Spindle Self-organization and Cytokinesis During Male Meiosis in asterless Mutants of Drosophila melanogaster

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Abstract. While Drosophila female meiosis is anastral, both meiotic divisions in Drosophila males exhibit prominent asters. We have identified a gene we call asterless (asl) that is required for aster formation during male meiosis. Ultrastructural analysis showed that asl mutants have morphologically normal centrioles. However, immunostaining with antibodies directed either to γ tubulin or centrosomin revealed that these proteins do not accumulate in the centrosomes, as occurs in wild-type. Thus, asl appears to specify a function required for the assembly of centrosomal material around the centrioles.

Despite the absence of asters, meiotic cells of asl mutants manage to develop an anastral spindle. Microtubules grow from multiple sites around the chromosomes, and then focus into a peculiar bipolar spindle that mediates chromosome segregation, although in a highly irregular way.

Surprisingly, asl spermatocytes eventually form a morphologically normal ana–telophase central spindle that has full ability to stimulate cytokinesis. These findings challenge the classical view on central spindle assembly, arguing for a self-organization of this structure from either preexisting or newly formed microtubules. In addition, these findings strongly suggest that the asters are not required for signaling cytokinesis.

Key words: centrosome • spindle assembly • cytokinesis • male meiosis • Drosophila

Chromosome segregation during both mitosis and meiosis is mediated by the spindle, a complex bipolar structure consisting of microtubules and associated proteins. Although the basic structure of the spindle is similar in all cell types of all higher eukaryotes, the routes through which the spindle assemblies can be substantially different (reviewed by Rieder et al., 1993; Merdes and Cleveland, 1997; Waters and Salmon, 1997).

In animal mitotic cells, spindle formation is mediated by the centrosomes. During prophase, duplicated centrosomes, while moving to the opposite poles of the cell, nucleate radial arrays of microtubules called the asters. After the breakdown of the nuclear envelope, the plus ends of astral microtubules are captured and stabilized by the kinetochores, allowing the formation of a bipolar spindle.

In contrast, higher plant cells and female meiotic cells of several animal species such as Caenorhabditis, Drosophila, or Xenopus, do not contain centrosomes (Smirnova and Bajer, 1992; Albertson and Thompson, 1993; Theurkauf and Hawley, 1992; Gard, 1992). In these systems, microtubules grow from multiple sites around the chromosomes and progressively self-organize into a bipolar spindle. Studies on Drosophila female meiosis and in vitro spindle assembly from Xenopus egg extracts have shown that microtubule focusing into spindle poles is mediated by minus-end–directed motor proteins. In Drosophila, the assembly and maintenance of a bipolar meiotic spindle requires the action of Ncd, a minus-end directed kinesin motor protein (Hatsumi and Endow, 1992; Matthies et al., 1996; Endow and Komma, 1997). Similarly, in Xenopus egg extracts spindle pole formation is mediated by cytoplasmic dynein, another minus-end–directed motor protein (Heald et al., 1996; Heald et al., 1997). Dynein forms a complex with NuMA (nuclear/mitotic apparatus protein) and dynactin, both of which are also necessary for proper microtubule focusing at the spindle poles (Merdes et al., 1996; Merdes and Cleveland, 1997). Thus, in acentrosomal spindles the minus-ends of the microtubules that grow around the chromatin move and converge towards the poles through the action of minus-end microtubule-based motors and their associated proteins.

Recent studies have shown that cells without centrosomes and cells with centrosomes share common mechanisms of spindle pole assembly (reviewed by Merdes and...
Cleveland, 1997). Inhibition of cytoplasmic dynein by a dynein-specific antibody disrupts spindle pole formation in both centrosome-free and centrosome-containing spindles (Gaglio et al., 1997; Heald et al., 1997). Furthermore, in the latter systems dynein depletion results in the detachment of centrosomes from the spindle poles (Gaglio et al., 1995; Eccher et al., 1996). These observations, and the finding that most intermicrotubule tetrads are not connected to the centrosomes (Mastronarde et al., 1993), suggested a model for pole formation in centrosome-containing spindles (Gaglio et al., 1997; Heald et al., 1997). It has been proposed that a substantial fraction of the microtubules nucleated by the centrosomes is released from these structures during the prometaphase search and capture process (Kirschner and Mitchison, 1986). The free minus-ends of these microtubules are then focused at the spindle poles through the action of the same structural and motor proteins that mediate pole formation in centrosomal systems. The translocation of the microtubule minus-ends towards the spindle poles, coupled with microtubule elongation at the plus-ends and microtubule shortening at the minus-ends, would then create a poleward microtubule flux that exerts force through the spindle (Waters and Salmon, 1996; Waters and Salmon, 1997). In addition, lateral interactions between the astral microtubules and the free minus-ends of poleward-migrating microtubules would tether the centrosomes to the spindle poles, restoring the connection between these organelles and the rest of the spindle.

