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Cytokinesis and Midzone Microtubule Organization in *Caenorhabditis elegans* Require the Kinesin-like Protein ZEN-4

William B. Raich,* Adrienne N. Moran,† Joel H. Rothman,‡ and Jeff Hardin*†§

*Program in Cellular and Molecular Biology and †Department of Zoology, University of Wisconsin-Madison, Madison, Wisconsin 53706; and ‡Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, California 93106

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Members of the MKLP1 subfamily of kinesin motor proteins localize to the equatorial region of the spindle midzone and are capable of bundling antiparallel microtubules in vitro. Despite these intriguing characteristics, it is unclear what role these kinesins play in dividing cells, particularly within the context of a developing embryo. Here, we report the identification of a null allele of *zen-4*, an MKLP1 homologue in the nematode *Caenorhabditis elegans*, and demonstrate that ZEN-4 is essential for cytokinesis. Embryos deprived of ZEN-4 form multinucleate single-celled embryos as they continue to cycle through mitosis but fail to complete cell division. Initiation of the cytokinetic furrow occurs at the normal time and place, but furrow propagation halts prematurely. Time-lapse recordings and microtubule staining reveal that the cytokinesis defect is preceded by the dissociation of the midzone microtubules. We show that ZEN-4 protein localizes to the spindle midzone during anaphase and persists at the midbody region throughout cytokinesis. We propose that ZEN-4 directly cross-links the midzone microtubules and suggest that these microtubules are required for the completion of cytokinesis.

INTRODUCTION

During cytokinesis, the final stage of cell division, a parent cell divides to form two daughters. Division normally occurs after chromosome separation, along a plane perpendicular to the mitotic spindle and equidistant from the segregated chromosomes. A large body of data suggests that the contraction of a transient actomyosin ring located immediately beneath the plasma membrane provides the driving force for propagation of the cleavage furrow (for review, see Rappaport, 1996), and cell division in the nematode *Caenorhabditis elegans* appears to be no exception (Strome and Wood, 1983; Hill and Strome, 1988).

The role of microtubules in the progression of the cleavage furrow is less clear, although a number of studies point toward a requirement for microtubules. In newt eggs, injection of the microtubule poisons colchicine, vinblastine, or nocodazole around a small initial furrow, or under the advancing furrow tip, blocks furrow progression or results in furrow regression (Sawai, 1992). In *Xenopus*, microtubule bundles arise in the cleavage furrows concomitant with new membrane assembly, and treatment with nocodazole or cold shock causes cleavage furrow recession (Danilchik *et al.*, 1998). In snail eggs, regression of polar lobes correlates with microtubule loss, in contrast with the persistent furrowing that correlates with taxol stabilization of microtubules (Conrad *et al.*, 1992).

One population of microtubules that could be involved in both furrow initiation and propagation are the midzone microtubules, which arise during anaphase and persist throughout cytokinesis. Midzone microtubules occur between the separating chromosomes and are oriented in an antiparallel manner. In cultured mammalian epithelial cells, reduction of midzone microtubules by nocodazole treatment results in inhibition or regression of furrowing (Wheatley and Wang, 1996). Insertion of a physical barrier between the mitotic spindle and the cell cortex prevents cyto-

§Corresponding author.

kinesis, but only if the barrier is placed at the equator of the spindle (Cao and Wang, 1996). In *Drosophila melanogaster* spermatocytes, mutations at the *diaphanous* and *KLP3A* loci disrupt the central spindle and the contractile ring, suggesting a cooperative interaction between the central spindle microtubules and ingression of the cleavage furrow (Giansanti *et al.*, 1998). A growing number of proteins that localize to the spindle midzone have been identified, but of particular interest is CHO1/MKLP1, which exhibits a variety of biochemical properties, suggesting that it may be crucial for midzone microtubule organization.

MKLP1 is a member of the kinesin family of motor proteins, which utilize energy derived from the hydrolysis of ATP to translocate along microtubules (Goldstein, 1993; Moore and Endow, 1996; Hirokawa, 1998). Phylogenetic analysis of the conserved kinesin motor domain has been used to construct kinesin subfamilies; currently, the MKLP1 subfamily consists of *Cricetulus griseus* CHO1 and *Homo sapiens* MKLP1. In dividing cells, CHO1 and MKLP1 tightly localize to the spindle midzone from anaphase to the completion of cytokinesis (Sellitto and Kuriyama, 1988; Nislow *et al.*, 1992). Unlike other microtubule motors, recombinant MKLP1 promotes the formation of antiparallel microtubule bundles in an ATP-dependent manner (Nislow *et al.*, 1992). When overexpressed in HeLa cells, CHO1 promotes the formation of microtubule bundles and is thought to have both ATP-dependent and ATP-independent microtubule-binding sites (Kuriyama *et al.*, 1994). Injection of a mAb recognizing CHO1 into PtK_1 cells before metaphase halts mitosis in a metaphase-like configuration. However, perturbation experiments have yielded little information on the role of CHO1 beyond metaphase, as injection of CHO1 antibody after anaphase onset has little effect on cell division (Nislow *et al.*, 1990).

In this article, we report the identification of a null mutation in a *Caenorhabditis elegans* kinesin-like protein called ZEN-4 belonging to the MKLP1 subfamily of kinesins. Deprivation of both maternal and zygotic ZEN-4 protein results in the formation of a single multinucleate cell, as embryos continue to cycle through mitosis but fail to complete cytokinesis. ZEN-4 protein localizes to the midzone of the mitotic spindle and persists in the midzone through the completion of cell division. In embryos deprived of ZEN-4, microtubules in the midzone of the mitotic spindle are severely disorganized. Given that MKLP1 has been demonstrated to bundle antiparallel arrays of microtubules (Nislow *et al.*, 1992), we propose that ZEN-4 directly bundles midzone microtubules and suggest that organized midzone microtubules are required for the progression of the cleavage furrow and the completion of cytokinesis.

MATERIALS AND METHODS

C. elegans Strains

The Bristol strain N2 was used as wt (Brenner, 1974). Nematodes were grown at 20°C in all experiments and were cultured as described by Brenner (1974). To map and balance *zen-4*, the following mutations and deficiencies were obtained from the *C. elegans* Genetic Stock Center: linkage group IV, *dpy-13(e184)*; *unc-5(e53)*; *unc-44(e362)*; *unc-44(e1260)*; *lag-1(q385)*; *pat-8(st554)*; *bli-6(sc16)*; *unc-24(e138)*; *unc-31(e169)*; *dpy-4(e1166)*; *nDf41*; *stDf7*; linkage group V, *him-5(e1467)*.

Genetics

w35 was isolated in a general, genome-wide screen for ethylmethane sulfonate (EMS)-induced zygotic embryonic lethal mutations (Ferguson *et al.*, 1996; our unpublished results). *w35* was mapped to LGIV between *unc-44* and *bli-6* by standard three-factor recombination experiments (Sulston and Hodgkin, 1988). Recombination between *unc-44* and *bli-6* placed *w35* 11/28 of the distance from *unc-44* to *bli-6*.

