LIN-5 Is a Novel Component of the Spindle Apparatus Required for Chromosome Segregation and Cleavage Plane Specification in Caenorhabditis elegans

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Abstract. Successful divisions of eukaryotic cells require accurate and coordinated cycles of DNA replication, spindle formation, chromosome segregation, and cytoplasmic cleavage. The Caenorhabditis elegans gene lin-5 is essential for multiple aspects of cell division. Cells in lin-5 null mutants enter mitosis at the normal time and form bipolar spindles, but fail chromosome alignment at the metaphase plate, sister chromatid separation, and cytokinesis. Despite these defects, cells exit from mitosis without delay and progress through subsequent rounds of DNA replication, centrosome duplication, and abortive mitoses. In addition, early embryos that lack lin-5 function show defects in spindle positioning and cleavage plane specification. The lin-5 gene encodes a novel protein with a central coiled-coil domain. This protein localizes to the spindle apparatus in a cell cycle- and microtubule-dependent manner. The LIN-5 protein is located at the centrosomes throughout mitosis, at the kinetochore microtubules in metaphase cells, and at the spindle during meiosis. Our results show that LIN-5 is a novel component of the spindle apparatus required for chromosome and spindle movements, cytoplasmic cleavage, and correct alternation of the S and M phases of the cell cycle.

Key words: lin-5 • mitosis • cytokinesis • microtubules • mitotic checkpoint

Introduction

During cell division, many biochemical processes must be accurately executed and well orchestrated to generate daughter cells that are genetically intact (for review, Nasmyth, 1996). First, the DNA should be replicated entirely during S phase of the cell cycle. Next, a bipolar spindle should be formed, attached to all chromosomes, and used to segregate the sister chromatids to opposite poles. Subsequently, the cytoplasm needs to be cleaved between the segregated chromosomes in a plane perpendicular to the spindle. Additionally, the spindle should be positioned correctly before cleavage, to control the direction of division and to determine whether cytoplasmic components are divided in a symmetric or asymmetric manner.

The molecular mechanisms involved in accurate chromosome segregation have been partially elucidated, mostly through genetic studies of yeast, biochemical studies of Xenopus and cell biological studies of mammalian tissue-culture cells. Meiotic and mitotic spindles have been shown to consist of microtubules and associated proteins (for review, Desai and Mitchison, 1997). The microtubules assemble by polymerization of αβ-tubulin dimers. Polymerization is usually initiated at microtubule-organizing centers (MTOCs). Microtubules are polar; they contain minus ends that usually remain associated with the MTOC and plus ends that extend into the cytoplasm and undergo rapid growth and shrinkage. Motor proteins of the dynein and kinesin families use the polarity of these microtubules to generate force in either the plus or minus end direction (for review, Hoyt and Geiser, 1996). The role of the MTOC in spindle formation is still unclear. MTOCs can vary greatly in morphology, as is evident when comparing centrosomes in animal cells and spindle pole bodies in yeast (Stearns and Winey, 1997). In addition, spindles can be formed without centrosomes in certain cell divisions, for instance during female meiosis in many animals.
Materials and Methods

Strains and Genetics

C. elegans strains were derived from the wild-type Bristol strain N2 and Bergerac strain RW7000 and cultured using standard techniques as described by Brenner (1974). We used the following mutations, descriptions of which can be found in Riddle et al. (1997) or this study: LGII, dpy-10(e128), lin-5(e1348), el457, n3066, n3070, ev571ts, rol-6(su1006), unc-4(e120), rol-1(e91), mnc1(dpy-10(e128) unc-52(e444)), mmD1100.

A line ev571ts was kindly provided by David Mertz and Joe Culotti (Mt. Sinai Hospital Research Institute, Toronto). Mutations n3066 and n3070 were isolated in this study. N2 animals were mutagenized with 50 mM ethyl methanesulfonate (EMS) as described by Brenner (1974). F1 progeny were placed on separate plates and examined for thin, sterile, and uncoordinated F2 self progeny. Mutants that at least partly resembled lin-5 mutants were examined for defects in cell division and polyplody, using Nomarski optics or fluorescence microscopy of Carnoy’s-fixed 4,6-diamidino-2-phenylindole (DAPI)-stained animals (Wood et al., 1988). Candidate mutations were recovered from siblings and were mapped either with recessive genetic markers or by PCR, making use of primers based on polymorphic sequence-tagged sites in the RW7000 Bergerac strain (Williams et al., 1992). lin-5 mutations mapped to two loci on the C. elegans genome (for reviews, Wills and Williams, 1991). lin-5-1 and lin-5-4 were mapped to lin-5(e1348) region.

Germline Transformation Rescue

Germline transformation experiments were performed as described by Mello et al. (1991). A nimals of genotype lin-5(e1348) unc-4(e120)/mncC1[dpy-10(e128) unc-52(e444)] were injected with cosmids or plasmids containing C. elegans genomic DNA (1–20 μg/ml) and prF4 (80 μg/ml), which contains the dominant marker rol-6(su1006). Rescue was examined in the F1 self progeny or in the F2 self progeny from animals that displayed the rol-1 phenotype. The presence of multiple unc rol rol progeny was at least partially confirmed; vulva was scored as rescue. Such animals typically contained a more extensive germ line than lin-5(e1348) mutants and several oocytes or embryos, but nearly invariably failed to produce viable progeny. Similar experiments were performed to examine rescue with lin-5 cDNA transcriptionally fused to the heat-shock promoters of plasmids pPD48-78 and pPD49-87 and injected at a concentration of 50 μg/ml, together with prF4 (Fire et al., 1990). A after establishing transgenic lines, we shifted embryos to 33°C for 1 h and scored rescue in the adults.

