KLP-18, a Klp2 Kinesin, Is Required for Assembly of Acentrosomal Meiotic Spindles in *Caenorhabditis elegans* ▼

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This article is dedicated to the memory of Christoph Segbert, an outstanding young scientist.

The proper segregation of chromosomes during meiosis or mitosis requires the assembly of well organized spindles. In many organisms, meiotic spindles lack centrosomes. The formation of such acentrosomal spindles seems to involve first assembly or capture of microtubules (MTs) in a random pattern around the meiotic chromosomes and then parallel bundling and bipolar organization by the action of MT motors and other proteins. Here, we describe the structure, distribution, and function of KLP-18, a *Caenorhabditis elegans* Klp2 kinesin. Previous reports of Klp2 kinesins agree that it concentrates in spindles, but do not provide a clear view of its function. During prometaphase, metaphase, and anaphase, KLP-18 concentrates toward the poles in both meiotic and mitotic spindles. Depletion of KLP-18 by RNA-mediated interference prevents parallel bundling/bipolar organization of the MTs that accumulate around female meiotic chromosomes. Hence, meiotic chromosome segregation fails, leading to haploid or aneuploid embryos. Subsequent assembly and function of centrosomal mitotic spindles is normal except when aberrant maternal chromatin is present. This suggests that although KLP-18 is critical for organizing chromosome-derived MTs into a parallel bipolar spindle, the order inherent in centrosome-derived astral MT arrays greatly reduces or eliminates the need for KLP-18 organizing activity in mitotic spindles.

INTRODUCTION

In eukaryotes meiosis allows the exchange of genetic material between parental chromosomes and leads to the formation of haploid gametes. Reliable segregation of meiotic chromosomes depends on the correct assembly of microtubules (MTs) into a bipolar spindle. In most animal systems, female meiotic spindles lack centrosomes and their MT nucleating activity (Sawada and Schatten, 1988; Gard, 1992; Theurkauf and Hawley, 1992; Albertson and Thomson, 1993). Interestingly, vertebrate cultured cells in which centrosomes have been destroyed can use a centrosome-independent pathway to build a functional bipolar spindle (Khodjakov et al., 2000). These and other results suggest that although the diastral mode of spindle assembly dominates when centrosomes are present, chromatin-based spindle assembly can be used when centrosomes are absent. The question of how meiotic MTs become organized into a bipolar structure remains largely unanswered.

Studies in *Xenopus* egg extracts have provided some important insights into acentrosomal spindle assembly. The observation that bipolar spindles can form around DNA-coated beads confirmed that chromatin itself can provide a platform for the nucleation, stabilization, and/or capture of MTs (Heald et al., 1996). Analysis of the effects of inactivating or depleting specific MT motor proteins from *Xenopus* extracts along with analysis of mutations that affect assembly of acentrosomal meiotic spindles in *Drosophila* (Merdes and Cleveland, 1997; Walczak et al., 1998; Walczak, 2000) have led to the following model for chromatin-based assembly of bipolar spindles. Chromatin-associated kinesins, e.g., *Xenopus* klp1, participate in the interaction of MTs with chromatin and may help push the minus ends of captured MTs away from the chromatin by walking toward plus ends (McKim and Hawley, 1995; Vernos et al., 1995; Matthies et al., 1996; Walczak et al., 1998; Antonio et al., 2000; Funabiki and Murray, 2000). Plus-end–directed BirC kinesins, e.g., *Xenopus* Eg5 and *Drosophila* KLP61F, participate in parallel bundling by lateral cross-linking of MTs. These motors also sort the bundled MTs by sliding antiparallel MTs such that plus ends move toward one another at the spindle equator, whereas minus ends move away from one another toward the poles (Sawin and Mitchison, 1995; Kashina et al., 1996; Sharp et al., 1999). The minus-end–directed motor cytoplasmic dynein then helps focus the poles, perhaps by cross-linking MT minus ends and by moving other motors and/or MT cross-linking proteins toward minus ends (Heald et al.,...
MATERIALS AND METHODS

C. elegans Strains and Alleles

Maintenance and handling of C. elegans were carried out as described previously (Brenner, 1974). Bristol N2 was used as the wild-type strain. The following mutations were used: LGI, goa-1(hm224); L1Gf, emb-27(q48); LGII, fem-2(b245ts1); LGIII, fem-27(g48ts1); LGIV, glp-4(bn2ts1); LGV, emb-27(g48ts1); LGVI, emb-27(g48ts1); LGVII, emb-27(g48ts1). Other phenotypes or mutations were obtained from the C. elegans Genetic Center (University of Utah, Salt Lake City, UT). All strains used in this study were derived from Bristol N2. All new mutations were confirmed by sequencing. E. coli DH5α was used for cloning, and E. coli strain HT115 (DE3), carrying IPTG-inducible T7 polymerase, was used for protein expression. Antibodies were used for analysis of KLP-18 localization in embryos and worms. Western blots for analysis of KLP-18 expression were performed using rabbit anti-KLP-18 antibodies and Chemiluminescence. 