Rescue

zen-4 maps between the cloned genes, *unc-44* and *smg-7* (*smg-7* maps to the right of *bli-6*), on linkage group IV. Cosmids from this region were obtained from the *C. elegans* Genome Consortium and were tested for transformation rescue by coninjection with the dominant marker *rol-6(su1006)* (Mello *et al.*, 1991). The following cDNAs were obtained from the *C. elegans* cDNA Project (Y. Kohara) because they mapped to the minimal *zen-4* rescuing genomic fragment: yk35d10, yk13d7, yk391b3, and yk2g4. We sequenced additional *zen-4* cDNA clones isolated from an embryonic cDNA library (J. Zhou, University of California, Santa Barbara). All sequencing was performed using ABI PRISM dye terminator cycle sequencing (Perkin Elmer-Cetus, Norwalk, CT). The cDNAs are predicted to encode two products, called ZEN-4a, and ZEN-4b, which are identical from amino acids 1 to 745 but contain differing C-terminal domains of 30 and 27 amino acids, respectively. Genomic sequence of cosmid M03D4, available from the *C. elegans* Genome Project, agrees with our cDNA sequence and indicates that the *zen-4* locus consists of eight exons included in the 7.0-kilobase (kb) rescuing fragment. An alternative splice site predicted within exon 7 supports our finding that the *zen-4* locus produces two transcripts.

The genomic region of the *zen-4* locus was amplified from homozygous *w35* embryos by the single-embryo PCR method (Williams *et al.*, 1992). The following sets of primers were used to amplify the genomic region in two overlapping fragments: CCCTCCGCCGCCAGTTTG, CTCTTGCTCTCCTTGCTCACAC and CTCTTCTTACTATGATTCGCC, GAAAATGCAGCAGGATGGAGG. All PCR products were subcloned into the pT7 vector (Novagen, Madison, WI). For each amplification product, three subclones of two independent reactions were sequenced.

Mosaic Analysis and RNA Interference

Two methods were used to deplete maternal ZEN-4 to ascertain the phenotype in the complete absence of ZEN-4, mosaic analysis, and RNA interference. Germline mosaic animals were identified from a homozygous *zen-4(w35)* strain carrying a minimal wt *zen-4* transgene on an extrachromosomal array. Animals that produced exclusively inviable progeny were assumed to have spontaneously lost the array from the lineages that generate the germline. Germline mosaics were recovered at a frequency of 1–2%, which is similar to the reported frequency of germline mosaics for other mutations (Costa *et al.*, 1998).

RNA was transcribed in vitro as described by Guo and Kemphues (1995), with the following modifications: T3 and T7 polymerase were used to synthesize both sense and antisense RNA (MEGA-

script kit, Ambion, Austin, TX). RNA was recovered by an ethanol ammonium acetate precipitation, and product concentration was assessed by OD_{260} and by electrophoresis on standard agarose gels. Single-stranded RNA was injected into the gonad of young N2 hermaphrodite adults at a concentration of ~1 μg/μl. Both sense and antisense RNA interfered with *zen-4* function. Specificity of interference can be inferred, because progeny obtained from germline mosaic animals displayed an identical phenotype to progeny of animals injected with *zen-4* RNA.

Phenotypic Analysis

Gravid hermaphrodites were cut transversely through the vulva. The extruded embryos were mounted on a 5% agar pad in M9 solution (Wood, 1988) and filmed using four-dimensional microscopy. Typically, 15–20 focal planes spaced at 1-μm intervals were recorded every 30–35 s. The four-dimensional system consists of a Nikon Optiphot-II microscope equipped with Nomarski optics, a Ludl Z-axis stage controller operated via a Ludl Mac2000 control box, and a Uniblitz electronic shutter on the transilluminator port. The shutter and Z-axis motor are controlled via serial cable connections to a Macintosh PowerMac 9600 equipped with a Scion AG-5 8-bit frame grabber. Image acquisition was accomplished using a modified version of NIH Image, originally developed by Wayne Rasband (available at zippy.nimh.nih.gov).

Antibody Production and Immunostaining

Rabbit polyclonal antibodies were raised and affinity purified against the following peptide, called ZEN-4N: SRDQVRRKKLSIEET (Quality Controlled Biochemicals, Hopkinton, MA). For Western blotting, 5 μl of a total worm protein extract were electrophoresed on a 7% SDS-PAGE gel and transferred to nitrocellulose. ZEN-4N antiserum was applied at 1:1000 in PBS with 0.1% Tween 20 (PBST)[1] + 1% BSA. Membrane preparation and protein visualization were carried out using NEN Western Blot Chemiluminescence Reagent according to the instructions of the manufacturer (New England Nuclear Life Science, Boston, MA).

A modified version of the freeze-crack method (Miller and Shakes, 1995) was used to process embryos for immunostaining. Briefly, 20–40 young gravid adults were placed in a drop of egg salts (Edgar, 1995) on a 0.01% poly-L-lysine–coated slide. The worms were covered with an 18 mm coverslip, and gentle pressure was applied on the coverslip directly over the center of each worm until the eggs were released from the vulva. The slides were quick frozen on dry ice for 20 min. After coverslip removal, the samples were fixed for 5 min in 100% methanol at −20°C, air dried for 2 min, and incubated in PBST + 1% BSA for 1–2 h. Affinity-purified ZEN-4 antiserum was added in a 1:200 dilution in PBST + 1% BSA, the β-tubulin antibody N357 (Amersham) was applied at a concentration of 2 μg/ml, the DNA antibody mAB030 (Chemicon, Temecula, CA) was applied at a 1:200 dilution in PBST + 1% BSA, and DAPI was added at a concentration of 1 μg/ml. All primary antibody incubations were performed overnight at 4°C. The samples were washed in PBST three times for 10 min and incubated in FITC or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in PBST + 1% BSA for 1 h at room temperature. The specimens were rinsed in PBST buffer and sealed in a drop of Slowfade antibleaching solution (Molecular Probes, Eugene, OR). Fluorescent images were obtained using a Bio-Rad MRC 1024 confocal laser scanning microscope or a Hamamatsu digital camera.

[1] Abbreviations used: EMS, ethylmethane sulfonate; PBST, PBS with Tween 20.

RESULTS

zen-4 Is Required for Embryogenesis

Embryogenesis in *C. elegans* can be divided into two stages: premorphogenesis, which consists of rapid cell proliferation with little change in embryo shape, and morphogenesis, which features little change in cell number but dramatic change in the shape of the embryo (Priess and Hirsh, 1986). Morphogenesis begins ~5 h after first cleavage, with the highly stereotyped tissue migration called ventral enclosure (Williams-Masson *et al.*, 1997). During this process, an epithelial sheet of cells called the hypodermis migrates from the dorsal surface of the embryo laterally and ventrally, wrapping the embryo in an epithelial monolayer. After standard EMS mutagenesis, we screened for mutations that failed to properly enclose in hypodermis. We identified a recessive, zygotic-lethal mutation called *zen-4* (zygotic enclosure defective). A complete characterization of the zygotic *zen-4* phenotype will be presented in a subsequent manuscript.

zen-4 Encodes a Kinesin-like Protein

zen-4(w35) was mapped to linkage group IV between *unc-44* and *bli-6*, a 0.4-genetic map unit interval corresponding to ~400 kb of DNA (Figure 1A). *zen-4(w35)* complements the lethal mutations *lag-1(q385)* and *pat-8(st554)* and fails to complement the deficiency *nDf41*. Cosmids covering this region were obtained from the *C. elegans* Genome Consortium and were tested for their ability to rescue *zen-4(w35)* using standard transformation protocols (Mello *et al.*, 1991). One pool of clones that rescued embryonic lethality was identified, and subsequently the injection of a 7.0 kb *Xho*I–*Kpn*I subclone of cosmid M03D4 was shown to be sufficient to rescue *zen-4* (Figure 1B).