Isolation of lin-5 cDNA

Standard molecular biological protocols were used as described by Sambrook et al. (1989). The genomic sequence of the lin-5 region was obtained by the C. elegans Genome Sequencing Consortium (1998). Subfragments were cloned based on these sequences and open reading frames (ORFs) predicted by GENEFINDER. A 2-kb segment from the minimal rescue fragment was used to screen embryonic and mixed-stage cDNA libraries (Ookema and Fire, 1994), 6 × 106 clones each. DNA sequences were obtained from four of the cDNA inserts, using an automated ABI 373A DNA sequencer (Applied Biosystems). The sequences of mutant alleles were determined by PCR-amplified genomic DNA. All primers were designed based upon the genomic sequences and are available upon request. The coiled-coil domain was predicted using MacStripe, a Macintosh version of the algorithm originally designed by Lupas et al. (1991), and was kindly provided by Alex Knight (University of Cambridge, MA).

RNA Mediated Interference

Plasmids containing full-length lin-5 cDNA were used for in vitro transcription. Genomic DNA containing part of the F03G10.5 sequence was obtained by PCR amplification with primers 5′-acctgctcatgagta and either primer 5′-ggggatccaccacctcagtctgatta or 5′-tccagctctatccagttg, resulting in fragments of 866 and 1,680 bp, respectively. Sense and antisense transcripts were generated using linearized templates and T3 and T7 RNA polymerases. RNA was extracted with phenol, precipitated with ethanol, and dissolved in TE (10 mM Tris-Cl pH 8.0, 0.1 mM EDTA) to a final
A 3’ fragment of the lin-5 C DNA (1,138 bp Sall–BamHI) was cloned into the pET19b expression vector (Novagen) and expressed in E. coli. The purified His-tagged LIN-5 COOH-terminal fragment was injected into mice. Fusion of splenocytes with myeloma Sp2 cells and selection on Hygromycin B was performed according to standard procedures (Harlow and Lane, 1998). Tissue culture supernatants from three independent LIN-5-reactive clones (hel-1, hel-2, and hel-3) were used in these studies, either individually or as a mixture with equal ratio. A rabbit antiboies produced by clones hel-1 and hel-2 are of the IgG1 isotype, whereas hel-3 produced IgG2a heavy chains. All light chains were of the k-type.

Immunostaining of C. elegans embryos was performed as described by Strome and Wood (1983) with minor modifications (Boxem et al., 1999). Immunostaining of larvae was performed according to standard procedures, using synchronously grown animals fixed in paraformaldehyde or Bouin’s reagent (Boxem et al., 1999). To visualize DNA synthesis, a solution of 5-bromo-2′-deoxyuridine (BrdU, final concentration 0.5 mg/ml; Sigma Chemical Co.) in S-medium was added to staged L1 animals at 6 h of L1 development. Animals were fixed 6–8 h later, washed three times in M9, and fixed in Bouin’s fixative (Boxem et al., 1999). Microtubules was determined by immunostaining with the anti-α-tubulin antibodies Y OL1/34 and Y L1/2. C. elegans larvae appeared highly resistant to all microtubule-interfering drugs tested, including nocardazole, colchicine, colchicinum, vinuclear, and vinblastine, even at concentrations 1,000-fold higher than usually applied to tissue-culture cells.

**Microscopy and Image Acquisition**

A Zeiss Axioplan II microscope was used for Nomarski differential interference and immunofluorescence microscopy. Confocal images were obtained using a Leica inverted microscope and Leitz imaging software. In addition, images were acquired with a Sensys cooled CCD camera (Photometrics), followed by image analysis and computational deconvolution with Openlab software (Improvision). Images were pseudocolored and merged using Adobe Photoshop.

**Results**

**lin-5 Is Required for Cell Divisions**

The C. elegans gene lin-5 was previously defined by two recessive mutations isolated in screens for cell lineage mutants (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981). Affects of the mutant phenotype have been described by A. Ibertson et al. (1978). Based on molecular and genetic criteria, the previously isolated lin-5 alleles, e1348 and e1457, and our newly isolated mutations, n3066 and n3070, are strong loss-of-function or null alleles (see below and data not shown). These mutations cause recessive phenotypes, including complete sterility, and are phenotypically indistinguishable. We have characterized the e1348 mutation most thoroughly. This mutation eliminates lin-5 function and is also referred to below as lin-5(0).