RNA-mediated Interference (RNAi)

To isolate a yk1676g cdna clone, phagemid DNA was prepared as described in the Stratagene ExAssist protocol. For in vitro transcription (RibozMax; Promega, Madison, WI), the template was linearized with EcoRI or Xhol. To generate double-stranded RNA (dsRNA), equal amounts of sense and anti-sense RNA were mixed in nuclease-free water, incubated at 37°C for 15 min, and precipitated by adding cold ethanol to a final concentration of 50%. dsRNA solution for RNAi was prepared from a 194-base pair PCR product derived from the middle of klp-18, a region absent from klp-30 (Figure 1C). For RNAi tests, dsRNA diluted to a final concentration of 0.5 µg/µl was injected into the gonads of either young adult wild-type hermaphrodites or young adult males (emb-27(q48) animals) (maintained at 15°C and shifted to 25°C after injection). After 24–48 h, the progeny of injected worms were scored for mutations present in the n2 background, as determined by examination of germ line transmission (Figure 1C). For RNAi tests, dsRNA diluted to a final concentration of 0.5 µg/µl was injected into the gonads of either young adult wild-type hermaphrodites or young adult males (emb-27(q48) animals) (maintained at 15°C and shifted to 25°C after injection). After 24–48 h, the progeny of injected worms were scored for mutations present in the n2 background, as determined by examination of germ line transmission (Figure 1C). For RNAi tests, dsRNA diluted to a final concentration of 0.5 µg/µl was injected into the gonads of either young adult wild-type hermaphrodites or young adult males (emb-27(q48) animals) (maintained at 15°C and shifted to 25°C after injection). After 24–48 h, the progeny of injected worms were scored for mutations present in the n2 background, as determined by examination of germ line transmission (Figure 1C). 

KLP-18 Antibody Production

PCR was used to amplify a 1.2-kb fragment of klp-18, encoding the C-terminal 425 aa of C. elegans KLP-18, with primers KLP-18F and KLP-18R: 5'-ATGCTCGTGGTCTGACTGACCTGTCGTC-3' and reverse, 5'-TCCACTGTCGTCCTGTCGTTGGGTTTGGTGA-3'. Amplification of a single colony overnight (37°C, LB medium) was used for bacterial growth. Lysates of purified Ni2+–NTA magnetic beads were used as a positive control and included in the Western blot analysis. 

Northern Blot Analysis

PolyA- RNA was prepared from mixed or synchronous worm populations by using the µMACS mRNA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). RNA was fractionated in formaldehyde-containing (1%) agarose gels, transferred to a Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ) by using 20× SSC and cross-linked with UV light. Digoxigenin (Roche Diagnostics)-labeled RNAs, generated from yk1676g or rpp-1 CDNA clones (Evans et al., 1997), were used as probes at a final concentration of 100 and 100 ng/ml, respectively. The membrane was prehybridized (30 min, 68°C) and hybridized (overnight, 68°C) in hybridization solution (5× SSC, 50% deionized formamide, 0.1% (w/v) sodium-laurylsarcosine, 0.02% (w/v) SDS, 2% (v/v) blocking reagent (Roche Diagnostics)) and washed at 2-5 min in 2× SSC/0.1% SDS at room temperature and 2-5 min in 0.1× SSC/0.1% SDS at 68°C. Detection was performed with horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibodies (dilution 1:10,000) by using BM chemilu-
adult hermaphrodites and temperature-sensitive mutants raised at restrictive temperature: *glp-4(bn2ts)* (in blocking buffer) and then on 2% agarose gel. Separated proteins were transferred to nitrocellulose membrane, incubated with blocking buffer containing TBT (Tris-buffered saline (25 mM Tris), plus 0.1% Tween 20 or Triton X-100) plus 1% bovine serum albumin, 3% nonfat dry milk powder, and 0.02% sodium azide for 1 h at room temperature, further incubated at 4°C overnight with rabbit anti-KLP-18 antibody (1:200; 10 min) in blocking solution and then on 2% agarose gel. Separated proteins were transferred to nitrocellulose membrane, incubated with blocking buffer containing TBT (Tris-buffered saline (25 mM Tris), plus 0.1% Tween 20 or Triton X-100) plus 1% bovine serum albumin, 3% nonfat dry milk powder, and 0.02% sodium azide for 1 h at room temperature, further incubated at 4°C overnight with rabbit anti-KLP-18 antibody (1:500) in blocking buffer, washed three times for 5 min each with TBT at room temperature, and finally incubated at room temperature for 1.5 h with anti-rabbit secondary antibody (HRP-conjugated, 1:10,000) in blocking buffer without sodium azide. After washing three times for 5 min each in TBT, detection was performed using BM chemiluminescence substrate (Roche Diagnostics). Sizes of transcripts were determined by comparison with RNA *M* standards (Promega).

**Western Blot Analysis**
Young adult hermaphrodites (100 or 20 worms per lane; Figures 1D and 4A, respectively) were transferred to 10 mL of M9 buffer and frozen in liquid nitrogen. Then 10 μL of 2× SDS sample buffer was added, and samples were immediately boiled (10 min), chilled on ice (5 min), and loaded on a 10% SDS polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane, incubated with blocking buffer containing TBT [Tris-buffered saline (25 mM Tris), plus 0.1% Tween 20 or Triton X-100] plus 1% bovine serum albumin, 3% nonfat dry milk powder, and 0.02% sodium azide for 1 h at room temperature, further incubated at 4°C overnight with rabbit anti-KLP-18 antibody (1:100) in blocking buffer, washed three times for 5 min each with TBT at room temperature, and finally incubated at room temperature for 1.5 h with anti-rabbit secondary antibody (HRP-conjugated, 1:10,000) in blocking buffer (without sodium azide). After washing three times for 5 min each in TBT, detection was performed using BM chemiluminescence substrate (Roche Diagnostics). Sizes of transcripts were determined by comparison with RNA *M* standards (Promega).

