E) to one side of the telophase nucleus and at the opposite side of the nucleus where it appears to be on the ends of bundles of microtubules on the borders of the central or mid-zone region of the spindle.

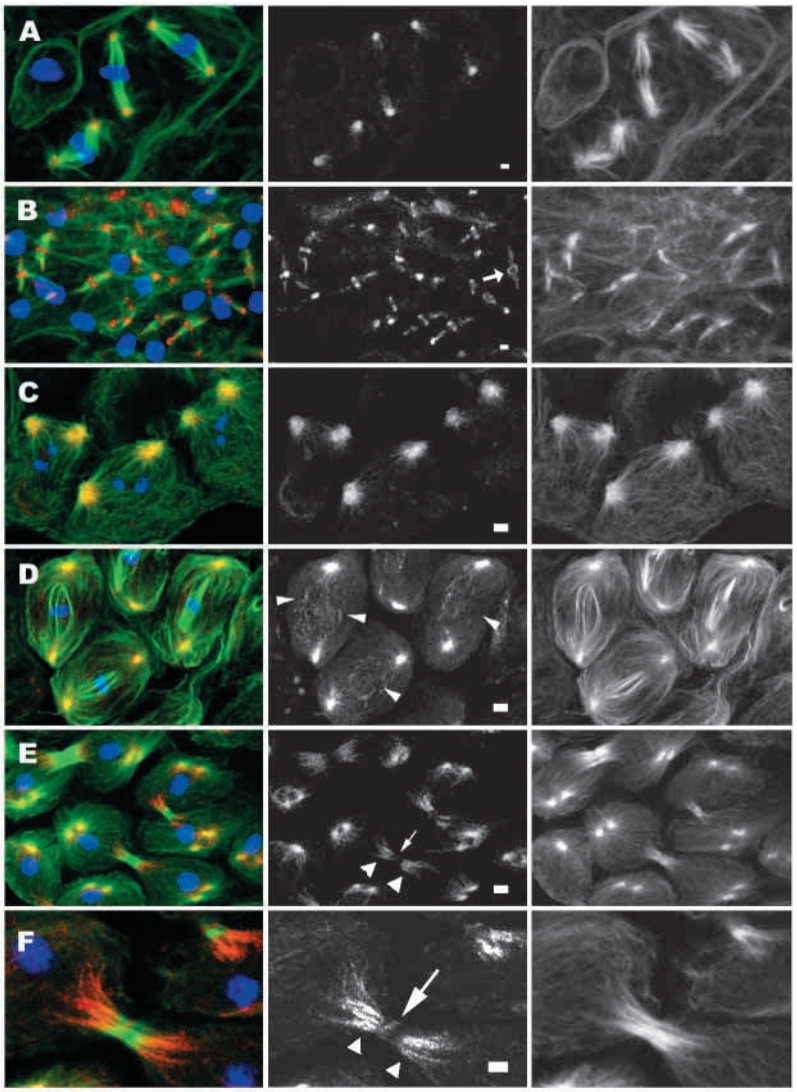

Fig. 1. Localisation of Asp protein in mitosis and meiosis during spermatogenesis. The merged image (left hand panels) shows DNA (blue), microtubules (green) and Asp (red). The channel showing Asp protein alone is shown in the central panels and microtubules alone in the right hand panels. (A) A four-cell cyst at metaphase and (B) an eight-cell cysts during late telophase; note the Asp ring between daughter nuclei (arrow). Progression through the first meiotic division: (C) Prometaphase meiotic spindles with Asp at the poles. (D) Metaphase meiotic cells with Asp staining strongly at the spindle poles and weakly in the midpoint of the spindles (arrowheads). (E) Telophase cells of meiosis I in which the Asp at the spindle poles falls into two clusters around the centrioles that are beginning to separate. In addition, these cells show prominent Asp staining along the putative minus ends of the central spindle microtubules, which are shown at higher magnification in (F) (arrowhead). The arrow in (E) and (F) point to a very central band of Asp staining that can also be seen in the central spindle. Scale bar is 10 μm.

Whereas meiotic spindle poles appear well focused in *asp* mutant meiosis, central spindle defects lead to a failure of cytokinesis

As mutations in *asp* are associated with disruptions in the compact nature of the MTOCs in third instar larval neuroblasts, resulting in broad spindle poles, we studied pole integrity during male meiosis in two *asp* mutant alleles. We examined asp^{1}/asp^{dd4}, asp^{d44}/asp^{dd4} and $asp^{dd4}/Df(3R)H\gamma^{\delta 1}$ (White-Cooper et al., 1996) males by immunostaining with antibodies that revealed tubulin and the centrosomal proteins Centrin, γ-tubulin and Centrosomin. The asp^{dd4} allele is a weak hypomorph that permits homozygotes to survive to adulthood. Such flies show rough eyes and wing defects characteristic of cell division cycle mutants, and both sexes show reduced fertility. It has previously been placed in an allelic series on the basis of the phenotypes seen in the sterile hemizygotes (White-Cooper et al., 1996). Here we observed comparable phenotypes in male meiosis in each of the allelic combinations studied. Our observations confirmed the meiotic spindle defects that have previously been reported for *asp*, principally, the long, wavy microtubules that are particularly evident at metaphase (Table 1). Examination of the spindle poles by immunostaining against Centrin (Figs 2A; 3AB), γ-tubulin (Figs 2B; 3C) and Centrosomin (Fig. 3D) revealed that they were well organised in discrete structures but were often irregularly positioned. Moreover, in contrast to the wild-type, the antibody against Centrin failed to recognise pericentiolar material and only stained the centrioles.