Homzygous lin-5 mutants derived from heterozygous hermaphrodites complete embryonic development, but fail to undergo postembryonic nuclear and cytoplasmic divisions (A. Ibertson et al., 1978: Fig. 1, A and B). Microscopic analyses using Nomarski optics revealed that cells entered mitosis at the normal times during larval development of lin-5(e1348) mutants, as manifested by disappearance of the nucleolus, condensation of the chromatin, and degradation of the nuclear envelope (A Ibertson et al., 1978). Chromosome movements towards the equatorial plane were initiated, but metaphase-plate formation was almost invariably incomplete, anaphase failed to occur, and neither nuclear division nor cell division was initiated (Fig. 2; and data not shown). Despite the lack of chromosome segregation, cells exited from mitosis without significant delay, the chromatin relaxed, and a nuclear envelope reformed (Fig. 3).

We examined phosphorylation of a histone H3 epitope to assay activity of the mitotic kinase NCC-1(Cdk1) (see Materials and Methods). Synchronously developing wild-type and lin-5(e1348) L1 larvae were found to contain sim-
of development. DNA synthesis appeared normal in lin-5 mutants, but was not followed by cell division. Incorporation of BrdU can be detected in all P cells and several other postembryonic blast cells. E–G, Single embryos: wild-type embryo (E), lin-5(ev571ts) embryo developed at the nonpermissive temperature (F), and lin-5(RNAi) embryo (G) stained with PI to illustrate the early embryonic arrest with polyploid nuclei in the absence of maternal lin-5 function. Bars, 10 μm.

lin-5 Mutants Exit Mitosis without Chromosome Segregation

As described above, mitoses become defective even before chromosome alignment at the metaphase plate in lin-5(0) mutants (Fig. 2). Based on studies of other eukaryotes, such defects might be expected to trigger surveillance mechanisms that delay exit from mitosis and initiation of the next S phase (for review, Skibbens and Hieter, 1998). We carefully determined the time in mitosis for P cells, P1–P12, of the ventral nerve cord in wild-type and lin-5 mutant L1 larvae. The P cells initiate their postembryonic lineages about half-way through the first larval stage. Only the first defective divisions were examined to avoid scoring secondary effects. The time between entry into and exit from mitosis was very similar for the P cells in wild-type and lin-5(e1348) animals, confirming the lack of mitotic arrest in lin-5(0) mutants (Fig. 3). However, partial loss-of-lin-5 function appeared to cause a delay. When lin-5(ev571ts) early L1 larvae were shifted from 15 to 25°C, a few P cells (12/105) completed division. If shifted to 23°C, a slightly higher number of P cells (22/105) divided. These data are in agreement with the incomplete loss-of-function and temperature-sensitive nature of the lin-5(ev571ts) allele. The duration of defective mitoses at 25°C was slightly longer in ev571ts mutants as compared with lin-5(e1348) mutants (Fig. 3, average time in mitosis ~24 and 19 min, respectively). Interestingly, P cells in lin-5(ev571ts) mutants remained in mitosis for significantly prolonged times at 23°C (Fig. 3, average time in mitosis 52 min). Thus, incomplete inactivation of lin-5 resulted in delayed exit from mitosis, whereas complete inactivation did not. These observations may reflect a role for lin-5 in preventing prema-
ture exit from mitosis in addition to its function in promoting chromosome segregation (see Discussion).

**The lin-5 Gene Encodes a Novel Protein**

To learn more about how lin-5 functions in cell division, we initiated a molecular analysis. The lin-5 gene was mapped previously between lin-26 and zyg-11 on chromosome II (Fig. 4 A). We identified two overlapping cosmids clones from this region, C03G3 and C30G9, that partially rescued the lin-5(e1348) mutant phenotype in germline transformation assays (Materials and Methods). We localized the rescue activity to an 8-kb genomic fragment present on both cosmids (Fig. 4 B). Based on genomic sequences and GENEFINDER predictions available from the <i>C. elegans</i> Sequencing Consortium (1998), we created further deletions and identified a single candidate gene. We used genomic DNA from this region to screen two different cDNA libraries, isolated ten clones, and determined the nucleotide sequences of four of these cDNAs. The sequences differed only in their 5' and 3' ends, indicating that the clones were independent and derived from identically spliced mRNAs. The longest ORF is predicted to encode a protein of 821 amino acids. This protein is likely the complete LIN-5 protein as the predicted initiator methionine is preceded in the genomic sequence by an in-frame stop codon and no consensus splice acceptor site is present between these stop and start codons. Moreover, Northern blot analysis and reverse transcriptase-PCR experiments confirmed that a single transcript of ~2,650 bp is the major product of this gene (data not shown).

