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The *Drosophila* Kinesin-like Protein KLP67A Is Essential for Mitotic and Male Meiotic Spindle Assembly

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Submitted May 29, 2003; Revised August 20, 2003; Accepted August 28, 2003

Monitoring Editor: Ted Salmon

We have performed a mutational analysis together with RNA interference to determine the role of the kinesin-like protein KLP67A in *Drosophila* cell division. During both mitosis and male meiosis, *Klp67A* mutations cause an increase in MT length and disrupt discrete aspects of spindle assembly, as well as cytokinesis. Mutant cells exhibit greatly enlarged metaphase spindle as a result of excessive MT polymerization. The analysis of both living and fixed cells also shows perturbations in centrosome separation, chromosome segregation, and central spindle assembly. These data demonstrate that the MT plus end-directed motor KLP67A is essential for spindle assembly during mitosis and male meiosis and suggest that the regulation of MT plus-end polymerization is a key determinant of spindle architecture throughout cell division.

INTRODUCTION

The proper assembly and positioning of the spindle during both meiosis and mitosis is essential for chromosome segregation and the correct placement of the division plane. Each step of spindle assembly seems to be facilitated by a stochastic state of microtubule (MT) polymerization and depolymerization with intermittent states of pause, termed "dynamic instability" (Mitchison et al., 1986). Cytologically, this is manifested as a rapid shrinking and growing of the MT plus ends (the MT minus ends are associated with the centrosomes and are intrinsically more stable). Measurements of MT dynamics in cultured cells (Salmon et al., 1984; Saxton et al., 1984), as well as in vitro studies with *Xenopus* egg extract (Verde et al., 1992), have shown that dynamic instability increases dramatically during entry into mitosis. The dynamic instability of MT plus ends has also been observed in real time during mitosis in yeast (Korinek et al., 2000, Lee et al., 2000). Together, these observations have led to a model in which dynamic instability allows the MT plus ends to "search and capture" appropriate anchorage sites such as the kinetochores or specialized sites at the cell cortex (Kirschner and Mitchison, 1986; Holy and Leibler, 1994; Desai and Mitchison, 1997; Schuyler and Pellman, 2001).

Many proteins have been discovered that regulate MT dynamic instability (Cassimeris, 1999). The biological role of several kinesin-like proteins in MT dynamics was first made evident by a series of elegant genetic analyses in *S. cerevisiae*. Mutants deficient for the kinesin-like protein *KIP3* were found to be viable but exhibit elongated spindles, and in the absence of dynein, resulted in failures in spindle alignment and nuclear migration (Cottingham and Hoyt, 1997; Straight et al., 1998; Cottingham et al., 1999). The two fission yeast Kip3p orthologs Kl5p and Kl6p also promote MT destabilization and mutations in these genes affect mitotic chromosome segregation and disrupt meiotic division (West et al., 2001, 2002). In vertebrates, the best characterized MT destabilizers are two kinesins of the *KcI* I family, the *Xenopus* kinesin catastrophe modulator (XKCM1), and its mammalian ortholog mitotic centromere-associated kinesin (MCAK). Inactivation of XKCM1 in *Xenopus* egg extracts results in the formation of large astral arrays of nondynamic, long MTs that are unable to form a bipolar spindle (Walczak et al., 1996), whereas overexpression in mammalian cells of specific XKC1M1 domains causes MT shortening and perturbs both spindle morphology and chromosome alignment (Kline-Smith and Walczak, 2002; Walczak et al., 2002). MCAK, however, may only function at anaphase, because both dominant-negative mutations as well as antisense depletion in cultured cells interfere with chromosome segregation (Maney et al., 1998). Although these studies clearly demonstrated that both XKCM1 and MCAK are MT-destabilizing enzymes required for spindle formation and chromosome segregation, they did not determine whether proper MT dynamics is continuously required throughout cell division in higher eukaryotes. This was mainly due to the intrinsic limitations of the systems analyzed (e.g., the...

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presence of the spindle checkpoint) and to the unavailability of leaky mutations that would allow mutant cells to progress through mitotic division.

Here, we show that the Drosophila kinesin-like protein KLP67A is a microtubule-destabilizing factor and that it is continuously required throughout both mitosis and male meiosis. Previous studies have shown that KLP67A is a plus end-directed motor associated with the ends of astral MTs, which is expressed only in proliferative tissues (Pereira et al., 1997). Consistent with these results, we show that mutations in Klp67A have striking effects on cell division. Our data indicate that proper regulation of MT plus-end polymerization is continuously required throughout cell division, allowing MTs to search and capture their appropriate targets during multiple steps of both mitosis and male meiosis.

MATERIALS AND METHODS

Isolation of Mutations in the Klp67A Gene

To isolate mutations in the Klp67A gene, we initially performed a vector screen (Egger et al., 1998), starting with I(3)30369I (Deak et al., 1997). We determined by plasmid rescue experiments that I(3)30369I contains a P element at 10 kb from the 3' end of the Klp67A gene. We recovered several new P elements; all localize within a "hot spot" 30 base pairs upstream of the transcription start of Klp67A. On completion of this screen, a P element insertion, Epi33514, was identified in the same location from the Rorth collection (Rorth, 1996) during the Drosophila genome-sequencing project (Spradling et al., 1995). Both our insertions and Epi33514 were founder homozygous viable. They also had no phenotype when made heterozygous with Df(3L)12AR that removes Klp67A.