Comparison of genomic and cDNA sequences revealed that the *zen-4* locus consists of eight exons (Figure 1C). All exon/intron boundaries are flanked by conserved *C. elegans* splice junction sequences. Exon 7 contains an alternative splice site that can result in the production of two alternatively spliced proteins, ZEN-4a and ZEN-4b, predicted to be 775 and 772 amino acids, respectively (Figure 1C). cDNAs corresponding to both transcripts have been identified and sequenced, indicating that both splice forms are produced in vivo. To verify that *zen-4* is encoded by this locus, we amplified and sequenced the region from *w35* homozygous eggs. A 5-base pair (bp) insertion in exon 1 that is predicted to result in a frameshift and premature stop codon following amino acid 91 was identified (Figures 1C and 2A).

ZEN-4 is similar to members of the CHO1/MKLP1 kinesin protein subfamily (Figure 2, B and C). Sequence alignment suggests that ZEN-4 has a conventional kinesin organization, with an N-terminal motor

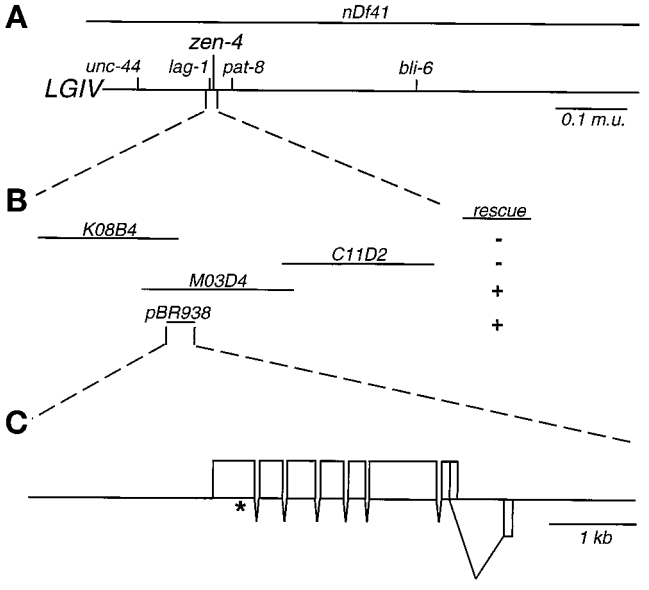


Figure 1. Cloning *zen-4*. (A) Recombination data from three-factor mapping placed *zen-4* 11/28 of the distance between *unc-44(e1260)* and *bli-6(sc16)* on LGIV. *zen-4* complements *lag-1(q385)* and *pat-8(st554)* but fails to complement the deficiency *nDf41*. (B) All *zen-4* phenotypes are rescued by germline transformation with the cosmid M03D4 and the 7.0-kb *Xho*I–*Kpn*I subclone pBR938. (C) *zen-4* is oriented 5′ to 3′; exons are boxed; exon 7 contains an internal splice site resulting in the formation of two products. The *w35* allele contains the insertion GATTT, which is predicted to result in six frameshifted amino acids and a premature stop codon following Arg 91 (asterisk).

domain followed by a central region predicted to mediate the formation of a stable homodimer through a parallel coiled coil. While the sequence identity with published MKLP subfamily members varies from 28 to 31%, a 326-amino acid stretch in the N terminus shows 51% identity to CHO1, and a 98-amino acid domain in the C terminus unique to the MKLP1 family is 48% identical to CHO1.

A number of motifs implicated in nucleotide or microtubule binding are highly conserved in ZEN-4 (Figure 2A). The nucleotide-binding motif N1 (or P loop) is 67% identical to the kinesin gene family consensus sequence, and the N2 motif (SSRSH) and N3 motif (DLAGSE) are 100% identical. As shown in Figure 2, the DLL and L12 microtubule-binding motifs are also conserved in ZEN-4. Note that the premature stop codon in *w35* occurs upstream of all of these sites, suggesting that *w35* does not encode a protein capable of binding microtubules or ATP (Figure 2A). Two additional lines of evidence demonstrate that *w35* is a null allele: absence of ZEN-4 staining in the offspring of germline mosaic animals (Figure 4D; see below) and the similarity between the offspring of *zen-4* germline mosaic animals and animals injected with antisense *zen-4* RNA (Figure 3; see below).

ZEN-4 Is Required for Cytokinesis

To determine whether ZEN-4 was required before embryo morphogenesis, two techniques were utilized to remove functional maternal and zygotic protein. Germline mosaic animals were identified in which an extrachromosomal array containing wt *zen-4* was lost only in the lineages contributing to the germline. Offspring of these germline mosaic animals have neither maternal nor zygotic *zen-4* mRNA, and 100% of the embryos display the cytokinesis defect described below. In addition, the complete absence of *zen-4* mRNA was achieved using the technique of RNA interference (Guo and Kemphues, 1995; Fire *et al.*, 1998), in which *zen-4* RNA is synthesized in vitro and injected into the hermaphrodite gonad of wt animals. Injected animals laid eggs that were indistinguishable from the progeny of *zen-4(w35)* germline mosaic animals (Figure 3), indicating that the interference was specific for *zen-4*.

Embryos depleted of *zen-4* display a highly reproducible defect in cytokinesis. In wt embryos, the maternal pronucleus completes meiosis, and the first and second polar bodies are extruded ~20 min after fertilization (Figure 3, A and M). Polar body extrusion separates the byproducts of meiosis from the fertilized egg and is considered to be a specialized case of cytokinesis. In embryos depleted of ZEN-4, the polar bodies fail to be extruded (Figure 3, B and C). Pronuclear migration and centrosome rotation occur normally, demonstrating that ZEN-4 is not necessary for these intracellular movements (Figure 3, D–I).

As is the case in wt, embryos deprived of ZEN-4 initiate the formation of a cleavage furrow after formation of the mitotic spindle (Figure 3, J–L). Time-lapse videomicroscopy reveals that the furrow continues to expand for ~2–3 min but stalls at the region of the spindle midzone (Figure 6). Shortly thereafter, the furrow begins to retract, ultimately relaxing to produce a single, large multinucleate cell instead of two smaller cells (Figure 3, Q and R). During the second mitosis, two mitotic spindles form, indicating that the centrosomes have duplicated, and the embryo forms two cleavage furrows. However, cytokinesis again fails, and the furrows retract upon reaching the spindle midzone. Ultimately, this produces a single cell with a large, central multinucleate mass (our unpublished results).