If the poles are assembled with similar mechanisms in both centrosomal and centrosome-less systems, centrosome-containing cells should be able to assemble a spindle even in the absence of centrosomes. In most cell types, however, this is not the case. For example, micromanipulation experiments carried out in echinoderm embryonic cells, vertebrate somatic cells, and grasshopper spermatocytes have clearly shown that removal of centrosomes from prophase cells prevents spindle formation (Sluder and Rieder, 1985; Sluder et al., 1986; Rieder and Alexander, 1990; Rieder et al., 1993; Zhang and Nicklas, 1995). However, if centrosomes are removed or lost during anaphase, the spindle poles remain focused, and chromosome segregation is not affected (for review see Waters and Salmon, 1997). On the other hand, experiments on spermatocytes of the crane fly Pales ferruginea indicate that in these cells spindle pole assembly is independent of the presence of centrosomes (Steffen et al., 1986). The reason why Pales spermatocytes can assemble spindle poles in the absence of centrosomes whereas the other systems cannot, is not understood. An intriguing possibility is that the requirement of centrosomes for spindle assembly simply reflects the fact that in some cell types these organelles are the only source of microtubule nucleation. Thus, in the absence of centrosomes, there would not be enough microtubules to be focused at the spindle poles, and spindle assembly would be prevented (Waters and Salmon, 1997).

In this paper we describe another centrosome-containing system that does not require centrosomes for spindle formation. While Drosophila female meiosis is anastral (Theurkauf and Hawley, 1992), both meiotic divisions in Drosophila males exhibit prominent asters (Cenci et al., 1994; see Fig. 2). We have genetically micromanipulated Drosophila male meiosis by means of mutations in aster-less (asl), a gene required for centrosome assembly and aster formation. In asl spermatocytes, despite the absence of functional centrosomes, microtubules grow from multiple sites around the chromosomes, and self-organize into peculiar anastral spindles. These spindles manage to mediate chromosome segregation, although in a very irregular way. Surprisingly, asl spermatocytes develop a morphologically normal anaphase–telophase central spindle. The finding that asl mutants are completely devoid of asters and have normal central spindles gave us the opportunity to test the relative role of these structures in signaling cytokinesis. Our results show that central spindles are fully able to induce cytokinesis, indicating that asters are not required for the cytokinetic signal.

Materials and Methods

Drosophila Stocks and Mutagenesis

To isolate the asl\textsuperscript{2} and asl\textsuperscript{3} mutant alleles we mutagenized \textit{ca} males with a 25-mM ethyl methane sulfonate (EMS)\textsuperscript{1} solution (Lewis and Bacher, 1991). We labeled them with \textit{cy-green-R} virgin females. The F1 \textit{e ca}\textsuperscript{+/+} females were crossed individually to asl\textsuperscript{2}/TM6C, \textit{Sb e Tb ca} females, and their e\textsuperscript{ca}asl\textsuperscript{2} e\textsuperscript{ca} males progeny were tested for fertility. The e\textsuperscript{ca}TM6C brothers of the sterile males were then mated to ap\textsuperscript{v}TM6C females to balance the putative asl alleles. The asl mutations (asl\textsuperscript{2}, asl\textsuperscript{3}, and asl\textsuperscript{4}) were kept over the TM6C balancer that carries the body-shape marker Tubby (Tb), allowing identification of homozygous asl larvae and pupae. All the balancers and markers used for mutagenesis and mapping are described in Lindsley and Zimm (1992). The flies were reared on standard Drosophila medium at 25 ± 1°C; dissections were performed at room temperature.

Immunofluorescence Microscopy

Cytological preparations were made with testes from third instar larvae or from young pupae. For tubulin immunostaining, KLP3A plus tubulin immunostaining, and anillin plus tubulin immunostaining, testes were fixed as described previously (Cenci et al., 1994; Williams et al., 1995). For phalloidin staining and tubulin immunostaining, testes were fixed according to Gunsalus et al., 1995. For \(\gamma\) tubulin plus tubulin immunostaining or centrosomin plus tubulin immunostaining, testes were dissected and frozen in liquid nitrogen as described (Cenci et al., 1994). Preparations were then fixed in cold methanol for 15 min and acetone for 30 s, and were then immersed for 10 min in PBS containing 0.1% Tween 20 and 0.1% acetic acid. Before incubation with antibodies, slides were rinsed several times in PBS containing 0.1% Tween 20.

Tubulin immunostaining and phalloidin staining plus tubulin immunostaining have been described previously (Cenci et al., 1994; Gunsalus et al., 1995). For double immunostainings, testes were first incubated overnight at 4°C with any of the following rabbit primary antibodies diluted in PBT (PBS containing 0.1% Triton X-100) containing 1% BSA: anti-\(\gamma\)-tubulin (1:200; Calliani et al., 1997); anti-centrosomin (1:1,000; Li and Kaufman, 1996); anti-KLP3A (1:500; Williams et al., 1995); or anti-anillin raised against amino acids 1–371 (1:300; Field and Alberts, 1995). These primary antibodies were detected by 2-h incubation at room temperature with TRITC-conjugated anti-rabbit IgG (Cappel Laboratories, Malvern, PA) diluted 1:100 in PBT. Slides were then incubated with a monoclonal anti-\(\alpha\)-tubulin antibody (Pharmacia Biotech, Inc., Piscataway, NJ) diluted 1:50 in PBS, which was detected by FLUOS-conjugated sheep anti–mouse IgG (Boehringer Mannheim, Mannheim, Germany) diluted 1:10 in PBS. After these immunostainings, testis preparations were air-dried and stained with Hoechst 33258 as described (Cenci et al., 1994).

All preparations were examined with an Axiosplan (Carl Zeiss, Oberkochen, Germany) microscope equipped with an HBO 50W mercury lamp for epifluorescence, and with a cooled charge-coupled device (CCD; Photometrics Inc., Woburn, MA). Hoechst 33258, FLUOS, and TRITC fluorescence were detected as described (Gunsalus et al., 1995). Gray-

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\textsuperscript{1} Abbreviation used in this paper: EMS, ethyl methane sulfonate.
scale digital images were collected separately using the IP Lab Spectrum software. Images were then converted to Photoshop 2.5 format (Adobe System, Inc., Mountain View, CA), pseudocoloured, and merged.