To obtain additional P element insertions into the Klp67A gene, animals of the genotype w; Epi33514/TM6B were crossed to a strain containing the P [Δ2-3] source of P transposable. Resulting male progeny of the genotype w; Epi33514/P [Δ2-3] were then crossed as single pair matings to w; Df(3L)29A6/TM6B females. To screen for local hoops, DNA was prepared from pools of w; Epi33514/TM6B progeny, and 200 mutagenized lines were screened by polymerase chain reaction (PCR) with primers specific for P and an internal region of Klp67A. A size change in the PCR product or its absence indicated a putative deletion. From 200 lines screened, we isolated 20 potential mutations. DNA sequence analysis of several of these lines revealed that they retained the P element but contained small deletions of ≤35 base pairs within the promoter region of Klp67A. One of these lines, designated Klp67A(30204A), was then chosen for further characterization. We have determined by reverse transcription (RT)-PCR analysis that in Klp67A(30204A) mutants transcription begins within the P element inserted upstream to the Klp67A coding sequence (our unpublished data). However, this change in the site of transcription initiation results only in a slight reduction of the KLP67A level in embryos (our unpublished data). However, this change in the site of transcription initiation results only in a slight reduction of the KLP67A level in embryos (our unpublished data). However, this change in the site of transcription initiation results only in a slight reduction of the KLP67A level in embryos. (Our unpublished data). However, this change in the site of transcription initiation results only in a slight reduction of the KLP67A level in embryos.

Rescue Experiments

A Klp67A cDNA was cloned into the BamHI/EcoRI sites of the P element transformation vector Paws2 (Heck et al., 1993) that allowed KLP67A to be expressed as a fusion protein with a myc epitope at the amino terminus. Germ line transformants with chromosome 3 insertions of Paws2 [myc-Klp67A] were recovered and kept as homozygous viable. For rescue screens, animals of the genotype w;Paws2 [myc-Klp67A]. Df(3L)29A6/TM6B were crossed to Klp67A(30204A)/TM6B; the resulting male progeny w; Paws2 [myc-Klp67A]; Klp67A(30204A)/Df(3L)29A6 were tested for fertility and found to be completely fertile. The Paws2 [myc-Klp67A] construct was also used to test rescue of lethal complementation groups in this region (Leicht and Bonner, 1988). None of these mutations were found to be allelic to Klp67A.

Immunofluorescence Analysis of Mutant Testes

Testes were dissected and fixed according to Cenci et al. (1994) for α-tubulin and either centrosomin or myosin II immunostaining, and according to Gunn-salus et al. (1995) for α-tubulin immunostaining followed by actin staining with phalloidin. Fixed preparations were rinsed several times in phosphate-buffered saline (PBS) and then incubated overnight at 4°C with one of the following rabbit primary antibodies, both diluted 1:300 in PBS: anti-centrosomin (and Klp67A, 1996), and anti-myosin II (kindly provided by Chris Garesse, Universidad Autonoma de Madrid, Spain). For Western blot analysis, equivalent concentrations of total cell proteins were separated on SDS gels and transferred to polyvinylidene difluoride (Bio-Rad, Hercules, CA). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Blots were probed with a rabbit anti-KLP67A antisera directed against the peptide YEFDQFQTSDEELHRFTPK. The protein concentration of different samples, blots, or primary antibody concentrations was determined by densitometry of the blots and normalized prior to analysis with a standard curve of recombinant α-tubulin (A. Bomohn, Gastroenterology, Spain). Blots were visualized using a laser scanner (Bio-Rad, Hercules, CA) and examined with a cooled charge-coupled device (Photometrics, Tucson, AZ). Grayscale digital images were collected using LabSpec Spectrum software and then converted to Photoshop 5.0 format, pseudocolored, and merged.

Live Confocal Imaging

For time-lapse confocal microscopy, early embryos (0–2 h) were manually dechorionated, transferred to adhesive-coated coverslips, and briefly dehydrated to accommodate the addition of injected material. The dehydrated embryos were then covered with halocarbon oil and transferred to the stage of an inverted confocal microscope. All imaging was performed using a Nikon TE300 microscope equipped with a 50-W mercury lamp for epifluorescence and with a cooled charge-coupled device (Photometrics, Tucson, AZ). Grayscale digital images were collected using LabSpec Spectrum software and then converted to Photoshop 5.0 format, pseudocolored, and merged.

RNA Interference

Drosophila cultured cells (DL2) were grown at room temperature in Schneider's Drosophila medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution. For transfection, cells were cultured at 2 × 105 cells/ml in a 24-well dish with and without coverslips for ~18 h before transfection. Cells were transfected by standard calcium phosphate method with 5 μg Klp67A dsRNA per well in 1 ml of medium. After 16–18 h of transfection, cells were washed with fresh medium and cultured for another 24 h before fixation or RT-PCR. Cells were fixed in methanol and immunostained with fluorescein isothiocyanate conjugated anti-α-tubulin (1:200 dilution; Sigma-Aldrich, St. Louis, MO), 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, and an antibody to ATP synthase (1:2000, gift of R. Garese, Universidad Autonoma de Madrid, Spain). For Western blot analysis, equivalent concentrations of total cell proteins were separated on SDS gels and transferred to polyvinylidene difluoride (Bio-Rad, Hercules, CA). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Blots were probed with a rabbit anti-KLP67A antisera directed against the peptide YEFDQFQTSDEELHRFTPK. The protein concentration of different samples, blots, or primary antibody concentrations was determined by densitometry of the blots and normalized prior to analysis with a standard curve of recombinant α-tubulin (A. Bomohn, Gastroenterology, Spain). Blots were visualized using a laser scanner (Bio-Rad, Hercules, CA) and examined with a cooled charge-coupled device (Photometrics, Tucson, AZ). Grayscale digital images were collected using LabSpec Spectrum software and then converted to Photoshop 5.0 format, pseudocolored, and merged.