Whereas all cells in wild-type cysts are generally at similar stages of meiotic progression, we found that meiocytes in *asp* cysts were at a range of stages (Fig. 2). Indeed we consistently observed a greater proportion of cells in all stages of the first meiotic division in *asp* mutants and a reduction of the proportion in the second division in comparison to wild type (Table 2). This observation is consistent with the abnormal spindles leading to a checkpoint response, which in male meiosis is seen as a delay rather than an arrest in the progression through prophase and metaphase (Savoian et al., 2000; Rebollo and Gonzalez, 2000). It may also reflect difficulties in exit from the first meiotic division cycle. In any event, excluding the unlikely possibility that the phenotype is a direct consequence of earlier defects, the ability of these cells to progress beyond metaphase reveals mutant defects later in the meiotic cycle that suggest additional functions of the Asp protein other than at the spindle poles. We found that in contrast to the well organised poles, the central regions of the spindles of the late meiotic spindles were not

Table 1. Frequency of spermatocytes with spindle abnormalities during meiosis I* in *asp* mutant males

	Late prophase		Metaphase		Late anaphase				Late telophase			
Genotype	No	% irregular†	No	% irregular‡	No	% wild-type	% mis-formed	%§ absent	No	% wild-type	% mis-formed	%§ absent
Oregon R	384	0.2	273	0.3	120	98.9	1.1	0	138	99.2	0.8	0
asp^{1}	472	81.4	391	46.3	175	64.4	21.3	14.3	181	61.9	32.8	5.3
asp^{dd4}	684	86.9	486	49.2	198	57.2	27.5	15.3	226	56.6	36.2	7.2
asp^{1}/*asp*^{dd4}	597	92.1	434	53.1	164	52.7	31.2	16.1	211	53.7	38.2	8.1
asp^{1}/*Df(3R)Hδ*^{γ1}	312	83.4	234	48.1	107	61.1	28.7	10.2	109	60.1	34.0	5.9
asp^{dd4}/*Df(3R)Hδ*^{γ1}	328	84.1	302	48.6	102	58.8	30.6	10.6	151	59.5	34.1	6.4

*Meiosis II is not considered in this analysis because secondary spermatocytes resulting from failure of cytokinesis during meiosis I have serious defects in spindle organization and are therefore more difficult to analyse.

†Irregularities in late prophase arise from the positioning of centrosomes, which in *asp* cells are contained in two cytoplasmic pockets over the nucleus.

‡We have scored two irregular features, namely the irregular long and wavy microtubules in *asp* spindles and the absence of distinct bundles of kinetochore microtubules.

§Abnormalities in anaphase and telophase are defined by microtubule staining and localisation of Pav-KLP, Actin and Peanut.

correctly organised at anaphase and telophase in *asp* testes (compare the *asp* cells indicated with an arrowhead in Fig. 3D with wild-type cells in Fig. 1E,F). Depending upon the allelic combination, we found that mid-zone microtubules were poorly organised in 20-40% of cells within the cyst and absent in 5-15% of cells (Table 1). The absence of the mid-zone correlates with a failure of cytokinesis, which in turn leads to tetranucleate cells at the end of meiosis II (e.g. the cell shown in Fig. 3B). We were able to quantify the extent of cytokinetic failure by counting the numbers of multinucleate spermatids (Table 3). We found that in each allelic combination studied, up to approximately half of the cells complete cytokinesis in both meiotic divisions, but about 35% fail one and up to 10% fail both divisions. The leakiness of this mutant phenotype probably reflects the hypomorphic nature of the alleles under study.

To further correlate the central spindle defects described above with abnormal cytokinesis, we studied the distribution of three proteins that associate with the contractile furrow: Actin, Pav-Klp and Peanut (Fig. 4). In wild-type cells (inserts to Fig. 4), all three proteins are found in a ring-like structure at the central region of the spindle. A spectrum of localisation patterns of these proteins was seen in the *asp* mutant cells, which reflects the extent to which the central spindle had formed again, probably reflecting the leaky nature of the hypomorphic mutant combinations studied. We found the localisation of Actin to be most dramatically disrupted and scattered throughout the central part of the cell in fibrous aggregates (Fig. 4A). Nevertheless, some cells managed to produce a ring-like structure able to constrict the telophase cell (e.g. cell in bottom left of Fig. 4A). Similarly when stained to reveal Pavarotti, some meiotic cells had normal contractile rings (Fig. 4B, large arrow), whereas in others in the same field Pav-KLP was more diffuse (Fig. 4B, small arrow). The presence of Pav-KLP in ring canals (Fig. 4B, arrowheads) indicated that cytokinesis had occurred correctly within the pre-meiotic rounds of mitosis. Staining to reveal the septin Peanut also showed that some cells are able to construct an apparently normal contractile ring (Fig. 4C, arrow) in association with an organised central spindle region (see the microtubule staining of the same cell, monochrome image). In other cells at a similar stage, however, both the distribution of Peanut and the central spindle microtubules were disorganised (Fig. 4C, arrowhead).