**Immunostaining of Embryos**
Gravid adults were transferred with a drawn-out pipette to a microscope slide coated with a thin layer of polylysine in a drop of sterile M9 buffer and cut with a scalpel. Embryos were immediately permeabilized by the freeze-crack method (Strome and Wood, 1983) and fixed in 100% methanol (10 min), 100% acetone (20 min), 90% ethanol (10 min), 60% ethanol (10 min), and 30% ethanol (10 min). Slides were washed twice for 10 min each with TBT (see above), incubated at 4°C overnight with primary antibodies (see below) in blocking buffer (TBT plus 1% bovine serum albumin and 1% nonfat dry milk powder), washed three times for 10 min each with TBT at room temperature, and incubated at room temperature for 1–3 h, with secondary antibodies (see below) in blocking buffer. Finally, slides were washed three times for 10 min each in TBT and mounted in Mowiol containing 1,4-diazabicyclo(2.2.2)octane (Sigma-Aldrich) as an antifade reagent.

The following primary and secondary antibodies were used at the dilutions (in blocking buffer) indicated: anti-KLP-18 (rabbit, 1:3,000–1:4,000), anti-β-tubulin (mouse, 1:40, 4A1; Piperno and Fuller, 1985), anti-PGL-1 (rabbit, 1:5,000, Kawasaki et al., 1998), anti-HEM-1 (rabbit, 1:40, Clark-Maguire and Mains, 1994a), and anti-ZYG-9 (rabbit, 1:25, Matheos et al., 1998). Secondary antibodies were Cy2, Cy-3 (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA), and Alexa 647 (1:200; Molecular Probes) conjugated. Yoyo-1 (1:20,000; Molecular Probes) and RNase A (1:200, 10 μg/μL) were usually added with secondary antibodies for DNA staining. To test specificity, anti-KLP-18 antibodies were incubated with an excess of 6× His-KLP-18 protein (0.5–1 μg/μL) in blocking solution and incubated for 30 min at room temperature.

**Microscopy, Live Imaging, and Image Processing**
Development of embryos and microinjections into the gonad of hermaphrodites were observed with Nomarski optics by using an Axiohot microscope (Carl Zeiss, Jena, Germany) equipped with a 100× PL Fluotar oil-immersion objective and a DM IB E inverted microscope (Leica, Wetzlar, Germany) equipped with a 60× PL Fluotar objective, respectively. Immunofluorescence analyses of embryos were performed on a confocal microscope (TCS-NT; Leica) equipped with a 100× PL Fluotar oil-immersion objective. Each recorded image represents a projection of a Z-series of 0.5-μm optical sections. For live imaging of green fluorescent protein (GFP)-tagged chromosomes during meiosis and mitosis, GFP-histone H2B-expressing embryos were grown to adulthood at room temperature on plates containing either nontransformed OP50 or HT115 bacteria expressing klp-18 dsRNA (see above). These “RNAi-fed” worms were placed in a solution of 0.01% tricane/0.01% tetramisole, an anesthetizing agent, for 15–20 min, and then on 2% agar pads on slides. Embryos inside the anesthetized worms were imaged using an
MRC600 laser scanning confocal system (Bio-Rad, Hercules, CA) with an argon/krypton laser. Images were collected every 3 μm on slow scan, by using a 60× objective. Temperature ranged from 21°C to 24°C. Images were manipulated to generate movies and figures in NIH Image (version 1.62, developed by Wayne Rasband at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Adobe Photoshop (Adobe Systems, Mountain View, CA) and Canvas (ACD Systems, Miami, FL).

RESULTS

Molecular Characterization of klp-18

The klp-18 gene (ORF C06G3.2; Figure 1A) is predicted to encode a kinesin-like protein (The C. elegans Sequencing Consortium, 1998; Siddiqui, 2002). To determine the sequence of full-length klp-18, we used 5′-RACE, PCR, and overlapping cDNAs. The klp-18 gene encodes a protein of 932 aa with a motor domain at its N terminus (Figure 1B). Based on sequence alignment of its motor domain, KLP-18 is most similar to Xenopus Xklp2 (43% identity/60% similarity; Boleti et al., 1996), Homo sapiens HKlp2 (45%/61%; Sueishi et al., 2000), and sea urchin KRP180 (43%/61%; Rogers et al., 2000). Furthermore, in a recent evolutionary analysis of kinesin-related proteins maximum likelihood methods indicate that KLP-18 is a member of the Klpl2 clade (Lawrence et al., 2002). Between its motor domain and C terminus, KLP-18 does not show significant sequence similarity to other proteins in the database. However, that region has a high probability of forming an extended α-helical domain with the potential to form coiled-coiled interactions (Figure 1B). Also, amino acids 507–510 (SPAR) in this “stalk” region provide a potential cdc2 kinase phosphorylation site ([IT/S][PXK]/R; Nigg, 1993). These features are similar to those of the stalk regions of other members of the Klpl2 subfamily (Boleti et al., 1996; Wittmann et al., 1998; Rogers et al., 2000).