ZEN-4 Localizes to the Midbody of Dividing Cells

The similarity between ZEN-4 and proteins capable of binding and organizing microtubules suggested that ZEN-4 might have a similar function. To test this, we raised antibodies to ZEN-4 to examine its temporal and spatial localization. After affinity purification, the antiserum recognized a major protein band of 93 kDa in extracts of total *C. elegans* proteins (Figure 4F) and gave a strong immunofluorescent signal. Four lines of evidence suggest that the staining obtained with the

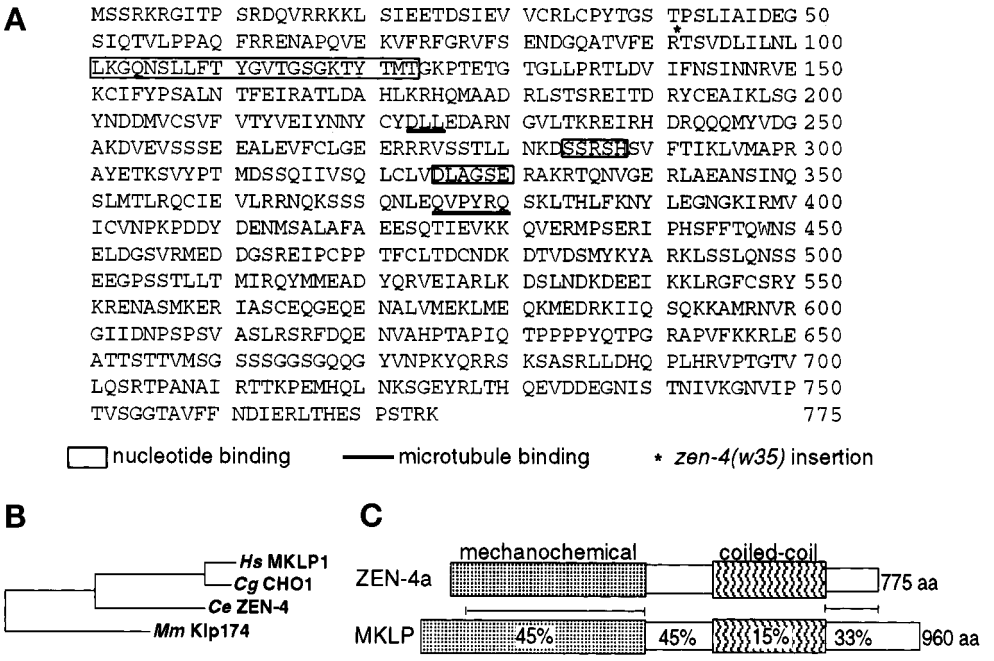


Figure 2. *zen-4* encodes a member of the MKLP1 subfamily of kinesin-like proteins. Because of alternative splicing, *zen-4* is predicted to encode a 775-amino acid protein (ZEN-4a) and a 772-amino acid (ZEN-4b) protein. (A) The predicted sequence of ZEN-4a. The putative nucleotide-binding motifs, N1, N2, and N3, are boxed; the putative microtubule binding sites, DLL and L12, are underlined. The location of the frameshift in the *w35* allele is marked with an asterisk. (B) ZEN-4 belongs to the MKLP1 subfamily of kinesin-like proteins. The tree shown was constructed by Moore and Endow (1996) and is based on sequence alignment of the conserved kinesin motor domain. An updated version of the kinesin tree can be found at: http://www.blocks.fhcrc.org/~kinesin/. (C) ZEN-4 sequence conservation with MKLP1 extends beyond the motor domain. A domain unique to the MKLP1 subfamily is 33% identical in ZEN-4 and MKLP1. While the coiled-coil domain is not well conserved, it contains the heptad repeats characteristic of a coiled coil. The complete sequence data for ZEN-4a and ZEN-4b are available from GenBank/EMBL/DDBJ under accession numbers AF057567 and AF057568.

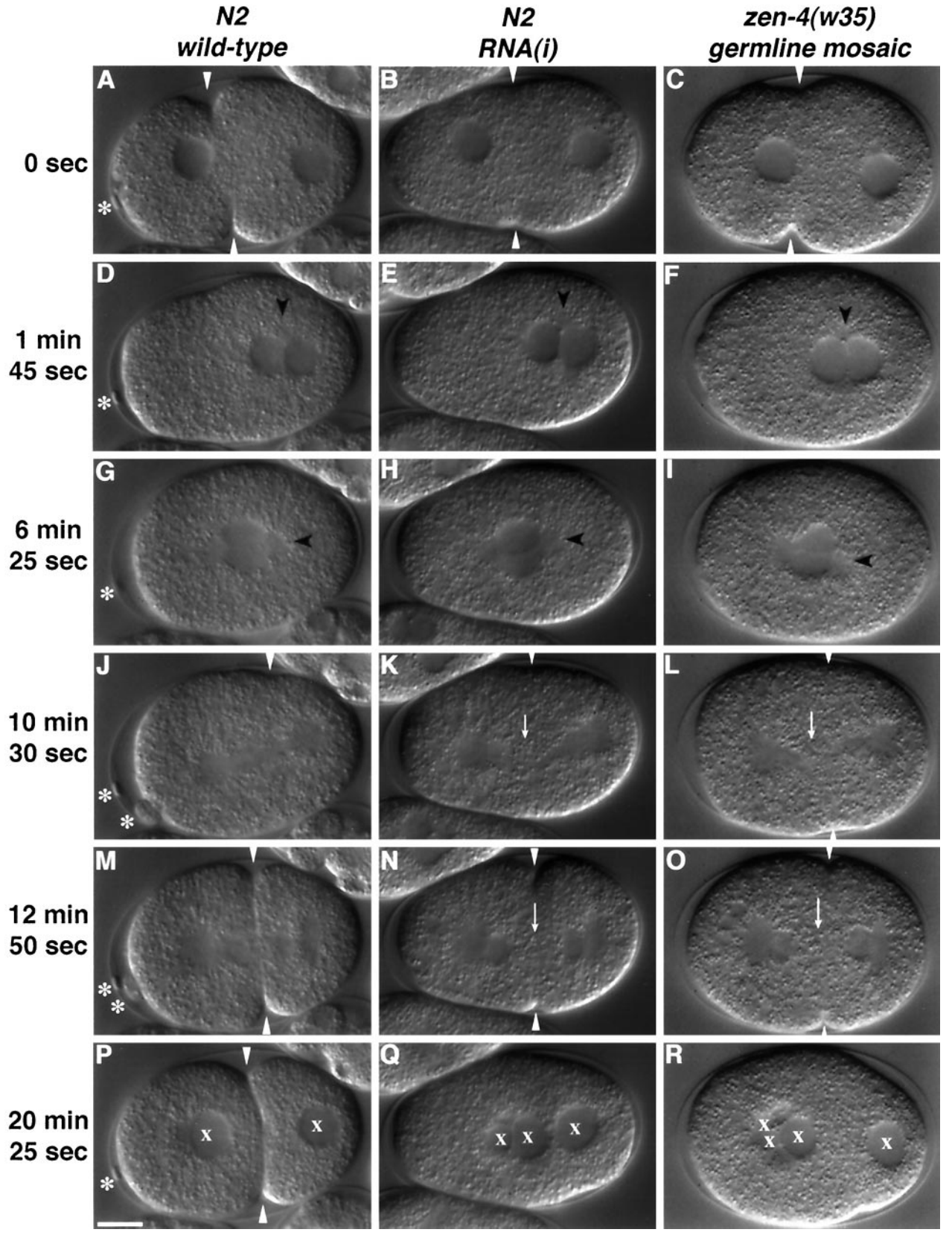
antiserum reflects the actual pattern of ZEN-4 localization. First, terminal *zen-4(w35)* embryos do not stain with the antibody but can be stained with other antibodies. This suggests that the decrease in staining is the result of a specific reduction in the level of ZEN-4 epitope. Second, addition of a 10-fold excess of the peptide against which the antibody was raised completely and specifically abrogates ZEN-4 staining. Third, RNA interference with *zen-4* RNA completely eliminates ZEN-4 immunostaining but does not reduce the levels of DNA detected with the anti-DNA mAb030 (our unpublished results). Fourth, the antibody does not stain offspring of *zen-4(w35)* germline mosaic adults, which do not express maternal or zygotic protein (Figure 4D). Together, these data indicate that the ZEN-4 antibody specifically recognizes endogenous ZEN-4 protein.