Several experiments confirmed that we indeed had cloned the lin-5 gene. First, expression of the longest cDNA under the control of heat-shock promoters hsp16-2 and hsp16-41 in transgenic lines could partially complement the lin-5(e1348) mutation. Specifically, exposure of such embryos to 33°C for one hour resulted in cell divisions in the P cell lineages in a significant percentage of lin-5 animals. As the P cell lineages were not completely restored, these divisions resulted in formation of abnormal protruding vulva. Animals with protruding vulva were found in three of three transgenic lines expressing the lin-5 cDNA and not among the progeny from control animals injected with only the dominant marker DNA. Second, in RNA-interference experiments, injection of dsRNA transcribed in vitro from the longest cDNA caused a Lin-5 phenotype in the F1 progeny. All progeny produced eight hours or more after injection were embryonic lethal and
closely resembled lin-5(ev571) embryos at the nonpermissive temperature (Fig. 1, F and G; Table I). In addition, some of the early progeny completed embryogenesis after injection, but failed larval cell divisions and resembled lin-5(0) mutants. This latter result is consistent with the interpretation that some of the first brood after RNA injection have maternal gene function, but lack zygotic gene activity (Tabara et al., 1998; Boxem et al., 1999). Finally, we identified the molecular lesions associated with the five existing lin-5 alleles (Fig. 4, C and D). Four of the five alleles contain G–C to A–T transitions, the most common mutation induced by ethyl methanesulfonate (Anderson, 1995). Alleles n3070 and e1348 contain amber mutations and n3066 contains an ochre mutation; these alleles should terminate translation after 52, 159, and 538 amino acids, respectively. The e1457 allele contains a missense mutation, changing the glycine residue at position 40 to glutamate. The conditional allele ev571ts contains a duplication of 9 bp, followed by a T→C transversion, which results in an insertion in-frame of three amino acids within the coiled-coil domain. Thus, the genetic strength of the five lin-5 alleles is largely in agreement with the lesions identified.

Searches of existing databases revealed that the LIN-5 protein is not closely related to any protein of known function. However, LIN-5 shares 42% amino acid identity with the protein product of F01G10.5, a gene predicted by the C. elegans Sequencing Consortium (1998; Fig. 4 D). We were unable to detect phenotypical consequences of F01G10.5 RNA interference, despite targeting two different segments of the gene and injecting wild-type animals as well as lin-5(ev571ts) mutants at a semipermissive temperature (20°C, data not shown).

A large central region of the LIN-5 protein, from amino acids 210 to 592, was predicted to form an α-helical coiled coil according to the algorithm of Lupas et al. (1991). Many proteins with myosin-type coiled coils were found in homology searches with LIN-5. We did not consider these proteins to be homologous, as the similarities were restricted in all cases to the residues that form the heptad repeats. Both the NH₂ terminus and COOH terminus of LIN-5 contain five serine/threonine residues followed by a proline, seven of which are conserved in the termini of F01G10.5. These residues are potential targets for phosphorylation by Cdk's and MAP kinases, which may regulate lin-5 function in mitosis.

**LIN-5 Protein Colocalizes with Components of the Meiotic and Mitotic Spindle**

To better understand the function of lin-5, we determined the subcellular localization of its product. Anti–LIN-5 mAbs were generated after immunizations with a COOH-terminal fragment expressed in and purified from E. coli. Using these antibodies in immunostaining experiments, we detected distinctly localized staining during both meiosis and mitosis (Table I). Four of the five alleles contain G–C to A–T transitions, the most common mutation induced by ethyl methanesulfonate (Anderson, 1995). A large central region of the LIN-5 protein, from amino acids 210 to 592, was predicted to form an α-helical coiled coil according to the algorithm of Lupas et al. (1991). Many proteins with myosin-type coiled coils were found in homology searches with LIN-5. We did not consider these proteins to be homologous, as the similarities were restricted in all cases to the residues that form the heptad repeats. Both the NH₂ terminus and COOH terminus of LIN-5 contain five serine/threonine residues followed by a proline, seven of which are conserved in the termini of F01G10.5. These residues are potential targets for phosphorylation by Cdk's and MAP kinases, which may regulate lin-5 function in mitosis.

**Table I. lin-5 Mutants Are Defective in Spindle Positioning**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Embryos</th>
<th>Spindle orientation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
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<tr>
<td></td>
<td></td>
<td>AB</td>
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<tr>
<td>Wild-type</td>
<td>27</td>
<td>92.6(25)</td>
</tr>
<tr>
<td>lin-5(RNAi)</td>
<td>26</td>
<td>34.6 (9)</td>
</tr>
<tr>
<td>lin-5(ev571) 26°C</td>
<td>26</td>
<td>34.6 (9)</td>
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Embryos were fixed with antitubulin and PI. The position of the spindle was examined in two-cell embryos with fully condensed chromosomes in P1 (see Rose and Kemphues, 1998; Materials and Methods). The few abnormally positioned spindles observed in wild-type embryos were likely caused by the fixation and staining procedure. The number of embryos scored for each orientation are in parentheses.
rescued the ORFs. Genomic fragments containing the second ORF partially rescued the ORFs by the als and Methods. The minimal rescuing fragment was predicted to be the lin-5 gene. Deletion mnD100 includes the lin-5 gene. B, Physical map and cloning of lin-5 by germline transformation rescue. Cosmids are shown below the physical map. Overlapping cosmids C03G3 and C30G9 partially rescued the lin-5(0) phenotype, as described in Materials and Methods. The minimal rescuing fragment was predicted by the C. elegans Sequencing Consortium (1998) to contain three ORFs. Genomic fragments containing the second ORF partially rescued the lin-5(0) phenotype. C, The predicted lin-5 mRNA and LIN-5 protein. The predicted protein contains a large central α-helical coiled-coil domain, as well as ten potential phosphorylation sites for proline-directed kinases, such as Cdc2/Cdk1. D, LIN-5 amino acid sequence and alignment with the C. elegans predicted ORF F01G10.5. Black boxes indicate residues of identity. The overlined region indicates the predicted α-helical coiled-coil region shared between LIN-5 and F01G10.5. The molecular lesions in the lin-5 alleles are indicated below the predicted protein in C and above the lin-5 sequence in D.