Database searches revealed that ~750 kb away from klp-18 in the C. elegans genome is a pair of predicted ORFs (C33H5.4 and C33H5.3) with high sequence similarity to the KLP-18 amino acid sequence. The C33H5.4 ORF, previously designated as klp-10 (Siddiqui, 2002), is separated from C33H5.3 by only 19 base pairs of genomic DNA. The two are colinear with KLP-18 (Figure 1C), suggesting that C33H5.3 actually encodes the C terminus of KLP-10. The high degree of sequence identity between klp-18 and the composite klp-10 ORF (85% identity at the DNA level and 90% identity/95% similarity at the amino acid level) suggests that they arose from a recent gene duplication event and that KLP-18 and KLP-10 could be redundant. However, the results of RT-PCR, Northern blots, and Western blots suggest that klp-10 is not expressed at appreciable levels (Figure 1, C and D). Furthermore, a KLP-10 protein would not be likely to engage in kinesin-like motor activity, because it lacks the highly conserved “switch I” amino acids “SSR,” which are thought to be critical for interaction with ATP (Kull et al., 1996; Sablin et al., 1996).

Despite these indications that klp-10 may not produce a MT motor, two recent studies reported that RNAi intended to deplete KLP-10 caused embryonic lethality (Piano et al., 2000; Kamath et al., 2003). Sequence comparison suggests that the dsRNAs used in those studies should be effective in eliminating KLP-18 as well as KLP-10. This raised the question of whether the embryonic lethality was due to depletion of KLP-10, KLP-18, or both. To address this issue, we obtained a deletion allele of klp-10 [klp-10(ok704)] from the C. elegans Gene Knock-Out Project at Oklahoma Medical Research Foundation (http://www.mutantfactory.ohsu.sc.edu/). The deletion removes codons for the predicted motor domain amino acids 178–662 of KLP-10. Homozygous klp-10(ok704) worms are viable and fertile, and their embryos show no obvious defects. We also designed a dsRNA that is specific for klp-18 (see MATERIALS AND METHODS). That specific probe, when tested by RNAi, caused 100% embryonic lethality. Consequently, we conclude that the embryonic lethality previously attributed to KLP-10 depletion was actually due to KLP-18 depletion.

Germline Expression of klp-18

In adult C. elegans, klp-18 mRNA expression is strongest in the female germ line. In glp-4 hermaphrodites, which have a severely underproliferated germ line (Bean and Strome, 1992), no klp-18 transcript is detectable (Figure 1E). In fem-2 hermaphrodites, which produce only oocytes (Kimble et al., 1984), klp-18 transcript is present at high levels, and in fem-3 gain-of-function hermaphrodites, which produce only sperm (Barton et al., 1987), it is present at approximately wild-type levels. Additional Northern blot analysis revealed increasing klp-18 transcript levels at progressively later stages of wild-type larval development (our unpublished data). This increase is likely due to a growing population of germ cells (also see Figure 2).

KLP-18 Localization in Mitotic Spindles

To investigate where KLP-18 is localized and may function, two antisera against the C terminus of the protein were generated, affinity purified, and used for immunofluorescence staining. Both antisera produced the same staining pattern (Figures 2 and 3). Both are specific for KLP-18, as demonstrated by a dramatic reduction of staining in embryos after preincubation of the antibody with an excess of KLP-18 fusion protein (our unpublished data). Staining was also greatly reduced or eliminated in embryos subjected to RNAi depletion of KLP-18 (Figure 4). KLP-18 staining was most concentrated in mitotic spindles toward the poles. During anaphase, KLP-18 staining also concentrated between the separating groups of chromosomes, with a dim zone at the spindle equator (Figure 2A). These patterns are similar to those seen for Xklpl2 in mitotic Xenopus cultured cells and KRP180 in mitotic sea urchin embryos (Boleti et al., 1996; Rogers et al., 2000), although a dim zone at the anaphase equator has not been reported previously.

After cytokinesis, KLP-18 accumulated somewhat around the nucleus and at the cortex near sites of cell-cell contact (Figure 2B). During later stages of embryogenesis, KLP-18 levels gradually diminished in somatic cells but increased in the primordial germ cells Z2 and Z3, presumably due to expression in the embryonic germ line (Figure 2, C–E). The germ line stained brightly for KLP-18 at all stages of larval development (Figure 2, F and G). Adult hermaphrodites contained high levels of cytoplasmic stain in the distal gonad arm, where germ cells proliferate and begin meiosis (Figure 2H). Adults also showed high levels of staining in the proximal gonad arm, where oocytes mature during the late stages of meiotic prophase I (Figure 2I).