Confocal microscopy was used to obtain a detailed description of the localization of ZEN-4 protein in dividing cells. ZEN-4 localizes to the spindle midbody of all dividing cells. Low levels of protein appear to localize to the region of the chromosomes of the metaphase mitotic spindle (Figure 5C). In anaphase cells, the antigen localizes in discrete lines along the equatorial region of the spindle (Figure 5, A and C). As mitosis progresses, the antigen condenses into a disk shape at the midzone of the intercellular bridge between daughter cells (Figure 5). Midbody staining intensifies during cytokinesis (Figure 5F) and gradually contracts to form an intense spot at the midzone of the mitotic spindle (Figure 5C). After the completion of cell division, staining persists at the remnant of the midbody (Figure 5, A and C). ZEN-4 staining in the remnant is intense and persists well after the midbody microtubules have dissociated.

In interphase cells, ZEN-4 can occasionally be seen localized to centrosomes (our unpublished results), but protein does not appear to stain the centrosomes in dividing cells (Figure 4). This is consistent with the known localization pattern for CHO1 antigen, but inconsistent with MKLP1, which has been reported to localize to the poles of the mitotic spindle as well as the spindle midzone (Nislow *et al.*, 1992).

Midzone Microtubule Bundles Are Disrupted in zen-4 Mutants

In wt embryos, bundles of microtubules are present in the midzone of the mitotic spindle. These microtubule


N2
wild-type
N2
RNA(i)
zen-4(w35)
germline mosaic
0 sec
1 min
45 sec
6 min
25 sec
10 min
30 sec
12 min
50 sec
20 min
25 sec
A
B
C
D
E
F
G
H
I
J
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L
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N
O
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Figure 3.

bundles persist from anaphase to late telophase (Figure 6). Time-lapse microscopy of mutant embryos suggested that ZEN-4 is required for the formation and/or maintenance of these midzone microtubules, as the clear area indicative of the central spindle was replaced with the granular material characteristic of cytoplasm (compare Figure 6C with 6D).

To confirm this observation, *zen-4(w35)* germline mosaic embryos were coimmunostained with the β-tubulin antibody N357 to visualize microtubules and DAPI to visualize DNA. Embryos deprived of ZEN-4 show diminished microtubules at the midzone of the mitotic spindle during anaphase, but the mitotic spindle appears qualitatively normal in other respects (Figure 4E). This demonstrates that ZEN-4 is necessary for the maintenance of midzone microtubule arrays and is consistent with in vitro experiments suggesting that MKLP1 promotes the formation of antiparallel bundles of microtubules (Nislow *et al.*, 1992).

ZEN-4 Is Not Required for Anaphase Spindle Pole Separation

MKLP1 has been proposed to be responsible for spindle pole elongation during anaphase B, as it slides antiparallel microtubules past one another at a rate consistent with spindle elongation (see DISCUSSION). However, spindle poles appear to separate as much in embryos deprived of ZEN-4 as in control embryos (compare Figure 6E with 6F).

Measurements of spindle pole lengths during the first cell division in wt and mutant embryos support this observation. In wt embryos, the distance between the spindle poles increased from a mean prophase length of 16 μm to a mean late-anaphase length of 27 μm. In embryos depleted of ZEN-4 protein by RNA interference, the mean spindle increased in length during the same time period from 16 μm to 29 μm, and offspring of germline mosaics display a similar increase (Table 1). This result demonstrates that ZEN-4 is not required for spindle pole separation during the first cell division.

DISCUSSION

Deprivation of maternal and zygotic ZEN-4 results in the formation of a multinucleate single-celled embryo, as embryos cycle through mitosis but fail to complete cytokinesis. The failure in cytokinesis is preceded by the premature dissociation of the microtubules between the separating chromosomes. ZEN-4 localizes to the midzone of the mitotic spindle, suggesting that ZEN-4 normally stabilizes midzone microtubules. This supports a growing body of evidence suggesting that the midzone microtubules are required for the progression of the cleavage furrow (Wheatley and Wang, 1996; Eckley *et al.*, 1997).

Identification of a C. elegans MKLP1 Homologue

Phylogenetic analysis of the conserved kinesin motor domain places ZEN-4 in the MKLP1 subfamily of kinesin-like proteins (Moore and Endow, 1996). While the *C. elegans* protein is more diverged than previously reported family members, ZEN-4 displays the same molecular organization and contains conserved microtubule- and nucleotide-binding sites. In addition, ZEN-4 contains a 98- amino acid stretch in its C terminus that is 48% identical with CHO1, and this domain is conserved exclusively in the MKLP1 subfamily. At the present time, the *C. elegans* sequencing consortium has identified 14 kinesin family members (for their placement in molecular phylogenies, see http://www.blocks.fhcrc.org/~kinesin/); only ZEN-4 meets all of these criteria.

Another striking similarity between ZEN-4 and MKLP1 is their localization in dividing cells. During anaphase, ZEN-4 and CHO1/MKLP1 localize to discrete short lines in the midzone region of the mitotic spindle. In CHO1 cells, these fibers gradually shorten and coalesce into a bright fluorescent dot in the bridge between daughter cells (Sellitto and Kuriyama, 1988), which is highly reminiscent of the localization of ZEN-4 shown in Figure 5. Given the similarities in

Figure 3 (facing page). Nomarski images selected from time-lapse recordings of embryos at successive stages between fertilization and first cleavage. Embryos deprived of ZEN-4 display a late defect in cytokinesis. The left column shows a wt offspring of an N2 animal, the center column shows the offspring of an N2 animal injected with antisense *zen-4* RNA, and the right column shows the offspring of a *zen-4(w35)* homozygote that lost expression of the rescuing transgene pBR938 in the germline. In all panels, anterior is to the left. (A–C) Pseudocleavage and pronuclear migration. The white arrowhead points to the pseudocleavage furrow. The asterisk in panel A highlights the visible polar body; both polar bodies are visible in panel M. Embryos deprived of ZEN-4 fail to extrude polar bodies. (D–F) Pronuclear contact. The timing and position of pronuclear contact in wt (D) and mutant (E and F) embryos is very similar and was used to normalize the time-lapse recordings. Centrosomes are visible as granule-free regions and are labeled with a black arrowhead. (G–I) Pronuclear migration and rotation are unaffected by loss of ZEN-4. (J) Late anaphase and the beginning of first cleavage. A white arrowhead points to the cleavage furrow. (K and L) Spindle elongation is unaffected in embryos deprived of ZEN-4. A white arrowhead points to a region in the central spindle apparently devoid of organized microtubules. (M) Telophase. Microtubules in the spindle midzone can be seen bisecting the maturing cleavage furrow. (N and O) The cleavage furrow fails to propagate in the region of the central spindle. A white arrow points to a granular region at the center of the mitotic spindle lacking organized microtubules. (P) A two-cell embryo. An "x" is used to label each nucleus. (Q–R) The cleavage furrow has completely relaxed. Nuclei aggregate in the center of the embryo. The failure to extrude polar bodies may explain the excess number of nuclei. Note that loss of a rescuing array and RNA interference result in an indistinguishable phenotype. Scale bar, 10 μm.