Figure 4. Molecular cloning of the lin-5 gene. A, Genetic map. The lin-5 region of linkage group II is indicated, as well as the closest cloned genes to the left and right of lin-5. Deletion mnD100 includes the lin-5 gene. B, Physical map and cloning of lin-5 by germline transformation rescue. Cosmids are shown below the physical map. Overlapping cosmids C03G3 and C30G9 partially rescued the lin-5(0) phenotype, as described in Materials and Methods. The minimal rescuing fragment was predicted by the C. elegans Sequencing Consortium (1998) to contain three ORFs. Genomic fragments containing the second ORF partially rescued the lin-5(0) phenotype. C, The predicted lin-5 mRNA and LIN-5 protein. The predicted protein contains a large central α-helical coiled-coil domain, as well as ten potential phosphorylation sites for proline-directed kinases, such as Cdc2/Cdk1. D, LIN-5 amino acid sequence and alignment with the C. elegans predicted ORF F01G10.5. Black boxes indicate residues of identity. The overlined region indicates the predicted α-helical coiled-coil region shared between LIN-5 and F01G10.5. The molecular lesions in the lin-5 alleles are indicated below the predicted protein in C and above the lin-5 sequence in D.

lin-5 RNA interference of control animals. Moreover, in agreement with the genetic strength of the alleles, staining was observed during abortive mitoses in mutant larvae homozygous for the lin-5(ts) allele, but not for any one of the four strong lin-5 mutations. We conclude that these antibodies specifically detect LIN-5.

C. elegans development is initiated by fertilization of oocytes that are in meiotic prophase I. After fertilization, the maternal pronucleus completes meiosis I and II and two polar bodies are expelled. Subsequently, the maternal pronucleus migrates towards the paternal pronucleus in the posterior end of the egg, the two nuclei migrate together to the middle, the nuclear envelopes degrade, and a first mitotic spindle is formed. In immunostaining experiments with anti-LIN-5 antibodies, we observed the following localization pattern: LIN-5 was present at the meiotic spindle in the fertilized egg during meiosis I and II (Figs. 5 A and 6 C). Staining associated with the maternal pronucleus disappeared after completion of meiosis and LIN-5 became localized at the duplicated centrosomes that adjoined the paternal pronucleus (Fig. 5 B). LIN-5 remained localized at the centrosomes until completion of the first mitotic division. In all subsequent mitoses, LIN-5 was associated with the centrosomes and detectable after separation of the centrosomes in prophase until decondensation of chromosomes in telophase (Fig. 5, C–E). LIN-5 was also diffusely present in the cytoplasm and at two other locations; LIN-5 appeared microtubule-associated in cells with metaphase-aligned chromosomes (Figs. 5 F and 6 I). This localization was limited to the region between the poles and did not overlap with DNA staining. Therefore, it appeared to reflect specific association of LIN-5 with kinetochore microtubules. Finally, LIN-5 was diffusely present at the cell cortex in most embryonic cells from the two-cell stage onward (Fig. 5, D–F). During the larval stages, LIN-5 localized to the centrosomes from prophase to telophase and to the cell periphery in late mitosis. In adult hermaphrodites, mitosis is restricted to germ presursor cells in the distal ends of the gonad. LIN-5 was detected at the centrosomes of such cells and, in addition, diffuse cytoplasmic LIN-5 staining was detected in oocytes (data not shown). Finally, strong membrane-associated staining was detected in the gonad at all stages of development (data not shown).

LIN-5 colocalized with several components of the spindle apparatus. Its predominant localization to the centrosomes could reflect association with the centrioles or pericentrosomal matrix or, alternatively, could indicate that LIN-5 associates with microtubules that radiate from the centrosomes. We used the microtubule-stabilizing drug nocodazole to distinguish between these possibilities. Triple-staining experiments were performed to detect DNA, tubulin, and LIN-5 in embryos after nocodazole or control (DMSO) treatment (Fig. 6). Incubation with 20 µg/ml nocodazole for 15 min resulted in elimination of mitotic spindle microtubules in 65% of the embryos (tubulin staining was either absent or restricted to a centrosomal dot in 52 of 80 embryos). Despite the fact that cells were mitotic, as was indicated by DNA condensation, LIN-5 was not localized to the centrosomes in these embryos (Fig. 6). LIN-5 localization to the meiotic spindle also disappeared after nocodazole treatment in 47 of 50 fertilized oocytes (Fig. 6). These results show that LIN-5 is not an
lin-5 is an integral component of the centrosome, but rather directly or indirectly associates with the microtubules emanating from the centrosomes.

In conclusion, consistent with its role in chromosome segregation, LIN-5 is localized at the spindle apparatus in meiosis and mitosis. Its localization at the meiotic spindle, mitotic centrosomes, and metaphase microtubules are all dependent on the presence of microtubules.

**lin-5 Appears Required for Spindle Force, but Not for Centrosome Duplication or Spindle Assembly**

As a component of the mitotic apparatus, LIN-5 could act in spindle assembly and, for instance, be required for centrosome duplication or nucleation of microtubules. Alternatively, LIN-5 could act more specifically in the generation of spindle force and affect either microtubule motor proteins or microtubule dynamics. We examined the spindle apparatus in lin-5 mutant embryos and larvae to test these possibilities.