KLP-18 Localization in Meiotic Spindles

In C. elegans, mature oocytes, and thus female meiotic spindles, lack centrosomes. After the oocyte is fertilized in the spermatheca (Figure 2I, sp), disorganized MTs accumulate around the chromosomes and subsequently become ordered into a bipolar metaphase spindle that lacks astral MTs (Figure 3). Immunostaining showed KLP-18 colocalized with the disorganized MTs during prometaphase (Figure 3A). As the MTs became ordered into a parallel bipolar array, KLP-18 concentrated most at the poles but was also present between...
the poles and chromosomes (Figure 3B). The concentration at poles persisted during metaphase and early anaphase (Figure 3, C and D), but then shifted to the interzone between the separating chromosomes during late anaphase/telophase (Figure 3E). Similar KLP-18 staining patterns were observed during meiosis II (Figure 3, C and F; our unpublished data). The concentration of KLP-18 at the spindle poles of female meiotic spindles, which lack centrosomes, supports the view that Klp2 kinesins are not core centrosomal proteins but instead concentrate toward MT minus ends (Rogers et al., 2000; Wittmann et al., 2000).

During the meiotic divisions that occur in the male germ line, anti-KLP-18 staining patterns were similar to those seen in female meiosis and mitosis. KLP-18 was concentrated at the spindle poles of male meiotic spindles, which lack centrosomes, supports the view that Klp2 kinesins are not core centrosomal proteins but instead concentrate toward MT minus ends (Rogers et al., 2000; Wittmann et al., 2000).

During the meiotic divisions that occur in the male germ line, anti-KLP-18 staining patterns were similar to those seen in female meiosis and mitosis. KLP-18 was concentrated at the spindle poles, which have centrosomes, and in the anaphase interzone (our unpublished data). There was also a significant amount of KLP-18 staining on male meiotic chromosomes. Attempts to deplete KLP-18 from the male germ line by RNAi failed, as has been observed for other proteins expressed during spermatogenesis (S. L’Hernault, personal communication). Consequently, the specificity of KLP-18 antisera in staining the male meiotic chromosomes remains uncertain.

Depletion of KLP-18 Impairs Formation of the Female Pronucleus and Causes Cortical Instability

To test the function of KLP-18, we studied the consequences of depleting it by RNAi. Double-stranded RNA, transcribed from a klp-18 cDNA clone or from a smaller region of the klp-18 gene that is not present in klp-10, was introduced into the syncytial gonad of young adult hermaphrodites either by injection or by feeding. Western blots of adults and immunostaining of embryos demonstrated that 24–48 h after dsRNA treatment, KLP-18 levels were dramatically re-
duced (Figure 4). This confirmed the specificity of the antibodies and showed that the RNAi depletion was effective. Most experiments used dsRNA derived from cDNA, which could deplete klp-10 transcripts if they were present. Two results suggest that dsRNA-induced embryonic defects and lethality were due solely to KLP-18 depletion. First, cDNA-derived RNAi and klp-18 gene-specific RNAi caused identical defects. Second, RNAi depletion of KLP-18 from wild-type and from klp-10(ok704) embryos caused identical defects (our unpublished data).

Figure 3. Localization of KLP-18 during female meiosis. Different stages of wild-type female meiosis I (A, B, D, and E) or meiosis II (C and F) stained with anti-KLP-18 (red), anti-α-tubulin (blue), and Yoyo-1 (green) to visualize KLP-18, MTs, and DNA, respectively. Anterior is to the left. (A) After nuclear envelope breakdown, KLP-18 colocalizes with MTs around the condensed chromosomes. (B–D) During prometaphase, metaphase, and anaphase KLP-18 is highly enriched at the spindle poles. (E) During telophase, KLP-18 and MTs are concentrated between separating chromosomes. Bar, 2.5 μm (A–E). (F) For comparison of meiotic spindle brightness relative to cytoplasmic levels an entire one-cell embryo (metaphase II) is shown. Bar, 10 μm.
We first recorded the consequences of KLP-18 depletion in live klp-18(RNAi) embryos by using Nomarski video differential interference contrast microscopy. In wild-type, completion of female meiosis results in the formation of two polar bodies and a female pronucleus at the anterior end of the newly fertilized embryo. The female pronucleus migrates to meet the posteriorly located male pronucleus, and the joined pronuclei move to the center of the embryo where they begin mitosis (Figure 5, A–D). In klp-18(RNAi) embryos (n = 12 movies), severe but variable defects were seen in meiosis. The defects ranged from the complete absence of a female pronucleus (Figure 5, E and F; 3 of 12) to the formation of multiple small female pronuclei (Figure 5, I and J; 9 of 12), only some of which fused with the male pronucleus. The defects in formation of the female pronucleus suggested a failure in meiotic chromosome segregation. However, in most cases, subsequent formation of a bipolar mitotic spindle seemed normal, even when klp-18(RNAi) was done in the klp-10 deletion background. Occasionally, extra nuclei became visible after mitosis in the anterior daughter cell AB (Figure 5L). Extra nuclei may have been remnants of extra female pronuclei. Later in development, klp-18(RNAi) embryos displayed various signs of tissue differentiation but did not undergo morphogenesis (Figure 5N).

It was interesting that after cytokinesis klp-18(RNAi) embryos usually displayed aberrant ruffling and blebbing of the plasma membrane, especially at sites of cell-cell contact (Figure 5M; n = 14 of 19). These defects and the accumulation of KLP-18 seen near the plasma membrane (Figure 2B) suggest a role for KLP-18 in cortex dynamics during post-meiotic development. It has been demonstrated that proper interactions of astral MTs with the plasma membrane are necessary for stabilization of the cell cortex in early C. elegans embryos (Hird and White, 1993).