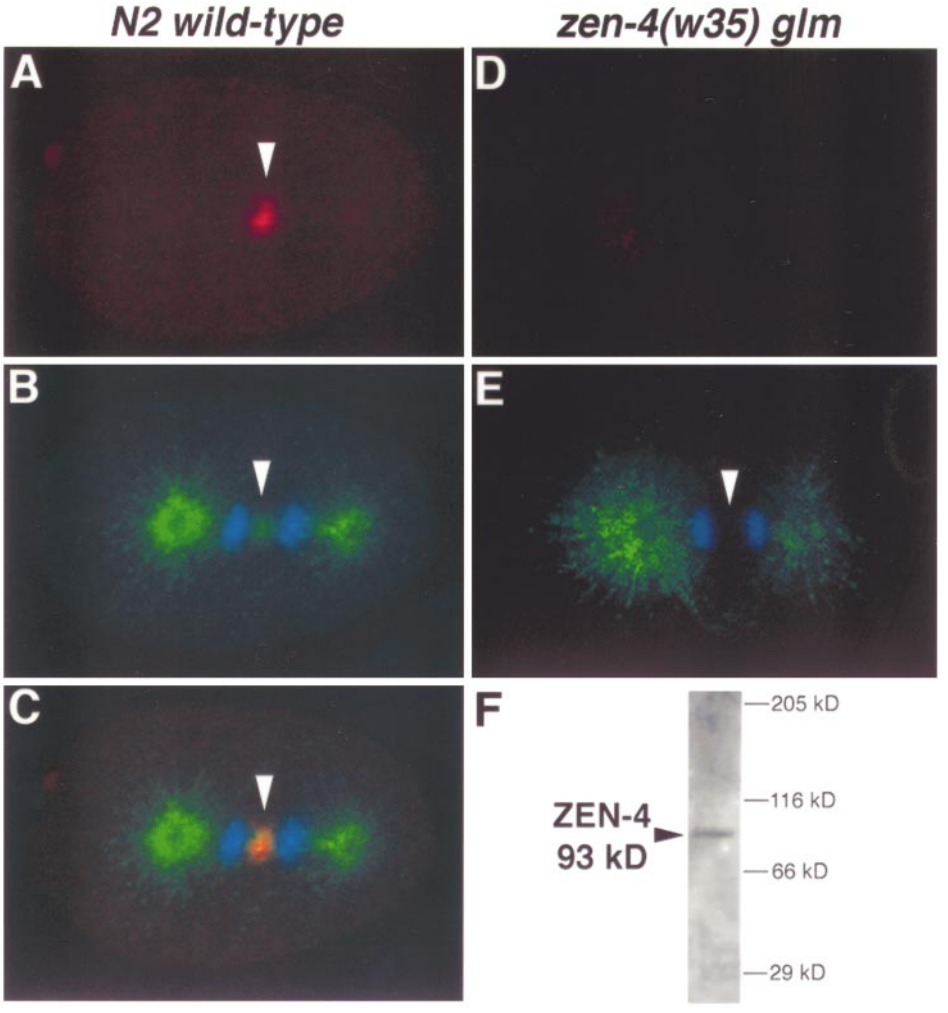

Figure 4. ZEN-4 is required for normal organization of the midzone microtubules. Embryos are coimmunostained with ZEN-4N to label ZEN-4 (red), N357 to label tubulin (green), and DAPI (blue) to label DNA. (A) ZEN-4 staining during the first cell division. ZEN-4 localizes in discrete short lines in the spindle midzone. (B) Tubulin and DNA staining in the same embryo shown in panel A. A white arrowhead points to organized microtubules in the spindle midzone. (C) An overlay of panels A and B, showing that ZEN-4 colocalizes with the microtubules between the separating chromosomes. (D) ZEN-4N does not stain the offspring of a germ line mosaic mutant, indicating that the *w35* allele encodes a protein null. (E) Tubulin and DNA staining of the same embryo shown in panel D. A white arrowhead points to the midzone, which has greatly reduced numbers of microtubules. (F) ZEN-4N recognizes a major band of 93 kDa in extracts of total worm proteins. This is slightly greater than the predicted size of 87 kDa for ZEN-4A and ZEN-4B.

sequence and localization, we propose that *zen-4* encodes an MKLP1 subfamily member.

ZEN-4 Organizes the Midzone Microtubules

Time-lapse Nomarski microscopy and tubulin staining reveal that embryos deprived of ZEN-4 show diminished midzone microtubule bundles, the first evidence documenting the reduction of midzone microtubules associated with the loss of an MKLP1 kinesin. This is consistent with experiments showing that CHO1 overexpression in Sf9 cells causes microtubule bundling (Kuriyama *et al.*, 1994) and in vitro evidence demonstrating that MKLP1 is capable of bundling antiparallel microtubules (Nislow *et al.*, 1992).

Other kinesins have been identified that are proposed to interact with midzone microtubules, primarily based on localization studies. These include Xklp1 (Vernos *et al., 1995)*, cut7 (Hagan and Yanagida, 1992), chromokinesin (Wang and Adler, 1995), CENP-E (Yen *et al.*, 1992), and KatAp (Liu *et al.*, 1996). Of particular interest is *Drosophila* KLP3A, which localizes to the equator of the central spindle. Mutations in the *Klp3A* gene disrupt the interdigitation of microtubules in spermatocyte central spindles, and KLP3A is required in the testes for cytokinesis (Williams *et al.*, 1995). Despite expression in larval brains, KLP3A does not appear to be necessary for somatic cell division. In addition, deprivation of maternal KLP3A by mitotic recombination techniques results in pronuclear migration defects (Williams *et al.*, 1997). These migrations are unaffected by loss of maternal ZEN-4; thus, it appears that the proteins have differing functions.

None of the four kinesin-like proteins characterized in *C. elegans* have been demonstrated to affect midzone microtubule organization. UNC-104 (Hall and Hedgecock, 1991) and OSM-3 (Tabish *et al.*, 1995) are neuron-specific motors. UNC-116, a member of the kinesin heavy chain subfamily, plays a role in both axon outgrowth and cell division, although it is currently un-