Asp associates with the spindle poles and the central microtubule organising centre in female meiosis

In *Drosophila* female meiosis, an unusual microtubule organising structure known as the central spindle pole body is

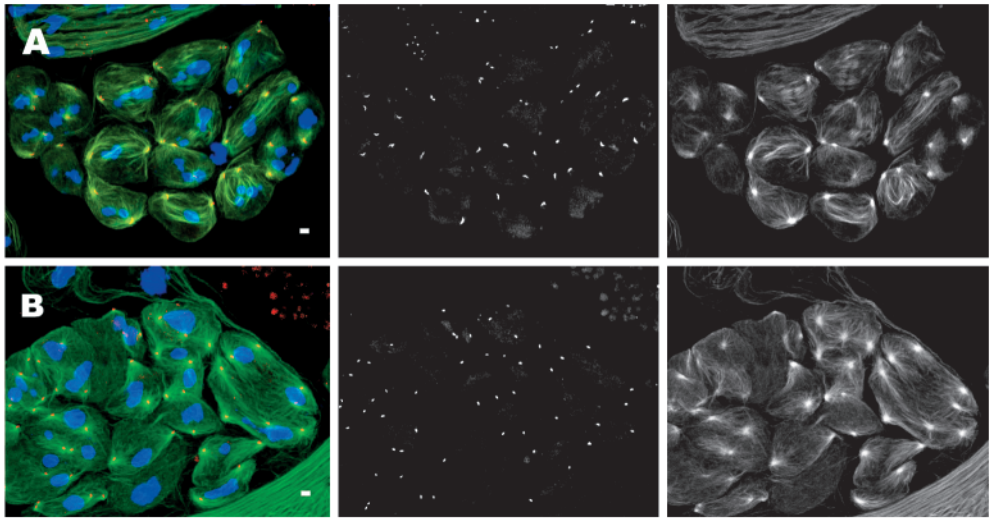

Fig. 2. Cysts of *asp*^{dd4} spermatocytes. The cells are stained to reveal DNA (blue), microtubules (green) and either Centrin (red; A) or γ-tubulin (red; B). The equivalent monochrome images for the centrosomal antigens and microtubules are shown in the respective central and right hand panels. Although the meiotic spindles look abnormal, their poles appear to be organised in discrete structures. Note the wide range of meiotic stages throughout the same cyst.

Table 2. Percentage of meiotic figures at different stages in testes of *asp* males

Genotype	Total cells scored	% Prophase I/ Prometaphase I	% Metaphase I	%Anaphase I/ Telophase I	% Prophase II/ Prometaphase II	% Metaphase II	% Anaphase II/ Telophase II
Oregon R	2816	21.3	9.7	9.3	26.4	12.8	20.5
asp^{1}	2126	31.1	18.4	17.2	15.2	7.1	11.0
asp^{dd4}	2381	33.7	20.4	18.3	12.4	6.3	8.9
asp^{1}/asp^{dd4}	2011	34.7	21.6	19.2	10.3	5.7	8.5
$asp^{1}/Df(3R)H\delta^{y11}$	1253	32.8	18.7	18.0	13.7	6.2	10.6
$asp^{dd4}/Df(3R)H\delta^{y1}$	1471	31.9	20.5	17.7	14.1	6.1	9.7

Table 3. Abnormal spermatids produced by mutant males carrying different allelic combination of asp

Genotype	Number of spermatids analysed	% spermatids with indicated numbers of nuclei		
		1 nucleus/cell	2 nuclei/cell	4 nuclei/cell
Oregon R	1813	99.8 (0)	0.2 (0)	0 (0)
asp^{1}	1375	55.7 (12.8)	32.1 (14.4)	12.2 (3.9)
asp^{dd4}	1297	50.3 (16.9)	35.7 (14.5)	14.0 (5.1)
asp^{1}/asp^{dd4}	1176	48.8 (18.1)	36.4 (15.1)	14.7 (6.0)
$asp^{1}/Df(3R)H\delta^{y11}$	1033	54.7 (13.9)	33.8 (12.2)	11.5 (3.9)
$asp^{dd4}/Df(3R)H\delta^{y1}$	1112	52.2 (14.5)	34.5 (13.9)	13.3 (4.7)

Variation in the nuclear size of spermatids is indicative of non-disjunction and has been previously described. The presence of multiple nuceli is an indication of the failure of cytokinesis.

formed between the daughter nuclei at the end of the first division. Female meiosis occurs without cytokinesis, and the shared pole of the second meiotic spindles is formed at the end of meiosis I in a position at which cytokinesis would be expected to occur in any other cell type. We therefore wished to determine the localisation of Asp with respect to the spindle poles at this unusual central microtubule organising center (MTOC). In female meiosis, the spindle is organised primarily by the condensed chromosomes and becomes focused at the poles by the concerted action of minus-end-directed motor proteins such as Ncd (Endow and Komma, 1996). Although these poles appear to lack γ-tubulin, by the criterion of immunostaining we found Asp protein at the poles at metaphase (Fig. 5A) and anaphase (Fig. 5B). At late anaphase