RESULTS

The Meiotic Phenotype of Klp67A Mutants

To determine the phenotypic consequences of reducing the level of KLP67A during meiotic division, we used males bearing the Klp67A(30204A) mutation over Df(3L)29A6, which removes Klp67A+. In these hemizygous males, the KLP67A level is reduced compared with homozygous Klp67A(30204A) males, leading to almost complete sterility. Df(3L)29A6+/+ males are completely fertile and do not exhibit any meiotic defect (see below), indicating that heterozygosity for Df(3L)29A6 does not contribute to the mutant phenotype. To determine the cellular basis of the sterility phenotype, testes

ratories, West Grove, PA). For actin plus tubulin staining, testes were first immunostained for α-tubulin and then incubated with rhodamine-phalloidin (Molecular Probes, Eugene, OR) according to Gunsalus et al. (1995). All preparations were mounted with Vectashield H-1200 (Vector Laboratories, Burlingame, CA) and examined.

A Zeiss Axiosplan microscope equipped with a 50-W mercury lamp for epifluorescence and with a cooled charge-coupled device (Photometrics, Tucson, AZ) was then microinjected into the embryos at a concentration of 5 μg/ml. The behavior of the labeled tubulin was visualized directly using a Leica TCS-SP laser-scanning confocal microscope as described previously (Theurkauf and Heck, 1999).

For Western blot analysis, equivalent concentrations of total cell proteins were separated on SDS gels and transferred to polyvinylidene difluoride (Bio-Rad, Hercules, CA). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Blots were probed with a rabbit anti-KLP67A antisera directed against the peptide YEFDQFQTSDEELHRFTPK. To standardize protein concentrations of different samples, blots, or primary antibody concentrations was determined by densitometry of the blots and normalized prior to analysis with a standard curve of recombinant α-tubulin (A. Bomohn, Gastroenterology, Spain). Blots were visualized using a laser scanner (Bio-Rad, Hercules, CA) and examined with a cooled charge-coupled device (Photometrics, Tucson, AZ). Grayscale digital images were collected using LabSpec Spectrum software and then converted to Photoshop 5.0 format, pseudocolored, and merged.
from Klp67A322b24/Df(3L)29A6 males were dissected, squashed, and examined cytologically for the presence of meiotic defects. As shown in Figures 1-3, a number of MT-related defects occur throughout all stages of the first meiotic division. First, in 89% (n = 74) of late prophase figures (stage M1 according to Cenci et al., 1994), the asters are not properly positioned at the opposite sides of the nucleus as in wild type (Figure 1A) but ectopically localized in the cytoplasm and close to each other (Figure 1B); occasionally, centrosomes and their associated asters seem to detach from the poles, giving rise to asters that freely float in the cytoplasm (Figure 1C). Despite this defect in aster localization and separation, most Klp67A primary spermatocytes (96%; n = 52) progressively assemble a bipolar spindle, which has the ability to mediate the formation of a metaphase plate (Figure 2C, arrow) and to proceed through anaphase (Figure 2C, arrow). However, mutant metaphase and anaphase spindles both display astral MTs that seem to be longer than their wild-type counterparts. In addition, in 12% of anaphase figures (n = 25), the two daughter nuclei have different sizes, suggesting an aberrant chromosome segregation (Figure 2C, arrow). Telophase I spindles of Klp67A spermatocytes are even more irregular. In 60% (n = 60) of these telophase figures, astral MTs are longer than in wild-type and the central spindle is either absent or much less dense than that seen in normal cells (Figure 2, D and E). An examination of secondary spermatocytes undergoing the second meiotic division revealed that only 18% of late prophase figures (n = 60) exhibit ectopic aster localization. However, cells in subsequent stages of meiosis II show the same defects observed in primary spermatocytes undergoing meiosis I (our unpublished data). Interestingly, 47% of anaphase II figures (n = 132) display two spindles within the same cytoplasm (Figure 2G), suggesting a failure of cytokinesis during the first meiotic division. In these “double” secondary spermatocytes astral MTs are also long, so that MTs from asters of different spindles often overlap, resulting in a highly disorganized spindle architecture (Figure 2G).

To characterize the cytokinetic phenotype of Klp67A mutants, spermocytes were stained for myosin II and F actin, two well-known components of the contractile ring. As can be seen in Figure 3, A and C, wild-type spermatocytes exhibit a robust central spindle and a clear acto-myosin ring. Mutant telophases with a normal central spindle also exhibit a regular contractile ring (our unpublished data). In contrast, mutant telophases with a poorly organized central spindle display a diffuse actin and myosin staining at the cleavage furrow rather than the typical tight band seen in wild-type cells (Figure 3, B and D). These results indicate that Klp67A telophases with a defective central spindle are unable to assemble a contractile ring and to undergo cytokinesis.