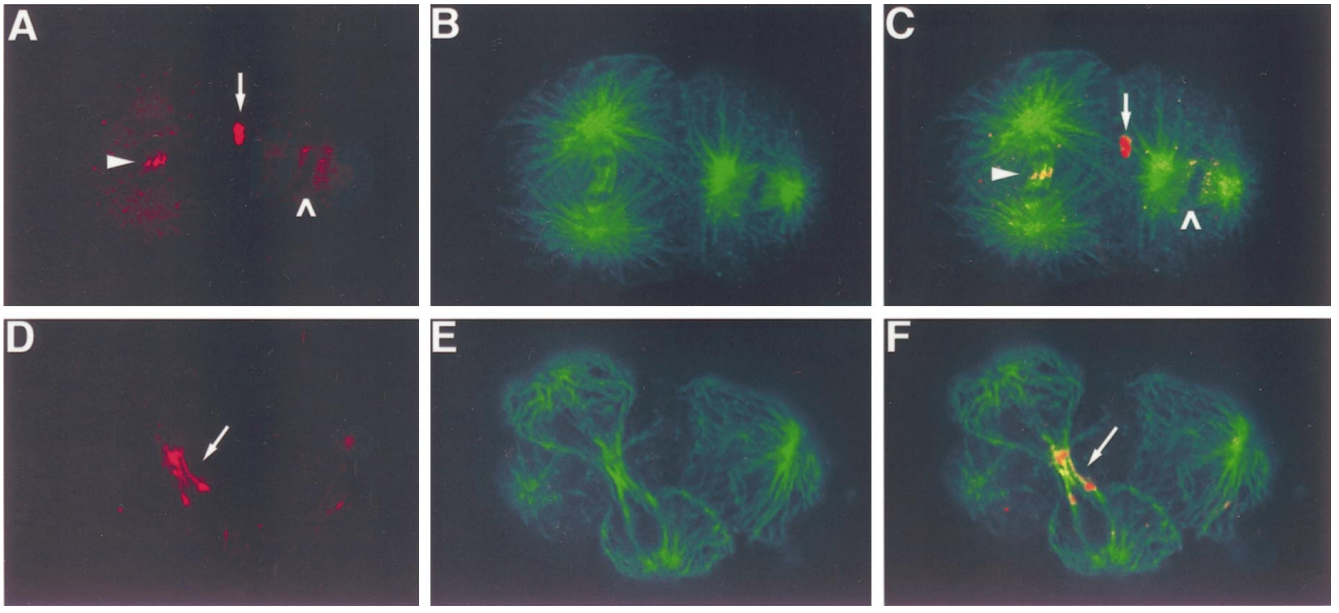

Figure 5. ZEN-4 localization is cell cycle dependent. Embryos are coimmunostained with ZEN-4N to label ZEN-4 (red) and N357 (green) to label the microtubules. Anterior is to the left in all panels. (A) ZEN-4 staining in a two-cell embryo. (B) Tubulin staining in the same embryo shown in panel A. (C) Combined images of panels A and B, showing the overlapping pattern in yellow. ZEN-4N strongly stains the midbody remnant from the first cell division (white arrow), and expression can also be seen at the periphery of the chromosomes in the more posterior cell and in the central region of the midzone in the anterior anaphase cell (white arrowhead). The chromatin can be seen as the black region of the spindle, which excludes both antibodies (white caret). (D) ZEN-4 staining in the midbody of a dividing cell. (E) Tubulin staining of the same embryo shown in panel D. (F) Overlay of panels D and E, showing that ZEN-4 colocalizes with the midzone microtubules at the cytoplasmic bridge between daughter cells.

clear how it functions during mitosis (Hall *et al.*, 1991). A C-terminal motor subfamily member *klp-3* is proposed to be involved in chromosome separation, as overexpression of *klp-3* reduces the incidence of males caused by nondisjunction of the X-chromosome, and depletion of *klp-3* transcript results in embryonic lethality and the production of polyploid cells. However, the subcellular distribution of KLP-3 and microtubule organization in KLP-3-depleted embryos have yet to be reported (Khan *et al.*, 1997).

ZEN-4 Is Not Required for Spindle Elongation

The sliding of midbody microtubules has been shown to participate in spindle elongation during anaphase in mammals, fission yeast, and diatoms (Saxton and McIntosh, 1987; Masuda *et al.*, 1990; Hogan *et al.*, 1992), and kinesin motor proteins are suspected to participate in this process. Antibodies recognizing the conserved kinesin peptide LAGSE block spindle elongation in isolated central spindles and detergent-permeabilized cells from the diatom *Cylindrotheca fusiformis* (Hogan *et al.*, 1993). This sequence is contained within ZEN-4 as well as a large number of other kinesin-related proteins.

Because MKLP1 has been shown to slide antiparallel bundles of microtubules past each other at a velocity of 4 μm/min, it has been proposed to be one of the motors involved in separating the spindle poles during anaphase (Nislow *et al.*, 1992). Perturbation experiments with MKLP1 antibody did not show defects when the antibody was added after the commencement of anaphase (Nislow *et al.*, 1990). However, it is possible that antibody perturbation is not equivalent to analyzing the null phenotype, as the antibody may not perturb MKLP1 function during anaphase spindle elongation. Thus, although MKLP1 promotes the antiparallel sliding of microtubules in vitro, it has yet to be demonstrated that MKLP1 participates in spindle elongation in vivo.

Our analysis suggests that ZEN-4 is not necessary for anaphase spindle elongation, as the spindle elongates to its full length at the normal rate whether or not ZEN-4 is present (Table 1). Although this is clearly the case in *C. elegans*, it should be noted that there are examples of kinesins differing in function that are grouped in the same subfamily by sequence analysis (Moore and Endow, 1996). Alternatively, there may be redundant proteins involved in spindle elongation in *C. elegans*, as multiple motors have been identified in *S. cerevisiae* that play partially redundant roles (Saunders and Hoyt, 1992; Saunders *et al.*, 1997). It should be noted that ZEN-4 deprivation results in *greatly* dimin-

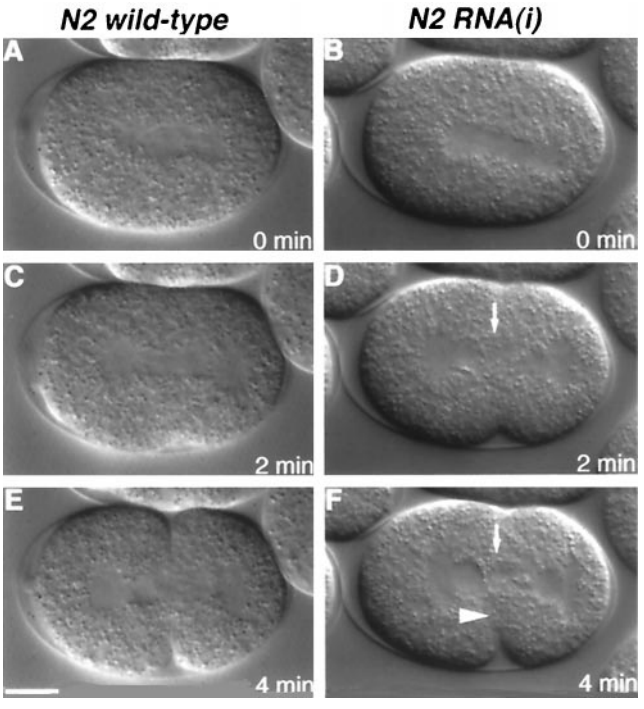

Figure 6. Time-lapse Nomarski images of first cleavage. The embryo in the left column is wt; the embryo in the right column is the offspring of an animal injected with antisense *zen-4* RNA. Anterior is to the left in all panels. (A) wt Metaphase embryo. (B) Metaphase embryo lacking ZEN-4. At this time, the spindle appears normal. (C) wt Anaphase embryo. Initiation of the cleavage furrow alters the ellipsoid shape of the embryo. (D) The cleavage furrow resembles the wt furrow (C), demonstrating that furrow initiation does not require *zen-4*. The white arrow points to the cytoplasmic granules in the spindle midzone, suggesting a lack of organized midzone microtubules. (E) wt Telophase embryo. The cleavage furrow is approaching the spindle midzone. (F) The white arrowhead points to the limit of furrow progression in an embryo lacking ZEN-4. Scale bar, 10 μm.

ished midzone microtubules, yet the spindle elongates to its full extent (Table 1). If a redundant mechanism exists, eliminating ZEN-4 would not affect spindle elongation, unmasking the subsequent defect in cytokinesis.

Table 1. Spindle elongation does not require ZEN-4

Strain	Experiment	n	Spindle elongation mean (μm)	SD (μm)
N2	Contol	5	10.3	0.9
N2	RNA interference	5	11.8	1.1
zen-4(w35)	Germline mosaic	5	10.8	1.3

Spindle length was measured as the distance between the spindle poles in time-lapse recordings of embryos undergoing first division. Spindle elongation was calculated as the absolute value of the difference in spindle length from prophase to late anaphase, a period of ~5 min. Deprivation of ZEN-4 by RNA interference or using germline mosaics does not reduce spindle length; in fact, spindle elongation is slightly enhanced in embryos lacking ZEN-4 ($p > 0.20$).