Staining with antitubulin antibodies revealed that centrosomes duplicated and migrated to opposite poles in lin-5(ev571ts) or lin-5(RNAi) mutant embryos and lin-5(0) L1 larvae. Mitotic microtubules were nucleated, at least in part, and bipolar spindles formed (Fig. 7). In fact, centrosome duplication continued and the number of cen-
trosomes doubled with each additional abortive division (Fig. 7 C). Thus, lin-5 is not required for centrosome duplication. Moreover, DNA replication and centrosome duplication both continued in lin-5 mutants in the absence of chromosome segregation.

As mentioned above, chromosomes failed to move towards or away from the metaphase plate during abortive mitoses in lin-5(0) mutants, indicating absence of spindle force. In addition, we observed defects in rotation of centrosomes and spindle positioning in lin-5 embryos and L1 larvae. Defects in spindle positioning occurred as early as the first division in lin-5(RNAi) and lin-5(ev571 ts) embryos (see below), whereas chromosome congression, segregation, and cytokinesis usually failed after two to four divisions.

The position of the spindle apparatus determines the cleavage plane of the cell, which is an important element in regulating symmetry and direction of cell division, two key aspects of animal development. Centrosome migrations in C. elegans have been well studied, in particular during the first two mitotic cleavages (for review, White and Strome, 1996). In wild-type embryos, the first mitotic spindle is oriented along the long axis of the egg (Fig. 8 B). The spindle migrates posteriorly during the first mitosis, causing the first cleavage to produce two unequal cells: a larger anterior cell, A B, and a smaller posterior cell, P1 (Fig. 8 C). After this division follows. In the P1 cell, the spindle rotates 90° and the subsequent division is longitudinal (Fig. 8 D; Table I).

To quantify the defects in spindle positioning and rotation in lin-5(ts) and lin-5(RNAi) embryos, we stained embryos with antitubulin antibodies and PI. In addition, we followed early embryonic development, using Nomarski optics and time-lapse video recordings, to further characterize the abnormalities. Both methods indicated apparently normal centrosome segregation and spindle formation in early lin-5 embryos, whereas spindle positioning frequently failed (Figs. 7, D and E, and 8). Deviations from wild-type development were seen as early as meiosis: in 7 of 25 lin-5(RNAi) embryos fixed before pronuclear fusion, two maternal pronuclei were formed at the anterior end rather than one (data not shown; and Fig. 8 E). Video recordings of such embryos showed that both maternal pronuclei migrated to the posterior and fused with the paternal pronucleus. The first division appeared symmetrical in 9 of 28 lin-5(RNAi) embryos fixed at the two-cell stage (data not shown; and Fig. 8 G). Video microscopy revealed that this defect was caused by failure of the first spindle to migrate posteriorly, after being formed at the correct position. The rocking movements that normally coincide with this migration did not occur during the defective divisions. Embryos in which the spindle was mislocalized nevertheless completed the first cycle of chromosome segregation and cytokinesis. At the two-cell stage, the position of the spindle was examined in embryos with fully condensed chromosomes in P1 (Table I). A prox-
mately half of the lin-5 embryos were found to have abnormally positioned spindles. In most of these embryos, the P1 spindle was transverse, indicating failure to rotate to the longitudinal position (Figs. 7 E and 8 H; Table I). Other spindle orientations were also observed with lower frequencies (Table I). These latter defects may have resulted indirectly from a symmetrical first division and coincident defects in the identity of A B and P1. P granules were correctly localized in 39 of 41 embryos that showed no detectable LIN-5 immunoreactivity. These granules segregate to the germline-precursor cells in a microfilament-dependent manner (Strome and Wood, 1983). Thus, the actin cytoskeleton and establishment of cell identities are at least partly functional in lin-5 embryos after RNAi treatment.

Chromosome movements, centrosome rotation, and spindle migration all are brought about by the spindle apparatus. We conclude that bipolar spindles can be formed in lin-5 mutants. However, the function of the spindle in generating the forces required for correct chromosome movements and centrosome positioning appear to depend on lin-5.

Discussion

lin-5 Is Required Generally for Mitotic Cell Division

In this study, we further defined the function of lin-5 in vivo, based on observations of the phenotype conferred by null mutations, the conditional ev571ts mutation and RNA interference. We cloned lin-5, characterized its genomic structure and nucleotide sequences, and identified the molecular lesions in the five existing mutant alleles. In addition, we determined the subcellular localization of the LIN-5 protein during different cell cycle and developmental stages. Finally, we showed that LIN-5 localization to the spindle depends on microtubules. Together, our data establish that lin-5 encodes a novel component of the spindle apparatus essential for multiple cell division events. We will discuss below how lin-5 may function.