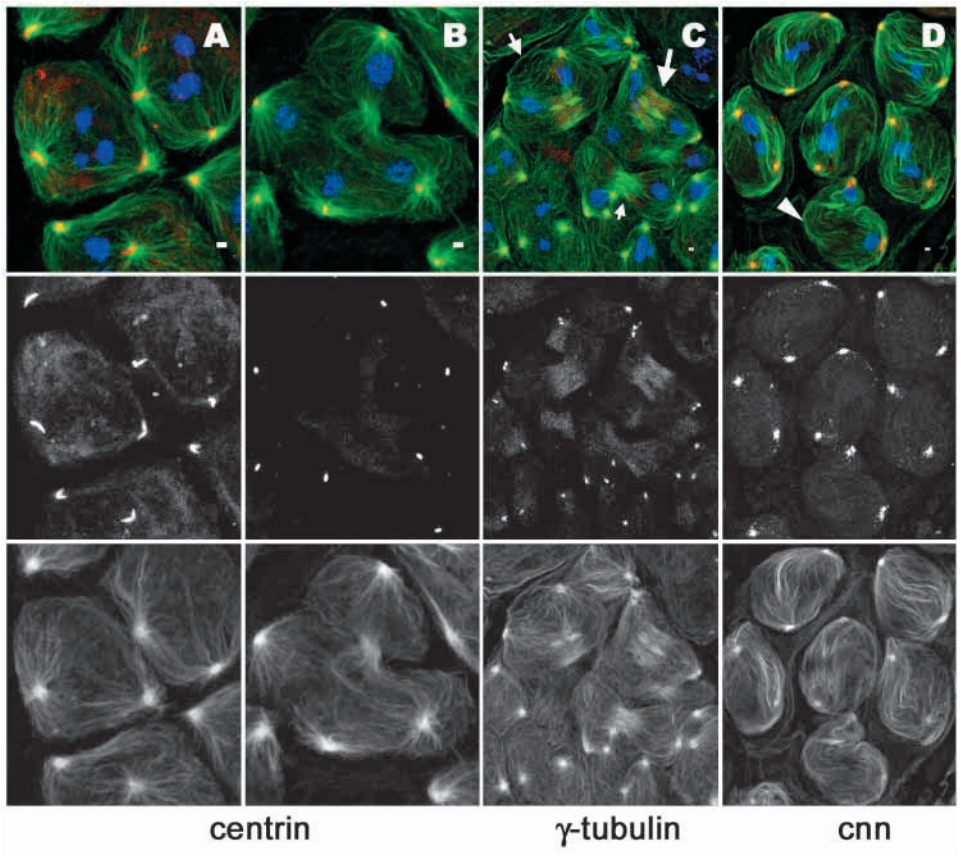

Fig. 3. asp^{dd4} meiocytes show well defined spindle poles and a range of central spindle defects. (A,B) Centrin (red in merged image and monochrome in central panels) is seen in a typical V-shaped pattern corresponding to adjacent centrioles at the spindle poles (A). The telophase tetrapolar cell in (B) shows no organisation of a central spindle. It has failed to undergo cytokinesis in meiosis I and appears set to fail the second meiotic division. (C) A cyst stained to reveal γ-tubulin (red in merged image, and monochrome in central panel). This illustrates the variation in the extent of formation of central spindle structures with this mutant allele. Central spindle structures are missing in several cells of the cyst. In this panel, three cells with central spindle structures are shown – in one it is poorly formed (small arrow), in another it is displaced (mid-sized arrow) and in the third it is comparable to wild type (large arrow). In all cases the merged image shows DNA in blue and microtubules in green. The corresponding monochrome images of microtubules are also shown in the bottom panels. (D) A cyst stained to reveal centrosomin (red in the merged image, and monochrome in the central panel). The arrowhead points to a cell lacking central spindle structure.