To determine the outcome of the spindle defects observed in Klp67A mutant spermatocytes, we analyzed spermatid morphology. In wild type, both chromosomes and mitochondria are equally partitioned between the two daughter cells at each meiotic division. After completion of meiosis, the mitochondria fuse to form a conglomerate called the nebenkern. Thus, each wild-type spermatid consists of a round phase-light nucleus associated with a single phase-dark nebenkern of similar size (Figure 3E). Defects in chromosome segregation result in differently sized nuclei (Gonzalez et al., 1989), whereas failures in cytokinesis give rise to spermatids that comprise a large nebenkern associated with either two or four normal-sized nuclei (Fuller, 1993). An examination of Klp67A mutant spermatids revealed that 41% of these cells (n = 254) have large nebenkern associated with two or four nuclei (Figure 3, F and G). In addition, we found that 24% of the nuclei display irregular sizes (Figure 3G). Together, these observations strongly suggest that Klp67A spermatocytes are defective in both chromosome segregation and cytokinesis.

Finally, it should be noted that we observed a few polyploid spermatocytes with four rather than two centrosomes (Figure 1D). This finding suggests that KLP67A is also required for cytokinesis during the gonial mitoses that precede meiotic division. Both the sterility and the meiotic phenotypes are completely rescued by a Pwum2 [myc: Klp67A] transgene (see MATERIALS AND METHODS).

Klp67A Maternal Effect on Embryonic Divisions

Females of the genotypes Klp67A322b24/Df(3L)29A6 exhibit reduced fertility compared with the parent strains Klp67A322b24/TM6B and Df(3L)29A6/TM6B. Forty percent of the eggs laid by Klp67A322b24/Df(3L)29A6 females hatch to first instars compared with 96% of the eggs laid by a wild-type strain. However, eggs laid by Klp67A322b24/Df(3L)29A6 females exhibit only a small decrease in the amount of the KLP67A protein compared with those from either the parent strains or wild type, indicating that the Klp67A322b24 mutation reduces gene expression only slightly (Figure 4A). Nonetheless, the reduction in KLP67A caused by this mutation is sufficient to lower female fertility and to cause mitotic defects in embryonic divisions (see below).

To determine whether the partial sterility of Klp67A322b24/Df(3L)29A6 females is due to a defect in the early blastoderm mitoses, eggs from Klp67A322b24/Df(3L)29A6 mothers crossed to wild-type males were collected and used for real-time analysis of mitosis in living embryos. Rhodamine-labeled tubulin was injected into living embryos and mitosis was allowed to proceed in real time. Time-lapse analysis showed that both spindle formation and architecture is severely abnormal through all stages of mitosis (Figures 4 and

Figure 1. Klp67A is required for aster migration to the opposite sides of spermatocytes (A-C). Wildtype (A) and Klp67A mutant spermatocytes (B-D) Primary spermatocyte preparations were stained for α-tubulin (green), centrosomin (red), and DNA (blue). (A) Prometaphase I spermatocyte showing two well-separated prominent asters closely apposed to the nuclear envelope. (B) Two mutant prometaphase I spermatocytes with ectopically located asters, not associated with the nuclear envelope and still close to each other. (C) A mutant primary spermatocyte showing the bulk of one of the centrosomes and its associated aster detached from the spindle pole (arrowhead). (D) Tetraploid mutant spermatocyte with four ectopically located asters, indicating a cytokinesis failure in a previous gonial division.

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5; see also Video 1 in Supplemental Material). Control embryos derived from Df(3L)29A6/TM6B mothers undergo normal mitoses, as shown in Video 2 (Supplemental Material).

The first important feature of the mutant phenotype is seen during prophase, when the majority of the centrosomes do not complete their migration to the opposite sides of the nucleus (Figure 4A, arrows). The average angle between prophase centrosomes (n = 60) was observed to be 145.2° in mutants and 164.2° in controls (n = 54). Although this difference is small, it is statistically significant according to the Student’s t test (p = 0.0002). Incompletely separated centrosomes then give rise to curved banana-shaped metaphase spindles (Figure 4B). The centrosome migration defect is sometimes seen even in wild-type embryonic cells, where it results in the same misshapen spindle phenotype observed in mutant embryos (Figure 4; Videos 1 and 2 in Supplemental Material). However, in mutant embryos this spindle malfunction not only occurs more frequently but it is also exacerbated. Videos 1 and 2 in Supplemental Material, from which Figures 4 and 5 are derived, also reveal that the distortion of the normal shape of the spindle occurs when dynamic MTs seem to reach the chromosomes. At this precise moment, the spindle becomes distorted and banana shaped to accommodate the extended MTs emanating from the spindle poles (see below). In addition, in some mutant prometaphase and metaphase spindles (video 1) centrosomes detach from the spindle poles, as occurs during male meiosis. The timing of centrosome separation is also affected by a reduction in KLP67A. The period of centrosome migration up until the point of nuclear envelope breakdown lasts for at least 3 min; 1 min longer than in wild-type embryos.