The presence of maternal product introduces a complication in studying genes that act both early and late in development (see O’Connell et al., 1998; Boxem et al., 1999). Animals homozygous for null mutations in lin-5 or other essential cell cycle genes have to be obtained from heterozygous parents. The single copy of the wild-type gene in a lin-5/+ mother provides maternal function and allows embryonic cell division to occur normally. Aalysis of the first round of defective postembryonic divisions provides the best information of the null mutant phenotype. However, conclusions based solely on these divisions may be misguided by persistence of maternal product in a subset of cells. In addition, analysis of the null phenotype neither reveals how lin-5 acts in embryogenesis nor takes advantage of the visibility of mitotic processes in the much larger embryonic cells. By combining experiments with null mutants, a conditional allele, and RNA interference, we have been able to address the function of lin-5 during various stages of development. These different studies have highlighted different roles. The essential roles of lin-5 in alignment and segregation of chromosomes, as well as cytokinesis, was most evident during abortive larval divisions in lin-5(0) mutants. A role for lin-5 in spindle positioning was most apparent in early embryonic divisions, although abnormal spindle locations were also detected in lin-5(0) larvae stained for tubulin.

Formation of two maternal pronuclei and defective spindle migrations in early lin-5 embryos implied roles for lin-5 in meiosis and the first mitotic divisions. Such functions are consistent with the prominent presence of LIN-5 at the meiotic spindle and mitotic centrosomes. However,
LIN-5 is a Novel Spindle Component

Figure 8. Nomarski images of living embryos. A–D, Selected images from a chronological time-lapse sequence showing early division events in a wild-type embryo. E–H, Images from multiple lin-5(RNAi) embryos illustrating defects in meiotic division and mitotic spindle positioning. A and E, Pronuclear migration and pseudocleavage. During meiosis in the wild-type, a single maternal pronucleus is formed (arrow) that migrates to the posterior. Meiotic defects can result in the formation of two maternal pronuclei (arrows) in lin-5(RNAi) embryos. Arrowheads indicate the sperm pronucleus with duplicated centrosomes. B and F, Formation of the first mitotic spindle. Asterisks are indicated by arrowheads; arrow points to an abnormally large polar body in the lin-5(RNAi) embryo. C and G, Two-cell embryos. The lin-5(RNAi) embryo has divided symmetrically. A arrowheads indicate cleavage plane. D and H, Preceding the second divisions, the centrosome/nucleus complex has rotated in the wild-type P1 blastomere, but not in the lin-5(RNAi) embryo. A arrowheads indicate spindle positions. Bar, 10 μm.

How Does lin-5 Act in Chromosome Segregation?

Based on the loss-of-function phenotype and protein localization data, some predictions can be made about how lin-5 functions in cell division. Below, we discuss how lin-5 could act by affecting microtubule dynamics, microtubule/cortex interactions, or the localization/activity of a microtubule motor.

The primary amino acid sequence does not reveal a function, as the only prominent feature of the LIN-5 protein is a central coiled-coil domain. This domain likely is used to form stable associations with either LIN-5 itself or with other proteins (Lupas, 1996). Coiled coils are found in proteins as diverse as transcription factors and keratins, and many coiled-coil proteins have been implicated in spindle function (e.g., Stearns and Winey, 1997). In database searches, we identified a large number of proteins with weak similarity to the coiled-coil domain in LIN-5. However, amino acid identities did not extend beyond the heptad repeats in any protein, with the exception of the product of predicted C. elegans gene F01G10.5.

Among the proteins that share similarity with the LIN-5 coiled-coil domain are structural components of the spindle pole bodies and centrosomes. LIN-5 is not an integral constituent of the centrosomes, but rather locates to the microtubules that emanate from the spindle poles. This conclusion is based on a number of observations. First, LIN-5 was associated with the meiotic spindle, which is
formed in the absence of centrosomes (Albertson and Thomson, 1993). Second, LIN-5 was not detected at the centrosomes until after their duplication and separation. Third, LIN-5 was detected at the kinetochore microtubules in metaphase cells. Finally, the localization of LIN-5 at the centrosomes and spindle was disrupted by microtubule depolymerization with nocodazole.

In the absence of lin-5 function, a bipolar spindle is formed that fails to position itself or to move the chromosomes. Spindle migration and chromosome movements depend on forces generated by a large number of motor proteins in concert with microtubule assembly and disassembly. The meiotic spindles and mitotic apparatus are disorganized and contain abnormally short microtubules in embryos mutant for zyg-9 (Matthews et al., 1998). ZYG-9 is a C. elegans MAP215-related protein and is thought to regulate microtubule turnover (Matthews et al., 1998). Such gross abnormalities in spindle structure were not detected in lin-5 mutants. In fact, in living lin-5(RNAi) embryos, mitotic spindles assembled with normal appearance and timing. Therefore, we do not expect that lin-5 primarily affects microtubule dynamics, although quantitative studies will be required to further investigate such a role.