Electron Microscopy

Testes dissected from asl\(^1\) adult males were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 1 h, washed four times in phosphate buffer (5 min each), and postfixed in 1% OsO\(_4\) in the same buffer for 1 h. After four washes in phosphate buffer (5 min each), testes were dehydrated with ethanol (30, 50, and 70% 3 times; 95 and 100%, 3 x, 10 min each at room temperature). Testes were embedded in Epon and, after sectioning, were stained with 3% uranyl acetate and lead citrate.

Results

Isolation and Characterization of asterless Mutations

The first asl mutant allele (asl\(^1\)) was isolated in the course of a cytological screen of a collection of 16 EMS-induced male sterile mutations kindly provided by Barbara Wakimoto (University of Washington, Seattle, WA). Living preparations of mutant testes were examined by phase contrast microscopy for defects in the onion stage spermatids. In wild-type, each spermatid contains one phase-light nucleus and one phase-dense mitochondrial derivative called the Nebenkern (reviewed by Fuller, 1993). At the onion stage of spermatid development, the nuclei and Nebenkern have spherical shapes and very similar sizes (Fig. 1). The regular size of both nuclei and Nebenkern depends on the correct execution of the meiotic process; abnormal-sized nuclei and Nebenkern are diagnostic of errors in chromosome segregation and in the partition of mitochondria, respectively (Gonzalez et al., 1989; Fuller, 1993). As shown in Fig. 1, asl\(^1\) spermatids are composed of nuclei and Nebenkern of very different sizes, suggesting that asl mutations disrupt both meiotic chromosome segregation and the correct distribution of mitochondria between the daughter cells.

asl\(^1\) is perfectly viable but sterile in both sexes. The phenotypes of male sterility, female sterility, and aberrant spermatids associated with asl\(^1\) were mapped using a ri Ki p\(^o\) chromosome by examining 44 recombinants between ri and p\(^o\) (these markers define an interval of 1 cM). This analysis showed that these three phenotypes comap just to the left of Kinked (Ki), from which they are separated by only one recombination event.

To determine whether the asl\(^1\) phenotype is specifically elicited by this mutation or is a general characteristic of lesions in the asl locus, we isolated two additional mutant alleles. We treated 2,360 chromosomes with EMS and tested them for allelism with asl\(^1\). This screen yielded two new mutations, asl\(^2\) and asl\(^3\), that are viable over asl\(^1\) and fail to complement asl\(^1\) for male and female sterility as well as for the aberrant spermatid phenotype. However, asl\(^1\)/asl\(^2\), asl\(^2\)/asl\(^3\), and asl\(^1\)/asl\(^3\) individuals are lethal; asl\(^2\)/asl\(^2\) and asl\(^2\)/asl\(^3\) larvae die at the larval pupal boundary, whereas asl\(^2\)/asl\(^3\) individuals have an earlier lethal phase. In addition, recombination experiments failed to separate the late lethal phenotype from asl\(^2\) and the earlier lethal phenotype from asl\(^3\). Thus, we conclude that asl is an essential locus required for viability. At present, however, we do not know whether asl\(^2\) is a null mutation. The fact that asl maps very close to Triple lethal (Tpl) prevented examination of the phenotype of asl\(^1\) over deficiency and its comparison with that of asl\(^2\)/asl\(^3\) individuals. In this context, it is of interest that the pattern and frequency of abnormal spermatids is very similar in all mutant combinations (asl\(^1\)/asl\(^1\), asl\(^1\)/asl\(^2\), asl\(^2\)/asl\(^2\), asl\(^2\)/asl\(^3\), and asl\(^3\)/asl\(^3\)), indicating that the three mutant alleles cause similar disruptions of the asl\(^+\) function during male meiosis.

**Figure 1.** Abnormal spermatids in asl mutants. Live testis squashes were viewed by phase contrast microscopy to examine onion stage spermatids. (A) Regular spermatids from Oregon-R controls with nuclei (white structures) and nebenkern (dark structures) of similar sizes. (B) Spermatids from asl mutants showing nuclei and nebenkern of various sizes. See text for details on the origin of these aberrant spermatids. Bar, 10 μm.

asl Spermatocytes are Devoid of Asters and have Defective Centrosomes

To define the primary lesion leading to the formation of aberrant spermatids in asl mutants, we analyzed cytologically the meiotic division. Testis preparations were stained with anti-tubulin antibodies and Hoechst 33258 for simultaneous visualization of both microtubules and chromatin. Examination of male meiosis in asl\(^1\)/asl\(^1\), asl\(^1\)/asl\(^2\), asl\(^2\)/asl\(^2\), asl\(^2\)/asl\(^3\), and asl\(^3\)/asl\(^3\) animals revealed that all these mutant combinations cause a common cytological phenotype. Whereas wild-type spermatocytes exhibit prominent asters throughout meiotic cell division (Fig. 2; see Cenci et al. 1994 for a detailed description of male meiosis), asl spermatocytes are completely devoid of asters (Fig. 3).