A second important feature of mutant metaphase spindles is their increased length compared with wild-type spindles. The average pole-to-pole distance in mutant metaphases (n = 60) is 15.6 μm (+1.5 SD), compared with 10.3 μm (+0.64 SD) in wild-type metaphases (n = 54). It is important to note that this increased spindle length is observed in all mutant spindles and is not a consequence of the defect in centrosome separation, because even the spindles with normal centrosome positioning are longer than their wild-type counterparts (Figure 5, 290 s; Videos 1 and 2 in Supplemental Material). In addition, in all types of spindles, the increase in pole-to-pole distance is associated with an increase in MT length. Thus, these results suggest that the activity of KLP67A is required for limiting the length of spindle MTs.

A third important feature of the Klp67A maternal effect phenotype is seen during telophase, when most spindles seem to be either missing a normal central spindle or have a greatly reduced number of midzone MTs (Figure 5, 370 s). Central spindle formation normally occurs during anaphase when the overlapping set of antiparallel interpolar MTs become bundled (Mastronarde et al., 1993). Although areas of MT overlap can be seen in the central region of most of these mutant spindles (Figure 5), their midzone MTs are not organized in the typical dense lateral array that characterizes wild-type central spindles. This suggests that the abnormally long, and often curved or bent, astral MTs are not able to interact properly to give rise to the ordered parallel array.
of central spindle MTs. Because these early blastoderm mitoses do not require MTs to form the pseudocleavage furrow (Stevenson et al., 2001), aberrant spindles are often able to proceed through telophase and two daughter spindles can form in the ensuing divisions. Chromosome behavior has also been observed in eggs laid by Klp67A322b24/Df(3L)29A6 females by using real-time analysis of eggs injected with Oligreen. Surprisingly, in spite of the structural abnormalities observed in the spindles of these eggs, chromosome segregation seems normal (Video 4, Supplemental Material).

Figure 3. Klp67A mutations disrupt central spindle and contractile ring assembly and cause failures in both cytokinesis and chromosome segregation. Primary spermatocytes from wild-type (A and C) and Klp67A322b24/Df(3L)29A6 (B and D) males. Preparations were stained for α-tubulin (green), actin (A and B; red) and myosin (C and D; red) and DNA (blue). (A and C) Wild-type telophase I spermatocytes showing a prominent central spindle and a regular actomyosin ring. (B and D) Mutant telophase I figures showing a severely defective central spindle and irregular patches of either actin (b) or myosin (d) at the cleavage furrow. (E-G) Phase contrast images of wild-type (e) and Klp67A322b24/Df(3L)29A6 (F and G) living spermatids. (E) Wild-type partial cyst in which each spermatid consists of a phase-dark nebenkern and a phase-light nucleus. (F) Klp67A mutant spermatids consisting of two equally sized nuclei associated with a large nebenkern (G) A Klp67A spermatid consisting of a large nebenkern associated with four nuclei of normal size (arrowhead) and two spermatids showing irregularly sized nebenkern associated with micro- and macronuclei (arrows). Bar, 10 μm.

To analyze spindle dynamics in Klp67A mutant embryos, Videos 1 and 2 in Supplemental Material were used to calculate the interval between nuclear envelope breakdown (t = 0 s) and the appearance of a central spindle (albeit drastically abnormal in the mutant embryo). As shown in Figure 5B, this period lasts 2 min longer in mutant embryos (360 s) than in wild-type embryos (240 s). This difference is primarily due to an increase in the amount of time spent in metaphase and anaphase B. Figure 5B also shows that, during metaphase, the mutant spindles continue to increase in length, whereas their wild-type counterparts reach a plateau. In addition, the final spike in pole separation at anaphase B occurs later (at 300 s) than in wild-type (at 220 s). Although wild-type and mutant spindles do not exhibit a noticeable difference in the speed of chromosome movement during anaphase A (Videos 3 and 4 in Supplemental Material), the mutant spindles take significantly longer to go through anaphase B (90 s in mutants versus 30 s in wild type). This slowing of anaphase B may be related to the unusual S-shaped conformation assumed by mutant spindles during pole separation (Figure 5A, 370 s).

Western blot analysis of eggs derived from Klp67A322b24/Df(3L)29A6 mothers demonstrated that the Klp67A322b24 allele lowers gene expression only slightly (Figure 4E). This suggests that MT polymerization and, as a result, spindle assembly is extremely sensitive to a slight decrease in the level of KLP67A during both male meiosis and embryonic mitosis.

RNAi of Klp67A

A genetic null allele of Klp67A would greatly facilitate the functional analysis of this gene. Because at this time, a null allele is unavailable, RNAi was used to create a severely
hypomorphic Klp67A mutant. These experiments were performed using the DL2 cell line of Drosophila. The flat morphology of these cells allows a clear view of individual MTs, permitting a reliable evaluation of the effect of KLP67A on MT length. For these experiments, cells were transfected with a 500-base pair double-stranded Klp67A RNA and examined 42–44 h after the beginning of dsRNA treatment (see MATERIALS AND METHODS). Depletion of Klp67A mRNA as well as the KLP67A protein was demonstrated by RT-PCR and Western blot analysis (Figure 6, E and F). Transfected and mock-transfected cells were fixed, stained for both tubulin and DNA, and analyzed for defects in mitotic division (Figure 6). The frequency of cells in the different stages of mitosis in treated versus control cells is shown in Table 1. It is evident from these data that RNAi causes a mitotic block at metaphase. This suggests that cells treated with Klp67A dsRNA are unable to progress to anaphase and telophase, probably due to the activation of the mitotic checkpoint that prevents cells with defective spindles to enter anaphase (see DISCUSSION).