Cytoplasmic dynein and at least seven different families of kinesin-related proteins have been found associated with the spindle (for reviews, Hoyt et al., 1997; Stearns, 1997). Several of these motor proteins show M phase-dependent localizations that partially overlap with LIN-5. Perhaps the best match in mutant phenotype and protein localization exists between lin-5 and cytoplasmic dynein motors. Dynein motors have been implicated in multiple mitotic functions, including nuclear migration, centrosome separation, spindle organization, spindle orientation, and cytokinesis (for reviews, Hoyt et al., 1997; Stearns, 1997; Karki and Holzbaur, 1999). The localization of cytoplasmic dynein is equally diverse and includes the meiotic spindle, mitotic centrosomes, and cell cortex. Dynein is present in the cell in large protein complexes with multiple subunits. Distinct functions of dynein motor complexes likely depend on specific subunits. For instance, a complex that includes NuMA and dynactin has been implicated in mitotic spindle assembly and is located at the centrosomes in mammalian cells (M erdes et al., 1996). A another component, DNC-1/p150\(^{\text{glued}}\), is localized at a cortical microtubule attachment site in early C. elegans embryos and acts together with DNC-2/dynamitin in cleavage plane specification (Skop and White, 1998). Importantly, staining of C. elegans embryos with antibodies that recognize the dynein heavy chain has shown a partial overlap in subcellular localization with LIN-5 (G\'onczy et al., 1999b). Most notable is the observation that both proteins are located at the cell cortex, in addition to the spindle, which has not been described for any member of the family of kinesin-related motor proteins. Based on the similarities in localization, we favor a model in which LIN-5 is required for the localization or regulation of a specific cytoplasmic dynein motor complex.

**Cytokinesis Depends on lin-5 Function**

Cytokinesis could either directly or indirectly require lin-5 function. On one hand, a significant percentage of lin-5 RNAi-treated embryos have multinucleated cells. This observation indicates that cytokinesis can fail despite completion of chromosome segregation, as seen in cytokinesis-specific mutants (Swan et al., 1998; G\'onczy et al., 1999a). On the other hand, the failure to undergo cytokinesis is generally preceded by defective chromosome segregation in lin-5 mutants. In fact, after lin-5-RNAi treatment, some early embryos formed extra ectopic division planes and anucleate cells. As the specification of the cleavage plane is dictated by the spindle, lin-5 might affect cytokinesis through its spindle function. The role of the spindle in cytokinesis likely involves two types of microtubules. The spindle pole position appears to be sensed by the cortex through an interaction with astral microtubules (reviewed by Strome, 1993). This interaction likely determines the position of the actomyosin ring. In addition, microtubules of the spindle midzone have important roles in the completion of cleavage (Wheatley and Wang, 1996). LIN-5 is not detected at the astral microtubules, the midzone microtubules, or the cleavage furrow. Therefore, it is unlikely that LIN-5 actively participates in the cleavage process. The lack of anaphase in lin-5 mutants should prevent the midzone localization of essential regulators of cytokinesis, such as kinesins and members of the Polo and Aurora kinase families (for review, Field et al., 1999). Thus, cytokinesis could fail as an indirect consequence of LIN-5 absence. However, LIN-5 is present at the spindle, as well as the cell periphery. This localization and the observed roles in polar body formation, spindle rotation, and chromosome movements may all be explained by a requirement for lin-5 in promoting spindle/cortex contacts that also affect cytokinesis.

**lin-5 May Be Required for a Mitotic Checkpoint**

In most animals studied, embryonic divisions lack certain cell cycle controls and consist of rapidly alternating S and M phases (for review, Hartwell and Weinert, 1989). C. elegans embryos treated with microtubule-interfering drugs continue DNA replication in the absence of chromosome segregation (Lauer et al., 1980; Strome and Wood, 1983), consistent with absence of a spindle assembly checkpoint. Therefore, checkpoint function must be examined during the conventional cell cycles later in development. It is presently unknown when C. elegans cell cycles acquire G1 and G2 phases and when a mitotic checkpoint might be established. Based on the time between embryonic divisions (Schierenberg et al., 1980; Sulston et al., 1983), switching to more conventional cycles may be gradual and lineage-dependent. We have been unable to study the spindle checkpoint directly during postembryonic development, as C. elegans larvae appeared highly resistant to all tested microtubule-interfering drugs (see Materials and Methods). The C. elegans Sequencing Consortium (1998) has identified homologues of several genes that act in the spindle assembly checkpoint in other species, including BUB1, BUB2, and MAD2, suggesting conservation of this checkpoint.

In lin-5 null mutants, blast cells continued to cycle without chromosome segregation, even during larval development. This observation indicates that lin-5 acts in a process not monitored by a mitotic checkpoint or that lin-5
is required to engage such a checkpoint. We observed that cells delayed their exit from mitosis after incomplete inactivation of lin-5. Together, these observations are consistent with a dual role for lin-5. Specifically, lin-5 might act both to promote chromosome segregation and to prevent premature exit from mitosis in response to defects in chromosome segregation. In this case, complete inactivation of lin-5 would remove both functions, whereas partial inactivation could have a differential effect, such as preventing chromosome segregation while still allowing a checkpoint-induced delay. Further studies will be required to test this hypothesis.

In summary, we propose that lin-5 has at least two direct roles in cell division: a role in spindle positioning and a role in chromosome movement. In addition, lin-5 is involved directly or indirectly in cytokinesis and in the coupling of DNA replication, centrosome duplication, and mitotic division. As outlined above, we favor the model that lin-5 brings about its functions by localizing or regulating a motor-protein complex and/or by promoting the connection between spindle microtubules and the cell cortex. Further studies of C. elegans should help define how lin-5 acts during cell division and provide further insights into the mechanisms that accomplish accurate chromosome segregation in animal cells.

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