To determine whether the absence of asters in asl mutants was the consequence of a primary defect in centrosome structure, we immunostained mutant testes with antibodies directed to either γ tubulin or centrosomin, two components of *Drosophila* centrosomes (Zheng et al., 1991; Li and Kaufman, 1996). In wild-type testes, anti-γ tubulin antibodies immunostain the centrosomes in primary spermatocytes and throughout meiosis (Fig. 4). In mature primary spermatocytes, the centrosomes are located near the plasma membrane (not shown). Before the first meiotic division they migrate to the periphery of the nuclear envelope where they nucleate prominent asters that move to the opposite poles of the cell (Fig. 4 A’). In anaphase and early telophase I there is a single centrosome at each spindle pole, which in late telophase I splits into two centrosomes that start migrating to the...
poles of secondary spermatocytes while nucleating new asters (Fig. 4, B and C). These centrosomes remain at the spindle poles throughout the second meiotic division and do not split into two separate entities in late telophase II so that each spermatid receives a single centrosome. In contrast, in \( asl \) mutants \( \gamma \) tubulin is not concentrated in the centrosomes during any phase of primary spermatocyte growth and meiotic cell division (Fig. 4, D–F). Instead, it is dispersed in multiple small aggregates that do not appear to have the ability to nucleate microtubules.

Similar but not identical results were obtained with centrosomin. In wild-type, centrosomin accumulates in the centrosomes of both premeiotic primary spermatocytes and meiotic cells, just as \( \gamma \) tubulin (Fig. 5). In \( asl \) mutants, antibodies directed to centrosomin fail to detect discrete centrosomal entities in most mature primary spermatocytes at the S5 stage (Fig. 5 F). However, in late prophase/prometaphase primary spermatocytes at the M1 stage, anti-centrosomin antibodies immunostain two structures located near the nuclear envelope. These structures are consistently paired, as are the wild-type centrosomes before their migration to the cell poles, but are much less fluorescent than regular centrosomes and fail to nucleate astral microtubules (Fig. 5, G and G'). During ana–telophase I, the centrosomin-enriched bodies are always detected at around the bivalents; this type of meiotic stage is never seen in the wild-type. (C) A metaphase I–like figure in which two large bivalents are associated with minispindles, and another large bivalent (upper right) is surrounded by microtubules that are not clearly polarized. Note that the tiny fourth chromosomes that have just begun to segregate are associated with very few microtubules. (D) An anaphase I–like stage in which three pairs of homologs (including the fourth chromosomes) have segregated, while a large bivalent (bottom left) is still unseparated. (E and F) An anaphase I–like figure showing segregation of sister chromatids; E shows only the chromosomes, while F shows both the chromosomes and the microtubules. See text for further explanation. (G) A telophase I figure showing a morphologically normal central spindle and scattered chromosomes at the poles. (H) A telophase I with a tripartite central spindle where the chromosomes have segregated only to two poles; in this cell the chromosomes are atypically congregated into discrete telophase nuclei. Bar, 10 \( \mu \)m.
only one of the cell poles, whereas the other pole is consistently devoid of them (Fig. 5, H–I’). In addition, although they usually appear as a pair of fluorescent spots (Fig. 5 H), they are occasionally resolved into four entities (Fig. 5 I). At telophase I, the centrosomin-positive bodies are transmitted to only one of the daughter cells, and are therefore inherited by only one half of the secondary spermatocytes. These bodies tend to remain associated either as doublets or quartets during the second meiotic division, and are usually transmitted together to one-fourth of the daughter cells, and are therefore inherited by only one half of the secondary spermatocytes. These bodies tend to remain associated either as doublets or quartets during the second meiotic division, and are usually transmitted together to one-fourth of the spermatids (Fig. 5 J). These observations strongly suggest that each element of the fluorescent doublets corresponds to a pair of centrioles, and that each element of the quartets consists of a single centriole. However, neither the doublets nor the quartets have nucleating ability, as they are never associated with astral arrays of microtubules.

To ascertain the presence of centrioles in asl spermatocytes and spermatids, we examined thin sections of testes by EM. This analysis revealed that asl spermatocytes have morphologically normal centrioles (Fig. 6). However, centriole separation is abnormal in that we observed that in some spermatids, Nebenkern are associated with two centrioles instead of a single one, as occurs in the wild-type (Fig. 6). Moreover, the two centrioles of the spermatid shown in Fig. 6, C and D, are lying parallel to each other instead of at a right angle, as do the parent and its daughter centriole in the wild-type. This spermatid may therefore contain four centrioles, with only two of them in the plane of the section.

**asl Mutants Organize Anastral Spindles that Mediate Chromosome Segregation**

Despite the absence of asters, asl primary spermatocytes develop a peculiar anastral spindle. After the breakdown of the nuclear envelope, microtubules grow from multiple sites near the chromosomes, and form radial arrays extending from each bivalent (Fig. 3 B). These microtubules then organize into bipolar bundles, creating minispindles associated with individual bivalents (Fig. 3 C). However, in many asl spermatocytes, not all the bivalents within the same cell develop clear minispindles; some bivalents remain associated with a nonpolarized or poorly polarized network of microtubules (Fig. 3 C). In addition, the bundles of microtubules associated with the bivalents are often oriented in different directions (Fig. 3, C–F; Fig. 4 E’).

As a consequence, the bivalents never congregate into a metaphase I plate during male meiosis in asl mutants. Interestingly, the network of microtubules associated with the tiny fourth chromosomes is always much smaller than that associated with the larger bivalents, indicating that microtubule growth around the chromosomes is promoted by the whole chromatin, and not by the kinetochores alone. It is worth noting that in most metaphase-like figures the fourth chromosomes exhibit precocious segregation (Fig. 3 C), as occurs in wild-type metaphase I female meiosis (McKim and Hawley, 1995).