Although the metaphase arrest of Klp67A dsRNA-treated cells precludes observation of the entire mitotic process, certain aspects of the phenotype of these cells resemble the Klp67A maternal effect observed in blastoderm embryos. The spindles of dsRNA-transfected cells are extremely elongated as in the blastoderm embryo. Several examples of these spindle malformations can be seen in Figure 6. The pole-to-pole distance in the control spindle shown in Figure 6A is less than half of that observed in the RNAi spindles shown in Figure 6, B–D (note that all these images are at the same magnification). These enlarged spindles also show interpo lar MTs that seem abnormally long and curved. In addition, the overall shape of these spindles is often curved as in the blastoderm mitotic divisions, again indicating incomplete centrosome separation. The metaphase chromosome configuration is also abnormal and a tight metaphase plate arrangement is rarely seen. All of these phenotypes are consistent with an increased MT length and stability caused by the ablation of the Klp67A gene activity. Furthermore, concentrations of nocodazole that destabilize MTs and perturb spindle assembly in control DL2 cells only slightly affect spindle architecture and have no effect on astral MT arrays in KLP67A-depleted cells (Gandhi and Pereira, unpublished data). The RNAi phenotype observed in cultured cells is likely to correspond to that elicited by a strong mutant allele.

RT-PCR analysis has shown that the Klp67A dsRNA treat-
ment results in a significant decrease in the \textit{Klp67A} mRNA after two days of treatment (Figure 6E).

Because previous studies showed that \textit{KLP67A} is associated with mitochondria at the plus ends of astral microtubules (Pereira \textit{et al.}, 1997), we also examined whether \textit{KLP67A} depletion affects mitochondria distribution in DL2 cells. An antibody to ATP synthase was used to visualize mitochondria in control and dsRNA-treated cells. In control cells, mitochondria surround metaphase spindles displaying a rather uniform distribution, consistent with their association with the ends of the astral MTs (Figure 7A). However, in \textit{KLP67A}-depleted cells mitochondria seem to be excluded from the cell poles and concentrated around the metaphase plate (Figure 7B). It is important to note that these cells are forced to assume an elongated shape to accommodate the enlarged mutant spindles, whereas control metaphase cells exhibit a typical round shape (Figure 7). As a consequence of this deformation, the astral MTs of \textit{KLP67A}-depleted cells are not found in their normal radial array but are forced inward, so that their plus ends are all oriented toward the chromosomes (Figure 7B). The characteristic distribution of mitochondria in \textit{KLP67A}-depleted cells is therefore likely to be a secondary consequence of the altered cell shape. In mutant precellular blastoderm embryos, where the astral arrays are not constricted by a cell membrane, this abnormal mitochondria distribution is not seen (Pereira, unpublished data).

\textbf{Double RNAi of \textit{Klp67A} and \textit{Klp61F}}

The increase in MT length observed in \textit{Klp67A} mutant cells could indicate a requirement for \textit{KLP67A} in the regulation of MT growth. Alternatively, \textit{KLP67A} could be required for maintaining normal spindle pole separation, and the increased pole-to-pole distance in \textit{KLP67A} mutant cells could result in an increased MT polymerization. To discriminate between these possibilities, we examined whether the absence of \textit{KLP67A} results in abnormal MT elongation in monopolar spindles. To perform this analysis, DL2 cells were cotransfected with both \textit{Klp61F} dsRNA and \textit{Klp67A} dsRNA. Previous genetic analyses (Heck \textit{et al.}, 1993) and antibody injection experiments (Sharp \textit{et al.}, 1999a) have shown that \textit{KLP61F} depletion prevents centrosome separation, resulting in monopolar spindles. As predicted, treatment of DL2 cells with \textit{Klp61F} dsRNA does indeed produce frequent spindles with unseparated asters. These monopolar spindles are associated with highly condensed chromosomes that seem to be in metaphase (Figure 8). Double RNAi treatment with \textit{Klp61F} and \textit{Klp67A} effectively reduced the expression of both mRNAs (Gandhi and Wentworth, unpublished data) and also resulted in monopolar spindles. However, these monopolar spindles display a dramatic increase in MT length compared with those observed in cells transfected with \textit{Klp61F} dsRNA alone (Figure 8). In addition, in cells doubly depleted for \textit{Klp61F} and \textit{Klp67A}, the individual MT fibers of monopolar spindles are not only much longer than those in \textit{KLP61F}-depleted monopolar spindles, but they are also often curved (Figure 8) as those observed in mutant \textit{Klp67A} embryos. It is also noteworthy that the effect...
of KLP67A on MT length is observed as early as in prometaphase and in some cases even in prophase (Figure 8, arrow). The finding that the activity of KLP67A is required as early as prophase indicates that the increased MT length seen in mutant spindles is not an indirect consequence of the metaphase arrest phenotype.