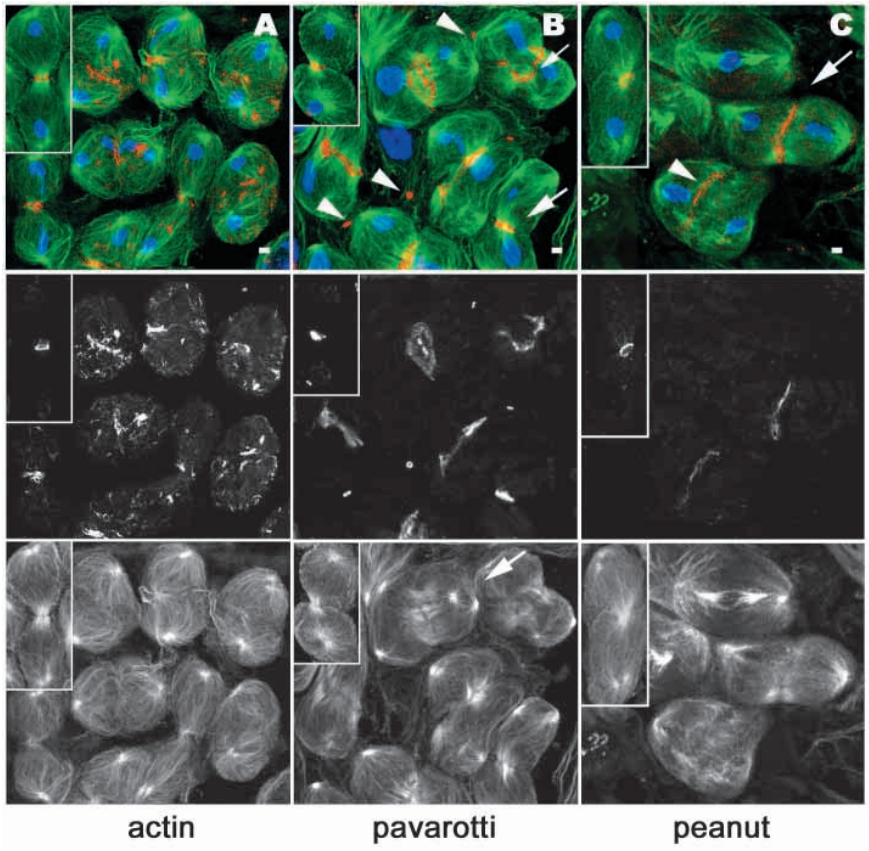

Fig. 4. Mislocalisation of mid-zone antigens in telophase cells of *asp*^{dd4} mutants. Localisation of actin (A), Pav-KLP (B) and Peanut (C) are shown in red in the merged image and in monochrome in the central panels. The DNA is blue and the microtubules are green. The corresponding images of microtubules are also shown in monochrome in the bottom panels. In each panel the inset shows the localisation of the corresponding proteins at an equivalent stage of wild-type meiosis. (A) Actin is not organised into a contractile ring as in the wild type but is dispersed in the central part of the cell. One exceptional cell in which the actin is sufficiently well organised to cause constriction of the cell is seen in the very bottom left of this field. Pav-KLP is present in ring-like structures in some cells (large arrow), but in the majority, it has a more diffuse distribution in the central part of the cell (the small arrow indicates one such example). It does stain distinct ring canals (arrowheads), indicating that cytokinesis has occurred in earlier division cycles. (C) In contrast to the wild-type telophase cell, Peanut does not accumulate in a contracted ring-like structure but remains in a wide band around the circumference of the mutant telophase cell. In some cells (arrow), the central spindle is better organised than others (arrowhead).

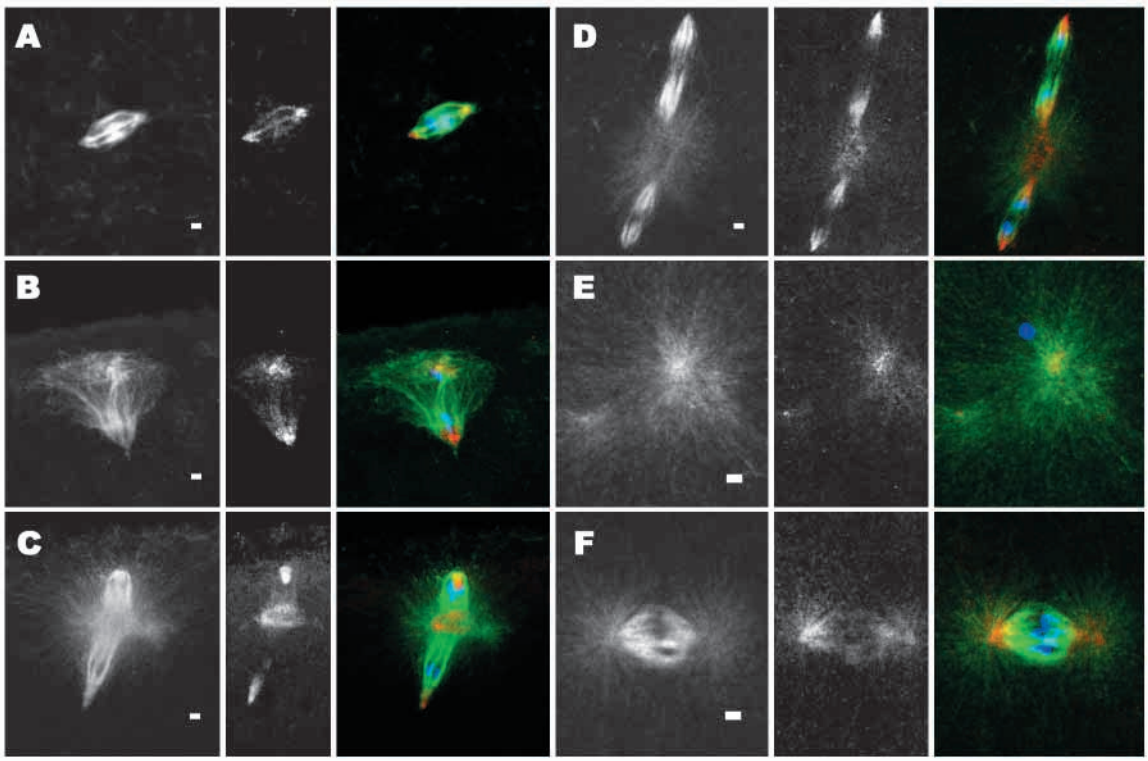

Fig. 5. Localisation of Asp protein (red) during meiosis in wild-type females. In all panels, microtubules are counter-stained green and DNA is blue. (A) Metaphase of meiosis I. Asp is present at the spindle poles. (B) Early telophase of meiosis I. (C) Late telophase of meiosis I. Note the accumulation of Asp in a ring-like structure in the central spindle around the central pole body. (D) Anaphase of meiosis II. Asp remains in the more diffuse structure connecting the two spindles and is in well defined regions at the spindle poles. (E) Asp at the centre of the sperm aster. (F) Asp at the poles of the gonomeric spindle of the first mitosis of the zygote.