**KLP-18 Is Required for Assembly of Acentrosomal Meiotic Spindles**

To analyze the formation and organization of female meiotic spindles in klp-18(RNAi) embryos, we imaged microtubules and DNA at the anterior end of fixed early embryos by confocal fluorescence microscopy. In klp-18(RNAi) embryos (n >40), MTs were clustered around female meiotic chromosomes, but those MTs were never seen in a bipolar array (compare Figure 6, A and B, with Figure 3), and meiotic chromosomes never showed signs of normal anaphase segregation.

**Figure 4.** Depletion of KLP-18 by RNA interference. (A) Equal amounts of protein from wild-type and klp-18(RNAi) young adults were immunoblotted after depletion of KLP-18 via injection or feeding of dsRNA. In both cases, KLP-18 was almost undetectable. The blot was reprobed with anti-α-tubulin antibodies as a loading control. (B–D) In comparison to wild-type, klp-18(RNAi) meiotic spindles at equivalent stages showed either greatly reduced levels of KLP-18 staining (C; n = 9) or no detectable staining (D; n = 9). Anterior is to the left. Bar, 5 μm.

**Figure 5.** klp-18(RNAi) embryos show defects in formation of a female pronucleus. Nomarski images of wild-type embryos (A–D) and two klp-18(RNAi) embryos (E–L). Anterior is to the left. Black and white arrowheads mark the female and male pronuclei, respectively. (A–D) The female and male pronuclei migrate to meet posteriorly (B), the first mitotic spindle forms (C), and the embryo undergoes cytokinesis to form a 2-cell (D). (E–H) In this klp-18(RNAi) embryo, a female pronucleus did not form (E and F), but mitosis and cytokinesis occurred apparently normally (G and H). Note the abnormal number of polar bodies (arrow in G). (I–L) In another klp-18(RNAi) embryo, three nuclear structures became visible in the anterior cytoplasm (J), and an additional nuclear structure formed in the anterior cell after cytokinesis (arrow in L). (M) Shortly after cytokinesis (e.g., two-cell stage) klp-18(RNAi) embryos often have transient membrane ruffling and blebbing (arrow). (N) They do not undergo morphogenesis. Bar, 10 μm.
The disorder of meiotic microtubules and chromosomes in klp-18(RNAi) embryos suggested an effect on initial acen- 
tosomal spindle assembly. However, judging a temporal se- 
cquence of events from fixed samples is uncertain. For ex- 
ample, a period of spindle assembly could have occurred 
followed quickly by a loss of order during anaphase. To test 
this possibility, we studied the effects of klp-18(RNAi) 
in emb-27 mutants, which arrest at meiotic metaphase I. The 
emb-27(g48ts) allele encodes a temperature-sensitive CDC-
16/APC-6 that at restrictive temperature is defective in 
pro-moting the metaphase-to-anaphase transition (Golden et al., 
2000). Examination of meiotic figures in klp-18(RNAi);emb-
27(g48ts) embryos (n = 14) revealed disordered spindles 
(Figure 6D) that were indistinguishable from those seen in 
klp-18(RNAi) embryos. These results confirm that KLP-18 is 
required during prometaphase/metaphase to organize chro-
matin-associated MTs into a bipolar spindle.

klp-18(RNAi) embryos often had a reduced accumulation 
of MTs around meiotic chromosomes (Figure 6B). This may 
reveal an additional role for KLP-18 in the initial nucleation, 
stabilization, or capture of MTs by chromatin. Alternatively, 
failure to organize a bipolar meiotic spindle may lead to 
subsequent loss of MTs. Future time-lapse analysis of MT 
behavior will discriminate between these possibilities.

**Depletion of KLP-18 Prevents Proper Meiotic 
Chromosome Alignment and Segregation**

To gain a clearer understanding of female meiosis and how 
KLK-18 depletion affects it, we studied chromosome dynam- 
ics in oocytes and early embryos. In fixed and stained klp-
18(RNAi) hermaphrodites, diakinesis-arrested oocytes were 
indistinguishable from their wild-type counterparts; they 
contained six properly condensed bivalents, suggesting nor-
mal execution of early meiotic events (Figure 7A). In live 
hermaphrodites expressing a GFP::histone H2B fusion pro-
tein, meiotic chromosome behavior was studied by time-
lapse confocal fluorescence microscopy (see on-line video 
supplement Movies 1–4). In wild-type embryos (Movie 1) 
the six bivalents initially congressed to a metaphase I plate 
perpendicular to the anterior cortex (Figure 7B). The meta-
phase plate then rotated 90° to orient parallel to the cell 
surface during the transition to anaphase I (Figure 7C). After 
separation (Figure 7D), the set of chromosomes near the 
cortex was expelled as a polar body, whereas the remaining 
set of chromosomes engaged in meiosis II. As in meiosis I, 
the meiosis II metaphase plate formed perpendicular to the 
anterior cortex (Figure 7E) and then rotated to a parallel 
orientation at the beginning of anaphase II (Figure 7F).

During telophase II, the set of sister chromatids near the cortex 
was expelled as a second polar body and the remaining set 
decondensed to form the female pronucleus.