Despite these problems in congression, asl meiotic chromosomes progress into a highly irregular anaphase A (Fig. 3, D–F; Fig. 4 E’). The homologs manage to segregate, but their separation is often asynchronous (Fig. 3 D; Fig. 4 E’). Moreover, in ~35% of anaphase I–like figures, the sister chromatids of one or more half bivalents split and separate from each other (Fig. 3, E and F). These peculiar anaphases are genuine anaphase I figures, and are not cells undergoing anaphase II. This conclusion is suggested by the finding that in telophase I figures we never observed abnormal segregations with all the chromosomes migrating to a single pole (see below). Thus, most if not all the di-

Figure 4. Failure of centrosome assembly in asl mutants. Cells were stained for α tubulin (green), γ tubulin (orange), and DNA (blue). Panels in black and white show only γ tubulin immunofluorescence; color panels show merged images. (A–C’) wild-type; (A, A’) prometaphase I; (B, B’) early anaphase I; and (C, C’) telophase I showing well-organized centrosomes that accumulate γ tubulin. In one of the telophases shown in C and C’, centrosomes have already started to separate in preparation for the second meiotic division. (D–F’) asl mutants; (D, D’) prometaphase I–like figure; (E, E’) anaphase I; and (F, F’) telophase I, showing no γ tubulin accumulations at the cell poles. Note that γ tubulin is dispersed in small aggregates that do not appear to have the ability to nucleate microtubules. Bar, 10 μm.
viding cells with a 2N complement are likely to be primary spermatocytes undergoing meiosis I, and not diploid secondary spermatocytes in meiosis II. The phenomenon of precocious sister chromatid separation observed in asl anaphase I figures is probably due to the structure of the kinetochore of their half bivalents. During wild-type prometaphase, each half bivalent has a single hemispherical kinetochore that differentiates into two planar kinetochores between late prometaphase I and early anaphase I (Goldstein, 1981). In asl mutants where spindle formation is likely to be delayed with respect to the wild-type (see below), kinetochore duplication may occur before the onset of anaphase I. As a consequence, some half bivalents may become connected to both poles through their dupli-

Figure 5. Centrosomin immunostaining of asl testes. Cells were stained for α-tubulin (green), centrosomin (orange), and DNA (blue). Panels in black and white show only centrosomin immunofluorescence. Color panels show merged images; tubulin immunofluorescence was not merged in the color images shown in E’ and J’. (A–E’) wild-type; (A’, A’) mature primary spermatocyte at the S5 stage showing a pair of centrosomes just under the plasma membrane; (B, B’) prometaphase I; (C, C’) telophase I; and (D, D’) late telophase I showing prominent centrosomin-decorated centrosomes. Note that in the telophase shown in D and D’, the centrosomes have already started to separate. (E and E’) Two spermatids, each consisting of a nucleus and a Nebenkern. The weak fluorescence of the Nebenkern (arrows) is due to the mitochondrial DNA they contain. Note that the anticentrosomin antibodies detect the basal body located between the nucleus and the Nebenkern. (F–J’) asl mutants; (F and F’) mature primary spermatocyte at the S5 stage showing no centrosomin accumulations; (G and G’) prometaphase I showing a doublet of centrosomin-enriched bodies near the nuclear envelope; (H and H’) telophase I with a pair of centrosomin-enriched bodies at one of the poles; (I and I’) telophase I showing a quartet of centrosomin-enriched bodies at one of its poles. (J and J’) Highly irregular spermatid associated with four centrosomin-containing entities (enlarged in the insert of J; the arrow points at the Nebenkern). Bar, 10 μm.
cated kinetochores, leading to separation of their component sister chromatids.

Regardless of the type of segregation they exhibit, anaphase I chromosomes of asl mutants are never organized into two discrete sets, but are instead scattered throughout the cell (compare Fig. 2C with Fig. 3, D–F and Fig. 4E). Most likely this irregular anaphase chromosome arrangement reflects both the poor polarization of the spindles and the asynchrony in chromosome segregation. After anaphase A, asl primary spermatocytes undergo anaphase B. Despite the aberrant configuration of anaphase A, ~85% of these cells develop a central spindle, which is indistinguishable from its wild-type counterpart (compare Figs. 2D and 4C with Figs. 3G and 4F; Table I). The remaining 15% of ana–telophases form tripartite or multipartite central spindles (Fig. 3H; Table I). Moreover, central spindles elongate normally and are pinched in the middle during cytokinesis (Figs. 3G, 4F, and 5H; I; see below). However, in about 80% of the ana–telophases with morphologically normal central spindles, these structures are asymmetrically located with respect to the cell poles, so that cytokinesis would produce two daughter cells of different size (Fig. 7; Table I). In addition, in most cells (60%) chromosomes also remain scattered during anaphase B and do not congregate into two daughter nuclei as in wild type (compare Figs. 2D and 4C with Figs. 3G, 4F and 5H, I; Table I).

About 70% of telophase I figures exhibit unequal chromosome segregation (Fig. 4F; Fig. 7C; Table I). In cells showing both an asymmetrically located central spindle and unequal chromosome segregation, there is no correlation between the size of the daughter cells and their chromosomal content, indicating that central spindle positioning and chromosome segregation are independent events.

As a consequence of the abnormal first meiotic division, asl secondary spermatocytes receive variable numbers of chromosomes. These cells undergo an anastral second

Table I. Types of Ana–telophases Observed in asl Mutants

| Meiotic division | Total ana–telophases scored | Multipartite central spindle | Bipartite central spindle |
|------------------|-----------------------------|-----------------------------|--------------------------|
|                  |                             | a | b | c | d | a | b | c | d |
| I                | 190                         | 26 |   |   |   |   |   |   |   |
| II               | 99                          | 7  |   |   |   |   |   |   |   |

a. Regular chromosome segregation; congregated chromosomes at the poles. b. Regular chromosome segregation; scattered chromosomes at the poles. c. Unequal chromosome segregation; congregated chromosomes at the poles. d. Unequal chromosome segregation; scattered chromosomes at the poles.