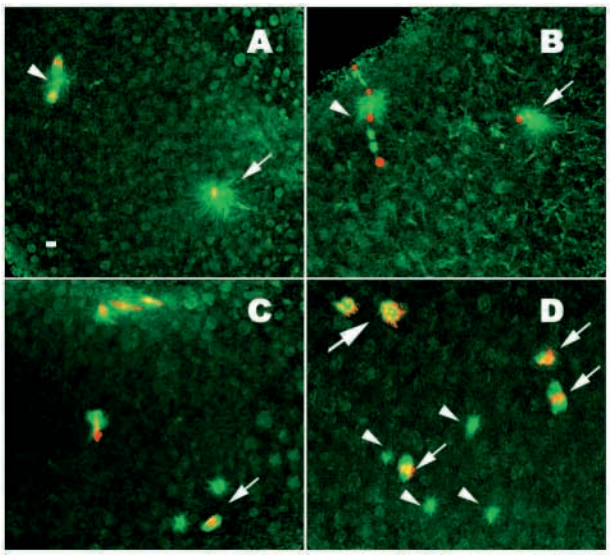

Fig. 6. Female meiosis in *asp*^{dd1}. DNA is stained red and microtubules are green. (A) This field shows the telophase spindle of meiosis I (arrowhead) and the male pronucleus with associated sperm aster (arrow). (B) Telophase of meiosis II (arrowhead). Unlike wild type, the sperm aster remains diminutive (arrow). (C) Dissociation of duplicated centrosomes from the male pronucleus (arrow). (D) This field shows polar bodies with condensed chromosomes (large arrow), and three haploid anastral mitotic spindles carrying DNA derived from the male pronucleus (arrows). There are also four independent asters of microtubules (arrowheads) derived from the sperm aster that has dissociated from the spindle.

or early telophase of meiosis I, we saw Asp as a well defined ring in the array of centrally nucleated microtubules (Fig. 5C). Prior to meiosis II, the region previously occupied by the plus ends of microtubules in contact with chromosomes has to become the site of microtubule minus ends at the shared central pole of the second meiotic spindle. At anaphase II, we found that Asp also became concentrated at the innermost spindle poles (Fig. 5D), and the ring-like staining at the central MTOC became more dispersed as meiosis II proceeded.

The sperm aster fails to grow and pronuclear fusion does not take place in *asp* mutant embryos

We then examined meiotic progression and behaviour of the pronuclei in eggs derived from *asp* mothers. We were only able to do this in oocytes derived from mothers homozygous for *asp*^{dd4} and transheterozygous for *asp*^{dd4}/*asp*^{1}. Meosis I progressed normally in such eggs, as can be seen by the spindle at early telophase (Fig. 6A, arrowhead), and the aster of microtubules that forms around the basal body, which was contributed by the incoming sperm, had begun to form (Fig. 6A, arrow). The second meiotic division also appeared to proceed normally (Fig. 6B, arrowhead). However, the sperm aster did not increase in dimension (Fig. 6B, arrow; Table 4). This should be contrasted with the sperm aster in wild-type eggs, where centrosomal proteins such as γ-tubulin and CP190 are recruited from the egg cytoplasm around the spindle basal body and the microtubules grow to reach the cortex of the egg cytoplasm. Immunostaining of wild-type eggs revealed that Asp was also recruited to this MTOC (Fig. 5E), which eventually contributes to both poles of the gonomeric spindles (Fig. 5F). In the *asp* mutant cytoplasm, the microtubules of the sperm aster never extended to the egg cortex. The sperm aster duplicated in the mutant eggs as it does in wild type, but it was frequently found to detach from the spindle that formed around the haploid male pronucleus (Fig. 6C, arrow). We found that these centrosome-associated asters continued to duplicate (Fig. 6D, arrowheads) as do the haploid male-derived nuclei that can undergo multiple rounds of haploid mitosis (Fig. 6D, small arrows; Table 4). However, the haploid products of female meiosis frequently appeared to condense to form typical polar body-like structures (Fig. 6D, large arrow).

Discussion

asp mutations highlight differences in centrosome organisation in mitosis and meiosis

The Abnormal Spindle protein forms a hemi-spherical cup-like structure overlying the spindle-facing side of centrosomes in larval cells. We show here that on the male meiotic spindle it extends much further along the tips of the minus end microtubules at the spindle poles. Moreover, unlike *asp* mutant neuroblasts where γ-tubulin is present in poorly organized bodies at broad spindle poles, in *asp* male meiotic spindles γ-tubulin has a similar distribution to wild-type and is present at well focused poles. Indeed, the only substantive difference in the localisation of centrosomal antigens that we were able to detect in male meiotic spindles was with centrin, which was more tightly restricted to the putative centrioles than in wild-type cells. While these observed differences between mitotic and meiotic centrosomes could reflect the leaky nature of the alleles studied or a more stringent requirement for Asp in one cell type rather than the other, they could also indicate differences in the organisation of centrosomes in mitosis and male meiosis. These could be related to the need to produce individual centrioles through the reductive divisions of the

Table 4. Failure of pronuclear migration and zygotic mitoses in *asp*-derived eggs