**DISCUSSION**

Our functional analyses have demonstrated a requirement for KLP67A in the regulation of MT growth and stability during both Drosophila mitosis and male meiosis. Depletion of this MT plus end-directed motor increases the length and perturbs the morphology of spindle MTs, beginning as early as prophase and extending through ana-telophase. Normally, mitotic MTs are known to be much shorter and less stable than interphase MTs (Salmon et al., 1984; Saxton et al., 1984). Studies with Xenopus egg extracts have indicated that the mechanism for this change in MT stability is an increase in the frequency of transitions from MT polymerization to depolymerization at the MT plus ends (reviewed in Desai and Mitchison, 1997). Thus, KLP67A may affect MT stability during both mitosis and male meiosis through either a direct or indirect effect on MT dynamics.

It has been proposed that the Kip3 family, which includes KLP67A, shares a close evolutionary relationship with the Kin I family and that they may be functional orthologs (Severin et al., 2001). However, other sequence homology studies strongly suggest a significant divergence (Endow and Kim, 2000; Miki et al., 2001). Based on sequence similarity, the Kip3 family can be subdivided into the fungal and metazoan subfamilies (West et al., 2001). The fungal subfamily of Kip3 is comprised of the Saccharomyces cerevisiae Kip3p and Schizosaccharomyces pombe Klp5p and Klp6p. The fungal members are further characterized by conserved domains at their amino terminal ends and in their tail domains that are not shared with their metazoan Kip3 relatives (West et al., 2001). Further evidence suggesting the functional divergence of the Kip3 family from the Kin I family comes from biochemical studies. Unlike the Kin I family kinesins, KLP67A does not depolymerize taxol-stabilized MTs nor does it have an internal catalytic domain (Pereira et al., 1997). In addition, whereas Kin I family kinesins, such as MCAK, diffuse along the MTs (Hunter et al., 2003), KLP67A is a directional motor that moves toward the MT plus ends (Pereira et al., 1997).

Although, the KLP67A and the fungal Kip3 members have a similar in vivo effect on MT stability and length (Connor and Hoyt, 1997; DesZwaan et al., 1997; Cottingham et al., 1999; West et al., 2001), the fact that they are in different subfamilies is likely to pertain to the specific requirements...
for MT stability in spindle assembly and function in metaphase. Unlike the yeast cellular phenotype, the Drosophila phenotype includes a dramatic and global increase in spindle size, problems in aster separation and chromosome segregation and defects in central spindle formation. Furthermore, whereas Kip3 is essential for yeast mitosis, the function of KLP67A is essential for somatic cell division, as a “knock-down” of Klp67A results in a nearly complete mitotic arrest. KLP67A is also required for male meiosis like the S. pombe Kip3 family members, but unlike the S. cerevisiae Kip3p.

We have analyzed the phenotypic consequences of KLP67A depletions in three different Drosophila cell types: blastoderm embryonic cells, spermatocytes, and DL2 cultured cells. Because the depletion of KLP67A in cultured cells results in an almost complete mitotic arrest, null mutations of Klp67A are predicted to be lethal. In contrast, flies bearing the Klp67A322b24 hypomorphic allele over a deficiency that removes Klp67A are viable, albeit partially sterile. Therefore, this allelic combination results in a level of KLP67A that is sufficient to sustain development to adulthood but is just at the threshold for normal MT dynamics and spindle assembly in spermatocytes and early embryos. At this time, the biochemical mechanism, which explains how a small decrease in the level of KLP67A in the embryo can lead to such a dramatic effect in MT behavior, is unknown. However, because male meiosis and precellular blastoderm mitosis are highly suitable systems for observing cell division in Drosophila, the availability of the hypomorphic Klp67A322b24 allele has been extremely advantageous to the functional analysis of KLP67A.

The absence of a stringent spindle checkpoint in embryonic cells and spermatocytes was also advantageous to our phenotypic analysis. We have shown that mutant embryonic cells and spermatocytes both proceed through anaphase and telophase, in contrast to KLP67A-depleted DL2 cells that arrest at metaphase. We believe that these findings reflect the different stringencies of the spindle checkpoint mechanisms that are operating in these three cell types. During male meiosis, the spindle checkpoint is known to be weak and only causes a small delay in the anaphase onset in response to the presence of univalent chromosomes (Rebloho and Gonzalez, 2000) and does not prevent spermatocytes with severely malformed spindles to undergo anaphase and telophase (Bonaccorsi et al., 1998; Sampaio et al., 2001; Wakefield et al., 2001; Riparbelli et al., 2002). Similarly, in the early blastoderm embryo, a stringent checkpoint is not operating until the midblastula (Sibon et al., 1997). In contrast, it is likely that DL2 cells employ a stringent checkpoint that prevents cells with defective spindles to enter anaphase. A similar checkpoint has been observed in larval neuroblasts that arrest in metaphase in response to the spindle defects caused by mutations in the abnormal spindle gene (Basto et al., 2000). Except for this checkpoint-mediated arrest, the three types of cells examined respond in similar ways to the depletion of KLP67A. All cell types display a substantial increase in MT length and an abnormal centrosome separation. In addition, blastoderm embryos and spermatocytes, the two systems with nonstringent checkpoints, fail to organize a normal central spindle. However, whereas blastoderm embryos exhibit normal chromosome segregation, a substantial fraction of both primary and secondary spermatocytes are defective in this process. We suggest that all these phenotypic abnormalities depend on the same primary defect in the regulation of MT plus-end polymerization.