In klp-18(RNAi) embryos containing GFP::histone H2B 
(Movies 2–4), bivalent chromosomes looked morphologi-
cally normal immediately after fertilization, but they did not 
algn on a metaphase plate. Instead, the bivalents formed a 
disorganized cluster near the anterior cortex (Figure 7G;
Movie 2). At a time when one would expect anaphase I, 
chromosomes moved slightly outward, making the cluster 
less compact, but did not separate into two sets (Figure 7, 
H and I). In most klp-18(RNAi) embryos, we observed more 
than six chromatin masses at this stage (Figure 7I), suggest-
ing that homologous chromosomes, or perhaps sister chro-
matids, did disjoin. Coincident with the period expected for 
meiosis II, the chromosomes clustered together again (Fig-
ure 7J). Part or all of the chromatin cluster then usually 
moved toward the cell surface in an attempt at polar body 
extrusion (Figure 7K; Movie 2). In 4 of the 18 embryos 
observed, the entire maternal complement of DNA was 
extruded (Figure 8, G–J; Movie 3). In 3 of the remaining 
embryos, the entire maternal complement of DNA remained 
in the embryo, forming one or more pronuclei. In the other 
11 embryos, some maternal DNA was extruded as a polar 
body and some was packaged into one or more pronuclei 
(Figure 8, K–N; Movie 4). These results and those described 
above show that KLP-18 is necessary during prometaphase 
I for the correct alignment of meiotic chromosomes and for 
their subsequent segregation. Prior tests of Xklp2 function in 
Xenopus extract-driven assembly of acen-trosomal spindles 
around chromatin beads revealed a minor contribution that 
overlaps with those of pole-focusing motors (Walczak et al., 
1998). In contrast, our in vivo tests of KLP-18, a C. elegans
KLP-18 is Not Required for Mitosis

To investigate the possibility that KLP-18 is also important for the assembly and function of centrosomal spindles, we examined the effects of KLP-18 depletion on mitotic spindle organization and chromosome behavior in embryos. The only prior report of in vivo function disruption tests of a Klp2 kinesin, KRP180 in sea urchin embryos, suggested that although it is not essential for mitosis, it helps maintain spindle pole separation in prometaphase and metaphase, perhaps by cross-linking and sliding overlapped antiparallel MTs (Rogers et al., 2000). To investigate this possibility in C. elegans, pole-to-pole distances were compared in Nomarski

Xklp2 homolog, indicate a major contribution to early stages of acentrosomal spindle assembly.
recordings of klp-18(RNAi) and wild-type one-cell embryos. The mean distance at metaphase in klp-18(RNAi) embryos (14.3 ± 0.7 μm; n = 11) was not significantly different from wild type (14.6 ± 0.8 μm; n = 11). Immunostaining of fixed embryos concurred that mitotic MT organization was normal in klp-18(RNAi) embryos (Figure 8, A and B) and in klp-10(ok704) klp-18(RNAi) embryos (our unpublished data). Even small mitotic spindles in older embryos, similar in size to female meiotic spindles, seemed normal, arguing against the possibility that it is small spindle size that dictates a need for KLP-18. Furthermore, comparison of wild-type (n = 12) and klp-18(RNAi) (n = 5) embryos (2- to 26-cell stage) revealed no significant differences in cell cycle timing, spindle orientation/position, or cytokinesis (our unpublished data). However, the observation of some binucleate cells highlighted the possibility of defective chromosome segregation.

To study mitotic chromosome behavior in more detail, the first embryonic cell cycle was studied by time-lapse imaging of GFP::histone H2B. In wild-type embryos, the female and male pronuclei migrate toward each other, meet in the posterior hemisphere, and move together to the center of the embryo (Figure 8, C and D; Movie 1). The nuclear envelopes then break down and the maternal and paternal chromosomes congress to a tight metaphase plate (Figure 8E). Sister chromatids separate cleanly into two distinct groups during anaphase (Figure 8F). As noted above, klp-18(RNAi) embryos displayed variable defects in formation of the female pronucleus. In embryos with a larger than normal complement of maternal chromatin in one or more female pronuclei (12 of 18 embryos), chromosome behavior during mitosis was defective: the maternal complement of chromatin failed to condense tightly during metaphase (Figure 8M) and failed to segregate normally, forming massive chromosome bridges during anaphase (Figure 8N; Movie 4). In embryos with no female pronucleus (4 of 18 embryos), chromosomes derived solely from the male pronucleus, although haploid, underwent a normal mitotic division (Figure 8, G–J; Movie 3). Thus, although depletion of KLP-18 resulted in severe meiotic defects in all cases analyzed, it resulted in mitotic defects only in those embryos that delivered an aberrant complement of maternal chromatin to the mitotic spindle. These results suggest that mitotic chromosome congression and segregation problems caused by KLP-18 depletion do not reflect a direct function for KLP-18 in the mitotic spindle. Rather, they are a consequence of the presence of maternal chromosomes that have suffered aberrant meiotic segregation. In summary, the assembly of centrosomal mitotic spindles is significantly less dependent on KLP-18 than is the assembly of acentrosomal female meiotic spindles. KLP-18 may function in mitotic spindles, but if so, its function is redundant.