Genotype	Total eggs scored	Pronuclear migration	Pronuclear apposition	First mitosis			Others
				Gonomeric	Haploid spindles	Irregular spindles	
Oregon R	937	2.8	7.7	77.1	0	0	12.4
asp^{dd4}/*asp*^{dd4}	422	11.7	1.9	4.5	48.1	16.2	17.6
asp^{1}/*asp*^{dd4}	369	14.3	0.8	3.9	46.4	16.7	17.9

The Table lists the proportion (%) of eggs at the indicated stages 20 minutes after deposition. Eggs were fixed for 5 minutes and stained with anti-tubulin antibody and Hoechst dye to score DNA and microtubules. Figures were scored as follows. Pronuclear migration: male and female pronuclei were not contacted. Pronuclear apposition: male and female pronuclei were in contact. First mitosis: stages ranging from prophase to telophase of the first nuclear cycle in which gonomeric, haploid or abnormal spindles are present. Others: various developmental stages after the first mitosis.

latter process that will eventually transform into the basal bodies in spermatids.

The spindle poles in female meiosis differ further from those of either mitosis or male meiosis. These spindles are initially nucleated by chromosomes, and the spindle poles are focused by the action of microtubule-associated motor proteins, particularly the *ncd*-encoded kinesin-like protein (Endow and Komma, 1996; Endow and Komma, 1997). Although the poles of the female meiotic spindle are not stained by antibodies that recognize γ-tubulin, progression through female meiosis shows some dependence upon this protein (Tavosanis et al., 1997; Wilson et al., 1997). In contrast to the low abundance of γ-tubulin, we find Asp to be abundant at the acentriolar poles of the female meiotic spindle. Nevertheless, meiosis appears able to proceed, at least for the mutant *asp* alleles we have selected for study. These are all hypomorphic and give leaky phenotypes that generally allow the premeiotic mitoses to proceed in both sexes and so permit the progression of both the primary spermatocytes and the oocyte to meiosis.

Microtubules are also nucleated in the fertilised egg by a centrosome that develops around the basal body of the sperm following its recruitment of centrosomal proteins such as CP190 and γ-tubulin (Callaini and Riparbelli, 1996; Riparbelli and Callaini, 1996). This sperm aster normally comprises short microtubules until metaphase II of female meiosis, at which time the microtubules begin to grow and make contact with the cortex of the egg. We have found that growth of the sperm aster does not take place in *asp* mutants. A similar phenotype is seen in eggs derived from *polo* mutant mothers (Riparbelli et al., 2000). It is possible that the dramatic effects of both *polo* and *asp* mutants on growth of the sperm aster may reflect the ability of Polo kinase to phosphorylate and activate the microtubule organising properties of Asp (Avides et al., 2001). In both *asp*- and *polo*-derived eggs, as a consequence of the failure of the sperm aster to grow, the female pronucleus cannot migrate to meet its male counterpart, leading to the failure of the first gonomeric division of the zygote. In both types of mutant cytoplasm, the centrioles associated with the sperm aster can dissociate from the male pronucleus and undergo autonomous replication cycles in the syncytium. The male pronuclei can also undergo several rounds of haploid mitoses in both mutants. One noteworthy difference between *asp*- and *polo*-derived eggs is that in *asp*, the female pronuclei remain arrested as the polar body conglomerate whereas in *polo* eggs they can escape from this arrest and also undergo several rounds of haploid mitosis (Riparbelli et al., 2000).

An MTOC at cytokinesis?

In late anaphase-telophase of male meiosis, Asp localises to the spindle mid-zone, but unlike other centrosomal antigens such as Pavarotti-KLP and Polo kinase that become associated with the central region of the spindle mid-zone, the majority of Asp decorates the very terminal regions of mid-zone microtubules. At this stage the microtubules are positioned between the telophase nuclei and the centre of the spindle. The terminal regions are likely to be the minus ends of microtubules that have been released from the centrosomes, which at this stage nucleate independent asters of microtubules. This association of Asp with the spindle mid zone appears to be required for the assembly of the correct structure of the late central spindle and in turn for cytokinesis. The central spindle plays an essential role during cytokinesis and there is a cooperative interaction between this structure and the actin-myosin contractile ring: whenever one of the structures is disrupted the other fails to assemble and function (Gatti et al., 2000). In keeping with this, we have found that many cells within *asp* mutant cysts have abnormal central spindles lacking the characteristic interdigitating microtubules. Moreover, molecules that participate in forming parts of the contractile ring, Pavarotti-KLP, the septin Peanut, Polo kinase and Actin, do not localise properly in *asp* mutant spermatocytes.

While our manuscript was in preparation, Wakefield et al. (Wakefield et al., 2001) have also analysed the role of the Asp protein at the spindle poles and in cytokinesis. We see no conflicts between their conclusion that Asp plays a role in microtubule bundling at the spindle poles and an earlier report that Asp contributes to the integrity of mitotic MTOCs (Avides and Glover, 1999). However, Asp may not be sufficient to bundle microtubules, as when centrosomal antigens are dispersed following the disruption of the γ-tubulin ring complex, spindle poles can be severely splayed even though Asp is present at their minus ends (Barbosa et al., 2000). Our present results confirm and extend the findings of Wakefield et