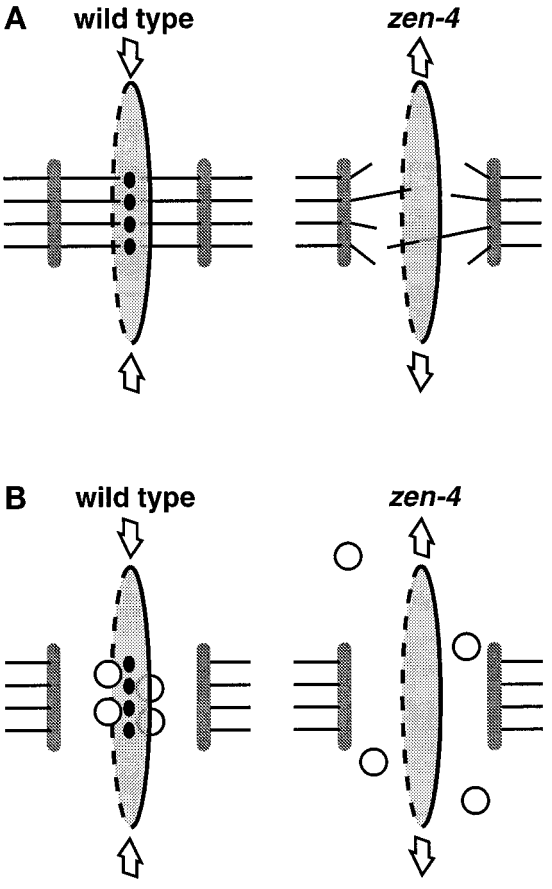

Figure 7. Models of ZEN-4 function during cytokinesis. Schematic diagram of the central spindle during cytokinesis in wt embryos and embryos lacking ZEN-4. (A) The microtubule organization model. ZEN-4 (black ovals) localizes to the spindle midzone, where it serves to directly or indirectly cross-link the midzone microtubules (shown as solid lines between the separating chromosomes). In the absence of organized midzone microtubules, the contractile actomyosin ring, shown as a black line ringing the midzone, halts its migration in the region of the mitotic spindle. In this model, the cytokinesis defect seen in embryos deprived of ZEN-4 is a secondary effect of the failure to organize the midzone microtubules. (B) The cargo model. ZEN-4 localizes to the equatorial region of the spindle, where it associates directly or indirectly with proteins localizing to the contracting actomyosin ring. In the diagram, ZEN-4 is shown to interact with the ring via a cargo protein shown as a white circle. In this model, the late cytokinesis defect results because the contractile ring is unable to associate with molecules in the midzone and is thus independent of the presence of absence of organized midzone microtubules. We stress that these models are not mutually exclusive (see DISCUSSION for details).

ZEN-4 and Models of Cytokinesis

At least three models can be invoked to explain why ZEN-4 deprivation results in a late cytokinesis defect (two of which are depicted in Figure 7). These include

1) the polar body model, 2) the cargo model, and 3) the microtubule organization model. These models are by no means mutually exclusive, as it is possible that the loss of ZEN-4 affects furrow progression in more than one way. In the polar body model, the defect in cytokinesis can be explained as a byproduct of failing to extrude the polar bodies. The excess chromatin could prevent cytokinesis directly or indirectly. However, other *C. elegans* mutations suggest that this is unlikely. Loss-of-function mutations in *mei-1* and *mei-2* prevent the formation of the meiotic spindle. Polar bodies either fail to be extruded or are unusually large; in either case, embryos are aneuploid but undergo cytokinesis (Mains *et al.*, 1990). This argues that the presence of extra chromatin is not sufficient to prevent cytokinesis.

In the cargo model, ZEN-4 would be required to localize its cargo to the midbody, and it is the delivery of this cargo that is necessary for cytokinesis. One candidate molecule is *PLK* (polo-like kinase), a serine-threonine kinase that phosphorylates MKLP1 in vitro and colocalizes with MKLP1 during anaphase, telophase, and cytokinesis (Lee *et al.*, 1995). In *Drosophila*, *polo* is required for normal mitotic and meiotic divisions (Llamazares *et al.*, 1991). A *C. elegans PLK* homologue has been identified by the sequencing consortium on cosmid K06H7. *C. elegans* PLK has a catalytic domain that is 55% identical to human *PLK*, but the molecular organization of the protein is substantially different, with the putative kinase domain located at the C terminus of the molecule. It is currently unknown whether or not this kinase interacts with ZEN-4. Other candidates include yet-to-be-identified PLK molecules or other proteins localizing to the midzone. It is also possible that ZEN-4 interacts directly or indirectly with proteins localizing to the leading edge of the contracting actin ring. *cyk-1*, a formin homologue, and the septin homologues, *sep-1* and *sep-2*, are candidates (Swan and Bowerman, personal communication; Nguyen and White, personal communication). In *Drosophila* spermatocytes, interactions between the central spindle and components of the contractile ring are proposed to involve the kinesin-like protein KLP3A (Giansanti *et al.*, 1998).

The microtubule organization model argues that ZEN-4 directly cross-links midzone microtubules, and that the defects seen in cytokinesis result from the absence of these microtubules. We believe our evidence supports this model, as ZEN-4 localizes to the midzone during anaphase and ZEN-4 deprivation eliminates the presence of midzone microtubules. In addition, this model is consistent with the previously reported ability of MKLP1 to cross-link microtubules in an antiparallel orientation (Nislow *et al.*, 1992). Mutations in another *C. elegans* gene, called *spd-1*, also result in defects in midzone microtubule organization (O'Connell and White, personal communication). *spd-1* mutants initiate cleavage furrow formation at the normal time, but the furrow fails to mature, lending further credence to the idea that midzone microtubules are required for the completion of cytokinesis in *C. elegans.*

An intriguing analogy can be drawn between the requirement for midzone microtubules and the role of the phragmoplast during cell division in higher plants (O'Connell, Skop, and White, personal communication). The phragmoplast consists primarily of a complex array of microtubules that arise from the mitotic spindle and are essential for cytokinesis. Microtubules in the phragmoplast interdigitate at their plus ends and are thus organized in an antiparallel manner (Staehelin and Hepler, 1996). Golgi-derived vesicles are transported along these microtubules to the equatorial region, ultimately forming a disk-like membrane-bounded structure called the cell plate. Vesicles continue to accumulate until the growing cell plate fuses with the plasma membrane of the parent cell. Several kinesins have been identified that localize to phragmoplast microtubules (Asada and Shibaoka, 1994; Liu *et al.*, 1996), although it is unclear whether they function to organize microtubules or function in vesicle trafficking. By analogy, midzone microtubule bundles could provide the structure by which motor proteins deliver vesicles to the furrow, suggesting that vesicle transport may be required for the progression of the cleavage furrow in the region of the spindle midzone. We hope to differentiate among these models as more genes become available that affect *C. elegans* cytokinesis.

Note added in proof. Recently, Adams *et al*. (1998. Genes Dev. *12*, 1483–1494) reported that mutations in the *Drosophila* homologue of MKLP1 result in defects in spindle organization during telophase and fail to undergo cytokinesis.

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