DISCUSSION

KLP-18 has an N-terminal kinesin-related motor domain that is most similar to members of the Klp2 kinesins (Lawrence et al., 2002). Like Klp2 homologs in other organisms, C. elegans KLP-18 concentrates in spindles, especially at spindle poles during prometaphase, metaphase, and early anaphase, and then in the interzone during late anaphase/telophase. Although this localization pattern is similar in both acentrosomal (female meiotic) and centrosomal (mitotic) spindles, KLP-18 depletion blocks assembly only of acentrosomal meiotic spindles. That assembly failure prevents normal segregation of maternal chromosomes, leading to embryos with either no maternal DNA or an abnormally small spindle. In the division cycle that follows meiosis, mitotic spindle assembly seems normal, but there are usually defects in congression and anaphase separation for some chromosomes. Time-lapse imaging suggests that the misbehaving chromosomes are maternal. Why chromatin from a defective female meiosis behaves aberrantly in the subsequent mitotic division remains an interesting unanswered question.

As described in the INTRODUCTION, it is thought that acentrosomal spindle assembly requires 1) chromatin-stimulated microtubule polymerization that leads to the accumulation of disordered MTs around chromosomes, 2) MT cross-linking that generates parallel bundles, 3) sorting of polarity by MT-chromatin and MT-MT force generation that moves minus ends away from chromosomes, and 4) focusing of minus ends to form more compact spindle poles (Theurkauf and Hawley, 1992; Albertson and Thomson, 1993; Heald et al., 1996). Excellent progress has been made in understanding the initial chromatin-stimulated polymerization, but subsequent steps are not as well understood. TPX2, a MT-binding protein, which is activated by high levels of RanGTP near the MT-Dynamitin stimulates MT polymerization (Wittmann et al., 2000; Gruss et al., 2001, 2002). TPX2 also mediates attachment of the nonmotor C-terminal tail of Xklp2 to MTs (Wittmann et al., 1998, 2000). It is reasonable to think that the N-terminal motor domain of Xklp2 then binds to neighboring MTs, forming force-producing cross-links that could contribute to the intermediate steps of acentrosomal spindle assembly, i.e., parallel bundling and MT-MT sliding. However, Xklp2 is dispensable for extract spindle assembly, making minor or redundant contributions (Walczak et al., 1998). Instead, a different MT cross-linking kinesin, the BimC homolog Eg5, is required for MT bundling/sorting. A chromosome-associated kinesin, Xklp1, also contributes to those intermediate assembly steps (Walczak et al., 1998).

Our results suggest a switch in the relative importance of kinesins in C. elegans meiotic spindle assembly. KLP-18 depletion leads to a failure in MT bundling and organization around female meiotic chromosomes. Our depletion/disruption tests of the worm BimC homolog (BMK-1) and of chromokinesin homologs (KLP-12 and KLP-19) suggest that they are dispensable in female meiosis (Saunders, Rose, Powers, Strome, and Saxton, unpublished data). The contrast between the Xenopus extract and worm results could be due to differences between the two organisms or between the extract versus in vivo approaches. The extract system can provide powerful new insights through the analysis of processes under defined conditions, but it cannot be expected to reveal all aspects of in vivo mechanisms. Thus, it is possible that in vivo acentrosomal meiotic spindle assembly in Xenopus oocytes does require Xklp2. Alternatively, Xenopus and C. elegans may have evolved different strategies for acentrosomal spindle assembly, emphasizing the use of different motors to accomplish the same MT accumulation, bundling, and sorting objectives.

Our results suggest that although KLP-18 concentrates in the mitotic spindles of early C. elegans embryos, it is dispensable for their assembly and function. MTs nucleated by mitotic centrosomes are well ordered relative to those organized by chromatin; their radial arrangement makes neighboring MTs nearly parallel, with most minus ends oriented toward the nearest centrosome. Hence, a KLP-18 MT bundling/sorting activity that is essential in acentrosomal meiotic spindles may be superfluous when centrosomes are present. Similarly, the human kinesin-related protein HSET localizes to both meiotic and mitotic spindles, perhaps cross-
linking neighboring MTs, but HSET inhibition seems to cause significant assembly defects only in acentrosomal meiotic spindles. Additional evidence that HSET is needed for taxol-induced, acentrosomal aster formation argues that it is the presence of centrosomes that eliminates the need for HSET during mitosis (Mountain et al., 1999). The Drosophila kinesins NCD and SUB also seem to play important roles in acentrosomal spindle assembly and lesser roles in embryonic mitotic spindle assembly (Matthews et al., 1996; Giunta et al., 2002).

The need for TPX2 activity in localizing Xklp2 to Xenopus spindle MTs and the role of TPX2 in chromatin-stimulated spindle assembly suggest that a TPX2 homolog and associated proteins might contribute to KLP-18 localization/function in C. elegans. An obvious homolog of TPX2 has not been found in searches of the C. elegans genome sequence (our unpublished data; Asksjaer et al., 2002). Furthermore, it was recently noted that RNAi of the worm homologs of Ran, RanGAP, and RanBP2, although sufficient to prevent organization of kinetochore and interpolar MTs in mitotic spindles, had no apparent effects on meiotic chromosome segregation of kinetochore and interpolar MTs in mitotic spin-

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