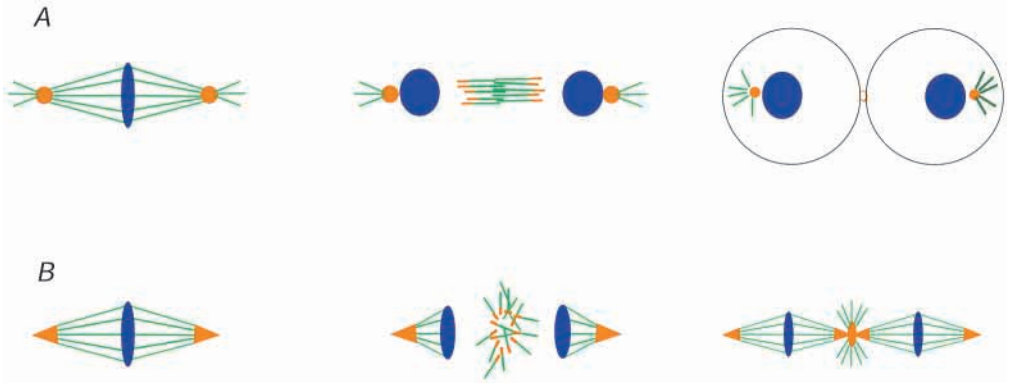

Fig. 7. Model of Asp function during mitosis/meiosis in males (A) and meiosis in females (B). Asp is depicted in orange and is localised at the minus ends of microtubules (green). DNA is shown in blue. (A) Asp is associated with the free microtubule minus ends at the metaphase spindle poles (left). At early telophase (centre), the central part of the spindle is reorganised, and a subpopulation of Asp molecules participate in nucleating central spindle microtubules. Following the completion of cytokinesis, Asp protein remains associated with the mid-body (somatic mitoses) or the ring canals (mitosis and meiosis in spermatogenesis). (B) Asp is at the acentriolar poles of the female meiotic spindles at metaphase I. The structure of the central MTOC starts to form at early telophase I by a reversal of the polarity of the central spindle microtubules. Asp is recruited to the central region at this time and is shown participating in the nucleation of the minus ends of these central spindle microtubules.

al. (2001) that Asp is required to organise microtubules at the central region of the late mitotic spindle to enable cytokinesis (Wakefield et al., 2001). We speculate that the minus ends of these microtubules have dissociated from the spindle poles and now rely on the Asp protein for their stability in order to form a mid-zone structure for the central spindle. Moreover, the idea that the central spindle results from de novo nucleation of microtubules by transient organising centres located in the region between the two daughter nuclei is substantiated by a requirement for γ-tubulin in the equatorial region at the time of central spindle formation (Julian et al., 1993; Shu et al., 1995).

Asp localisation in female meiosis – a unifying model?

Female meiosis differs from male meiosis and mitosis in *Drosophila* not only in that the spindle poles lack centrioles but also in that the first division is not followed by cytokinesis. Instead the central spindle has been postulated to undergo reorganisation to reverse the polarity of microtubules around an unusual central spindle pole body and so form two tandemly oriented spindles for meiosis II. A model for how this occurs has been presented by Endow and Komma (Endow and Komma, 1998) and is modified in Fig. 7B. This central spindle pole body recruits centrosomal antigens, including CP190 and γ-tubulin. It is also striking that it additionally recruits molecules that are known to function in cytokinesis. These include Pav-KLP (Riparbelli et al., 2000) and Asp (this paper).

We note that this specialised central pole body of female meiosis in *Drosophila* has several features in common with the central spindle of conventional divisions, and we speculate that Asp may be one of several centrosomal/central-spindle-associated proteins that play common roles in setting up these structures. In late anaphase, during both the conventional divisions and female meiosis I, a subpopulation of microtubules have to become detached from the initial poles. In effect the two half spindles of female meiosis I separate but remain focused at their original poles. The parallel step in male meiosis or in a conventional mitosis is seen by the ability of the original centrosomes to continue to nucleate asters of microtubules. In contrast, microtubules in the central part of the female meiosis I spindle must be reorganised such that their polarity is reversed to form the linked central poles of the tandemly arranged meiosis II spindles. The Asp-containing central pole body that develops in this region appears first as a ring like structure that nucleates a broad mid-zone region of microtubules that only later becomes independently focused as the meiosis II spindles migrate apart. In mitosis or male meiosis, Asp appears to be at the ends of the reorganised microtubules that form the central spindle. As telophase develops, this mid-zone becomes compacted as it coordinates the formation of the contractile ring. Indeed in male meiosis, it will ultimately be incorporated into the ring canal.

Microtubule-nucleating centres that reorganise the late central region of the spindle may be a highly conserved feature of cell division. In fission yeast, a transient MTOC nucleates a post-anaphase array of microtubules in the central part of the cell before the initiation of septation (Hagan and Hyams, 1988). This is probably the fission yeast counterpart of the central spindle region, as its formation is promoted by overexpression of the fission yeast homologue of Polo kinase encoded by *plo1*, and it drives septation at inappropriate stages of cell cycle progression (M.G.R., G.C., D.M.G. and M.d.C.A., unpublished).

Progression through M-phase appears to require the coordination of the activities of several MTOCs. The chromosomes themselves can provide nucleating centres for the plus ends of microtubules, and centrosomes, if present, can contribute a minus-end organising activity. Our results suggest, however, that components of such a minus-end organising activity may not be restricted to the poles but may later in M-phase be regrouped to participate in organising the spindle mid-zone in order to successfully execute cytokinesis.

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