Our data demonstrate that there is a defect in centrosome separation during both mitosis and male meiosis in Klpl67A mutant cells. Two other Drosophila MT motors have previously been shown to participate in centrosome separation. Mutations in the cytoplasmic dynein heavy chain gene Dhc64D result in a maternal effect phenotype that includes incomplete centrosome separation and frequent centrosome loss (Robinson et al., 1999). Because Dhc64D is cortically located, it has been suggested to power centrosome separation during prophase by exerting a minus-end directed “reeling in” force on astral MTs (Sharp et al., 1999a). Another Drosophila motor required for centrosome separation is the bimC family member, KLP61F (Heck et al., 1993; Sharp et al., 1999a,b). Antibody injection experiments in Drosophila embryos have led to the suggestion that this bipolar kinesin is not needed for powering aster migration but for maintaining aster separation (Sharp et al., 1999c). Our data suggest that the centrosome separation defect in KLP67A-depleted cells results from changes in MT dynamics and disproportionate MT growth that is likely that in these cells the improper behavior of the plus ends of astral MTs prevents the MT–cytokinesis interactions that mediate centrosome migration to the opposite poles of the nucleus. Thus, even though Dhc64D and KLP67A play distinct roles in centrosome separation, defects in either function would reduce astral pulling forces resulting in incomplete centrosome separation.

Chromosome segregation in the Klp67A mutant blastoderm seems normal, but this process is affected in both meiotic divisions of Klpl67A mutant males. The finding that spermatocytes require KLP67A for chromosome segregation is not surprising, as the abnormal MT behavior observed in the mutants can account for problems in chromosome segregation. However, we do not understand why blastoderm cells, despite the defect in MT dynamics, normally segregate their chromosomes. The simplest explanation for this discrepancy is that spermatocytes and blastoderm cells have different requirements for normal chromosome segregation. These cell-specific requirements may be related to the duration of cell division in these two types of cells. In the extremely rapid mitotic process of blastoderm cells chromosome segregation could indeed be mediated by molecular mechanisms that are partially different from those used during meiotic divisions.

We have found that Klp67A mutations disrupt central spindle formation in both blastoderm embryos and spermatocytes. Central spindle formation is known to be mediated by plus-end-directed MT cytoplasmic kinesins, such as Pavarotti (Adams et al., 1998), the Drosophila homolog of MKLP1. It is thus likely that the cross-linking activities of these kinesins are necessary for the function of KLP67A to ensure proper MT plus-end dynamics and morphology during central spindle assembly. However, although we favor the view that KLP67A only acts as a MT-depolymerizing factor, our results do not exclude the possibility that KLP67A has an additional MT bundling activity, promoting central spindle assembly in concert with the other MT-cross-linking kinesins. In blastoderm embryos the defect in central spindle does not result in a failure to separate the two daughter nuclei, because the formation of the pseudocleavage furrow is a MT-independent process (Stevenson et al., 2001). However, in spermatocytes the defect in central spindle is accompanied by a failure to assemble a compact spermatocyte apparatus and to undergo cytokinesis. This is consistent with a large body of data indicating that in animal cells, including Drosophila, proper central spindle assembly is an essential prerequisite for contractile ring formation (Gatti et al., 2000).
Previous observations on Drosophila embryos showed that KLP67A is associated with a population of the mitochondrial plus ends of astral microtubules (Pereira et al., 1997). We found that in KLP67A-depleted cells mitochondria concentrate at the plus ends of MTs (Figure 7), consistent with previous studies, indicating that mitochondria use multiple motors to attach themselves to MTs (Nangaku et al., 1994; Tanaka et al., 1998). However, mitochondria seem to have different distributions in control and in KLP67A-depleted metaphase cells (Figure 7). In the latter cells, they are more highly concentrated in the center of the spindle than in control cells. This is most likely a secondary effect of the abnormal aster morphology in KLP67A-depleted metaphases, with the long astral MTs extending inward rather than radially (see RESULTS). However, our results do not exclude the possibility that mitochondria mispositioning may contribute to the increased MT stability observed in mutant cells.

To summarize, our data suggest that the plus end-directed KLP67A motor at the MT plus ends where it either directly or indirectly promotes MT destabilization. We propose that KLP67A activity is required for spindle MTs to interact properly during centrosome migration, metaphase spindle formation, chromosome segregation, and central spindle assembly when MT ends must dynamically search and capture their appropriate targets. Further studies are underway to define the precise effect of KLP67A on MT dynamics during cell division.

ACKNOWLEDGMENTS

We thank Bill Theurkauf for generously allowing us to use his confocal microscope. We especially thank Byeong Cha for technical assistance during the embryo injections, and Paul Furbigin (University of Massachusetts Bio Imaging Core Facility) for technical help in imaging. We also thank Tom Kaufman, Chris Field, and R. Ganesse for the gift of the cmn, myosin, and ATP-synthase antisera, respectively. This work was supported by the National Institutes of Health (ROI521411) as well as an American Heart Association Award (525314) to A.J.P., and by grants from Fondo per gli investimenti di Ricerca di Base (RBNE01KXC9-004) and Centro di Eccellenza di Biologia Molecolare to M.G. A.J.P., and R.G. thank Paul R. Dobner and Aminabh Mohanty for invaluable assistance. A.J.P. also thanks William S. Gelbart (Harvard University) for laboratory space during the initiation of this project.

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Mitotic and Meiotic Functions of KLP67A