Figure 6. Presence of morphologically normal centrioles in asl mutants. (A) Cross-section through the proximal part of a centriole in a mature primary spermatocyte. (B) A pair of centrioles lying at approximately right angles, located at the periphery of a primary spermatocyte. (C) Cross-section through a Nebenkern of an onion-stage spermatid, irregularly associated with two basal bodies (arrow). (D) Higher magnification of the basal bodies in C, consisting of nine peripheral triplets of tubules. (A) 28,000×; (B) 22,000×; (C) 13,000×; (D) 60,000×. Bars, 0.2 μm.

Figure 7. Central spindle formation and cytokinesis in asl mutants. Cells were immunostained for tubulin (green); DNA (blue); and either actin (orange; A and B), anillin (orange; C), or KLP3A (orange; D). (A) A wild-type telophase I and (B) an asl telophase I showing similar actin bands in the middle of their central spindles. (C and D) asl telophase I figures showing normal anillin (C) and KLP3A (D) accumulations in the spindle midzone. Note that in the cells in B and C, the central spindle is asymmetrically located with respect to the cell poles. Bar, 10 μm.
meiotic division that has the same features reported above for mutant first meiotic divisions. Mutant secondary spermatocytes form an irregular apolar spindle that mediates chromosome segregation, and eventually assemble an apparently normal central spindle (data not shown).

To obtain insight into the dynamics and timing of the meiotic process in asl mutants, we determined the frequencies of the various meiotic figures in asl testes, and compared them with those observed in wild-type controls (Table II). An inspection of Table II reveals that the frequencies of late prophase/early prometaphase I and anaphase/telophase I figures found in asl testes are only slightly higher than those observed in controls. In contrast, the frequencies of prometaphase/metaphase I and early anaphase I figures are much higher in asl than in controls. Because the frequency of each meiotic stage should be proportional to its duration in vivo, these findings suggest that the duration of asl prophase/prometaphase I is only slightly increased with respect to the control. However, prometaphase I, metaphase I, and especially early anaphase I appear to last much longer in asl mutants than in the wild-type. A likely explanation of this observation is that the process of spindle organization in asl mutants lasts longer than it does in wild-type because of the absence of astral microtubules nucleated by the centrosomes. The fact that asl mutants and wild-type controls exhibit similar frequencies of ana–telophases with a well-formed central spindle strongly suggests that most if not all the asl cells that enter meiosis I progress until ana–telophase I.

The frequencies of meiosis II figures are substantially lower in asl than in the control, but the ratios between interphase/early anaphase II cells and anaphase/telophase II figures are similar in both mutants and control. Because there is no reason to postulate that the second meiotic division is more rapid in asl than in the wild-type, the most straightforward explanation for these results is that only a fraction of asl secondary spermatocytes has the ability to organize an anastral spindle. However, once this anastral spindle is assembled, the cells can progress to ana–telophase and complete the second meiotic division.

The Central Spindle has the Ability to Stimulate Cytokinesis

An open question about cell cleavage in animal cells is the source of signals that stimulate contractile ring formation and cytokinesis. At present it is unclear whether these signals emanate from the asters or from the central spindle (reviewed by Fishkind and Wang, 1995; Glotzer, 1997; Goldberg et al., 1997). The fact that asl mutants form a central spindle in the absence of asters provided us with a unique opportunity to discriminate between these alternatives. We stained asl testes with rhodamine-phalloidin, which detects the actomyosin contractile ring during male meiotic cytokinesis (Gunsalus et al., 1995). In addition, we immunostained asl testes for KLP3A (Williams et al., 1995) and anillin (Field and Alberts, 1995), two proteins that concentrate in the cleavage furrow during wild-type meiotic cytokinesis (Williams et al., 1995; Hime et al., 1996). As shown in Fig. 7, both symmetrically and asymmetrically located central spindles exhibit a regular actin-based contractile ring and normal accumulations of both KLP3A and anillin. Regardless, the positioning of the central spindle within the cell, actin, anillin, and KLP3A are always localized in the middle of this structure, as occurs in the wild-type. In addition, in correspondence with the localization of these proteins, the central spindle is pinched, suggesting regular execution of cytokinesis.

Discussion
asl Mutants are Defective in Centrosome Assembly

We have identified a gene we call asterless (asl), that specifies a function necessary for aster formation during Drosophila male meiosis. In interphase primary spermatocytes and meiotic cells of wild-type males, centrosomes are enriched in γ tubulin. In contrast, in the same cell types of asl mutants this protein does not accumulate in the centrosomes but remains dispersed in multiple cytoplasmic aggregates that do not have microtubule-nucleating ability. Most likely, this primary defect in centrosome assembly prevents aster formation throughout meiotic cell division in asl mutants.

A similar but not identical situation has been observed in the acentriolar Drosophila cell line 1182–4, established from aploid embryos produced by the female sterile mutant mh 1182 (Gans et al., 1975; Debec, 1978; Debec et al., 1995). In control embryonic cell lines, γ tubulin accumu-

Table II. Frequencies of Meiotic Figures Observed in asl† and Control (Oregon R) Testes

| Genotype     | Total cells scored | L. Prometaphase I (M1a, b) | Prometaphase I (M2; M3) | E. Anaphase I (M4a) | Anaphase I (M4b,c; M5) | Telophase I (M6; M7; M8; M9; M10a) | Interphase II Prometaphase II | Anaphase II (M10b; c; M11) |
|--------------|--------------------|-----------------------------|------------------------|---------------------|------------------------|----------------------------------|------------------------------|-------------------------------|
| asl†/asl†    | 820                | 21.6                        | 9.0                    | 13.0                | 23.2                   | 11.1                             | 12.1                         |
| Oregon R     | 1,058              | 14.8                        | 5.8                    | 0.9                 | 19.5                   | 31.8                             | 27.2                         |
| Ratios       | 1.5                | 3.3                         | 1.2                    | 1.2                 | 0.3                    | 0.4                              |                              |

The stages of wild-type meiosis are described in detail by Cenci et al. (1994; see also Figs. 2, 4, and 5). Some of the equivalent stages observed in asl mutants are shown in Figs. 3, 4, 5, and 7. For example, the meiotic figures in Fig. 3 A, B, C, D–F, and G correspond to the M1, M2, M3, M4a, and M5 stages of the wild type, respectively. In asl mutants, early anaphase II is difficult to distinguish from metaphase II because in both types of cells some chromosomes exhibit sister chromatid separation. Therefore, these stages have been grouped together. Ratios are between the frequencies of meiotic figures observed in asl and in control. The numbers of meiotic figures have been determined by examining 11 Oregon R testes and 10 asl† testes. E, early; L, late; Prometa, prometaphase.
lates in both interphase and mitotic centrosomes. In 1182-4 acen- 
triolar cells γ tubulin fails to associate with the inter-
phase centrosomes, but it concentrates in the spindle poles 
where it exhibits different patterns of accumulation (De-
bec et al., 1995). However, the γ tubulin polar spots seen 
in the acen- 
triolar cells are not true centrosomes in that 
they readily disappear upon microtubule disassembly with 
either cold or colchicine treatment (Debec et al., 1995). 
Based on these results, Debec et al. (1995) suggested that 
centrioles play an important role in the assembly of cen-
trosomal material.

We have shown that in wild-type testes, antibodies di-
rected to centrosomin immunostain the centrosomes in 
mature primary spermatocytes and throughout meiosis. In 
asl mutants these antibodies detect either doublets or 
quartets of discrete structures that are present in all late 
prophase/prometaphase primary spermatocytes, but are 
transmitted to only one half of the secondary sperma-
tocytes and to one fourth of the spermatids. The behavior 
of these centrosomin-enriched bodies seen in asl mutants can 
be easily explained if one assumes that they correspond to 
the centrioles.

In wild-type, each mature primary spermatocyte con-
tains two pairs of duplicated centrioles, with the daughter 
centriole lying at a right angle with respect to its parent. In 
preparation of meiosis I, both pairs of centrioles migrate 
together from the plasma membrane to the nuclear enve-
lope, become associated with centrosomal material, and 
move to the cell poles while nucleating astral microtu-
buluses. Thus, during meiosis I each centrosome contains a 
pair of duplicated centrioles. However, there is not cen-
trirole duplication before the second meiotic division; in 
secondary spermatocytes each pair of centrioles splits into 
two single centrioles that migrate to the opposite cell 
poles. Therefore, each spermatid inherits a single centriole 
that becomes the basal body of the elongating axoneme (reviewed by Fuller, 1993).

Based on centriole behavior in the wild-type, we pro-
pose that the centrosomin-enriched doublets seen in asl 
primary spermatocytes correspond to the centrioles. The 
fact that these doublets are occasionally resolved into four 
entities further suggests that each element of the doublets 
does in fact consist of a pair of centrioles. In addition, we 
propose that the two pairs of centrioles, due to the absence 
of astral microtubules (Waters and Salmon, 1997), fail to 
separate and migrate to the cell poles during both meiotic 
divisions of asl mutants. Thus, during each meiotic division 
they are transmitted together to only one of the two 
daughter cells. This model for centriole behavior in asl 
mutants is supported by the results obtained by EM. EM 
analysis has shown that asl cells contain morphologically 
normal centrioles that in several cases fail to separate 
properly. We have observed several Nebenkern associated 
with two instead of a single centriole. Moreover, in some 
asl spermatids, these two centrioles are lying parallel to 
each other instead of at a right angle, as do the parent and 
itself daughter centriole in wild-type. This parallel centriole 
arrangement is consistent with the possibility that the two 
centrioles in the plane of the section belong to different 
pairs of centrioles that have been transmitted together to 
the sectioned spermatid.

Centrosomin immunostaining and EM analysis clearly 
indicate that asl meiotic cells contain centrioles of regular 
morphology that duplicate normally. Thus, the asl1 
function does not appear to be required for either centriole 
structure or duplication. However, the observation that 
asl centrioles are never associated with γ tubulin and 
accumulate much less centrosomin than their wild-type 
counterparts, strongly suggests that asl specifies a function 
required for the assembly of centrosomal material around 
the centrioles. The identification of such a function must 
await the molecular analysis of asl, which, however, may 
turn out to be particularly difficult. We have not suc-
sceeded in isolating asl alleles by P-mutagenesis, and 
molecular cloning of asl by chromosome walking is hampered 
by its vicinity to the Tpl locus.

**Spindle Assembly in asl Mutants**

We have shown that despite the absence of asters, asl 
mutants assemble a peculiar anastral spindle. Meiotic chro-
mosomes appear to play an important role in this process, 
acting as microtubule-organizing centers and promoting 
formation of bipolar minispinides. This finding was antici-
pated by micromanipulation experiments showing that 
*Drosophila* male bivalents detached from the spindle can 
trigger the formation of minispinides (Church et al., 1986).

The aberrant meiosis observed in asl males has many 
similarities with naturally occurring anastral divisions, 
such as those accompanying female meiosis in mice, *Ca-
enorhabditis*, *Xenopus*, and *Drosophila* (reviewed in 
McKim and Hawley, 1995). The asl spindle formation 
pathway is also reminiscent of the in vitro spindle assem-
bly induced by DNA-coated beads in *Xenopus* egg ex-
tracts (Heald et al., 1996; Heald et al., 1997). In all these 
systems, chromatin can induce microtubule nucleation and 
stabilization. These microtubules are initially randomly 
oriented; their minus-ends then focus at the spindle poles 
through the action of minus-end-directed motors and their 
associated proteins (Hatsumi and Endow, 1992; Heald et al., 
1996; Matthias et al., 1996; Merdes et al., 1996; Heald et al., 
1997). However, the minispinides associated with the asl 
bivalents are not always clearly organized into a bipolar 
array. Moreover, when they do exhibit a bipolar configu-
ration, the poles are broad and are never as focused as 
those observed in *Drosophila* female meiosis or in the *Xe-
nopus* in vitro systems. This result suggests that *Drosoph-
ila* spermatocytes do not have sufficient minus-end motor 
avtivity to complete spindle polarization in the absence of 
centrosomes.

Our results on asl mutants indicate that cells in which 
spindle assembly is normally driven by centrosomes none-
theless have the ability to form anastral spindles. Similar 
findings have been obtained with crane fly spermatocytes 
(Dietz, 1966; Steffen et al., 1986), but not with grasshopper 
spermatocytes where both the chromosomes and the cen-
trosomes are essential for spindle formation (Zhang and 
Nicklas, 1995). In addition, a series of studies has clearly 
shown that spindle assembly during mitotic division of a 
variety of vertebrate cell types invariably requires the 
presence of functional centrosomes (reviewed in Rieder 
et al., 1993). Together, these findings raise the question of 
why the ability to form anastral spindles in cells that nor-
mally contain centrosomes is restricted to a few meiotic
systems. It is possible that this property reflects different types of interaction between chromosomes and microtubules. In vertebrate mitotic cells and in grasshopper spermatocytes, the chromosomes can only capture and stabilize the microtubules nucleated by the centrosomes, and do not appear to have the ability to stimulate microtubule growth (Rieder et al., 1993; Zhang and Nicklas, 1995). In contrast, in Drosophila male meiosis and most likely also in Pales meiosis, the chromosomes act as microtubule-organizing centers, even in the absence of centrosomes (Fig. 3 B; see also Church et al., 1986). Thus, we suggest that anastral spindles are assembled only in those centrosome-containing systems where the chromosomes can induce formation of a sufficient number of microtubules. In systems where the chromosomes are unable to promote substantial microtubule growth, there would not be enough microtubules to form a bipolar spindle.

### asl Mutants Form a Normal Central Spindle that is Fully Able to Induce Cytokinesis

One of the most remarkable features of asl male meiosis is the formation of a morphologically normal central spindle in most anae–telophases. This finding challenges the classical view of central spindle assembly through interaction of antiparallel polar microtubules. Our results argue for a self-organization of the central spindle using either preexisting or newly formed microtubules (Masuda and Cande, 1987). Most likely, central spindle formation during male meiosis is mediated by microtubule cross-linking, plus-end–directed kinesin-like motors (reviewed in Sawin and Endow, 1993; Ault and Rieder, 1994; Hoyt, 1994). This hypothesis is supported by the finding that mutations in KLPSA, a Drosophila gene encoding a kinesin-like protein that concentrates in the central spindle midzone during male meiosis, disrupts central spindle formation and cytokinesis (Williams et al., 1995; Giansanti et al., 1998).

An open question about cell cleavage in animal systems is the source of signals that stimulates contractile ring formation and cytokinesis (reviewed by Fishkind and Wang, 1995; Glotzer, 1997; Goldberg et al., 1998). It has been suggested that these signals may be provided either by the metaphase chromosomes (Earnshaw et al., 1991) or the asters (Rappaport, 1961; Hiramoto, 1971; Rappaport, 1986) or the central spindle (Rappaport and Rappaport, 1974; Cao and Wang, 1996; Fishkind et al., 1996). Our results clearly show that the asters are not needed for the cytokerin signal. Moreover, the fact that asl chromosomes are scattered within the cell and never congress into a metaphase plate strongly suggests that chromosomes cannot dictate the positioning of the cleavage furrow. This conclusion agrees very well with the results of recent microinjection experiments showing that cytokinesis can occur in the absence of chromosomes in grasshopper spermatocytes (Zhang and Nicklas, 1996). Thus, of the three components of the anaphase spindle—the asters, the chromosomes, and the central spindle—only the latter appears to be required for signaling cytokinesis. In this respect, we would like to point out that our findings rule out the possibility of the central spindle merely accumulating cytokinetic signals originating from the asters.

We have recently shown that during Drosophila male meiosis, there is a cooperative interaction between the central spindle and the contractile ring; when one of these structures is disrupted the other one is also affected (Giansanti et al., 1998). Thus, the central spindle appears to play an essential role during cytokinesis. The asters, however, may be important for symmetrical positioning of the central spindle between the two daughter cells.

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