Centrosome Maturation and Mitotic Spindle Assembly in *C. elegans* Require SPD-5, a Protein with Multiple Coiled-Coil Domains

Danielle R. Hamill,[1,3] Aaron F. Severson,[1]
J. Clayton Carter,[1] and Bruce Bowerman[1,2]
[1]Institute of Molecular Biology
University of Oregon
Eugene, Oregon 97403

Summary

The maternally expressed *C. elegans* gene *spd-5* encodes a centrosomal protein with multiple coiled-coil domains. During mitosis in mutants with reduced levels of SPD-5, microtubules assemble but radiate from condensed chromosomes without forming a spindle, and mitosis fails. SPD-5 is required for the centrosomal localization of γ-tubulin, XMAP-215, and Aurora A kinase family members, but SPD-5 accumulates at centrosomes in mutants lacking these proteins. Furthermore, SPD-5 interacts genetically with a dynein heavy chain. We propose that SPD-5, along with dynein, is required for centrosome maturation and mitotic spindle assembly.

Introduction

In dividing animal cells, bipolar mitotic spindles are organized by two centrosomes, one at each pole (reviewed in Wittmann et al., 2001). These microtubule-organizing centers nucleate the polarized assembly of microtubules (MTs), which capture and segregate chromosomes during mitosis. Centrosomes also organize MTs during interphase and within cilia and flagella, and can influence cell polarity (reviewed in Goldstein, 2000; Rieder et al., 2001). They are roughly spherical structures composed of many different proteins, and are highly dynamic (reviewed in Kellogg et al., 1994). Each centrosome has at its core a pair of short, orthogonally opposed MT-based cylinders called centrioles embedded within an electron-dense material called the pericentriolar material (PCM), or centromatrix (reviewed in Palazzo et al., 2000). In mature centrosomes, γ-tubulin ring complexes associate with the centromatrix and directly nucleate MT assembly (Keating and Borisy, 2000; reviewed in Gunawardane et al., 2000).

In most animals, centrioles are eliminated during oogenesis. Upon fertilization, the sperm provides a single centriole pair with little or no associated centromatrix (reviewed in Palazzo et al., 2000). After duplication of the single centrosome donated by a sperm, centrosomes increase in size. Upon entry into mitosis, centrosomes mature, further increasing in size and MT-nucleating activity (Khodjakov and Rieder, 1999). Duplication, growth, and maturation of the sperm-donated centriole into a functional pair of mitotic centrosomes appear to require maternally contributed centromatrix and other centrosomal proteins (reviewed in Palazzo et al., 2000).

Genetic and biochemical studies have led to the identification of proteins that regulate key steps in centrosome duplication and maturation (Palazzo and Schatten, 2000). For example, the *C. elegans* ser/thr kinase ZYG-1 associates with centrioles and is required for their duplication in early embryonic cells, but is not required for centrosome growth and maturation (O'Connell et al., 2001). In mammals, cell division kinases (CDKs) regulate the initiation of centriole duplication, providing coordination with the cell cycle (reviewed in Fry et al., 2000). A kinase called NEK-2 appears to target for destruction a tether that physically links the two centrioles after duplication. Polo kinase and Aurora A kinase also promote centrosome separation and maturation (reviewed in Fry et al., 2000). Although the composition of the centromatrix and its mechanism of growth remain unclear, vertebrate pericentrin and Cep135 are coiled-coil proteins that may be part of the centromatrix and are required for centrosome maturation (Dictenberg et al., 1998; Doxsey et al., 1994; Ohta et al., 2002).

With large accessible cells and powerful genetics, the early *C. elegans* embryo is a useful model system for studies of cell division, and several genes required for mitotic spindle assembly have been identified (reviewed in O'Connell, 2000). However, genes that encode centromatrix components remain unknown. Here we describe our isolation of a conditional mutation in a maternally expressed gene called *spd-5*, (*s*pindle-*d*efective). *spd-5* encodes a centrosomal protein with multiple predicted coiled-coil domains. It is required for other proteins to localize to centrosomes, and for mitotic spindle assembly. In contrast to recent studies in other cell types (Bonaccorsi et al., 2000; Khodjakov et al., 2000; Megraw et al., 2001; Vaizel-Ohayon and Schejter, 1999; reviewed in Raff, 2001), we conclude that centrosomes are required for bipolar mitotic spindle assembly in the early *C. elegans* embryo.

Results

Maternal Expression of *spd-5* Is Required for Mitotic Spindle Assembly

To identify genes required for mitotic spindle assembly, we screened mutagenized populations of nematodes for conditional embryonic lethal mutants, which we then examined at the restrictive temperature for defects in cell division (see Encalada et al., 2000). Among 1600 conditional mutants, we identified alleles of several genes known to be required for spindle assembly or stability. These include *zyg-1* (O'Connell et al., 2001), a protein kinase gene called *zyg-8* (Gönczy et al., 2001), the XMAP215-related gene *zyg-9* (Matthews et al., 1998), and *spd-2*, a gene of unknown identity required for the assembly of sperm asters and for mitotic spindle assembly (O'Connell et al., 2000). One mutant, *spd-5(or213ts)*, did not map near these genes. In *spd-5* mutants, spindles were undetectable in time-lapse Nomarski DIC recordings of the first two attempts at mitosis (Figure 1;

[2]Correspondence: bbowerman@molbio.uoregon.edu
[3]Present address: Department of Zoology, Ohio Wesleyan University, Delaware, Ohio 43015.
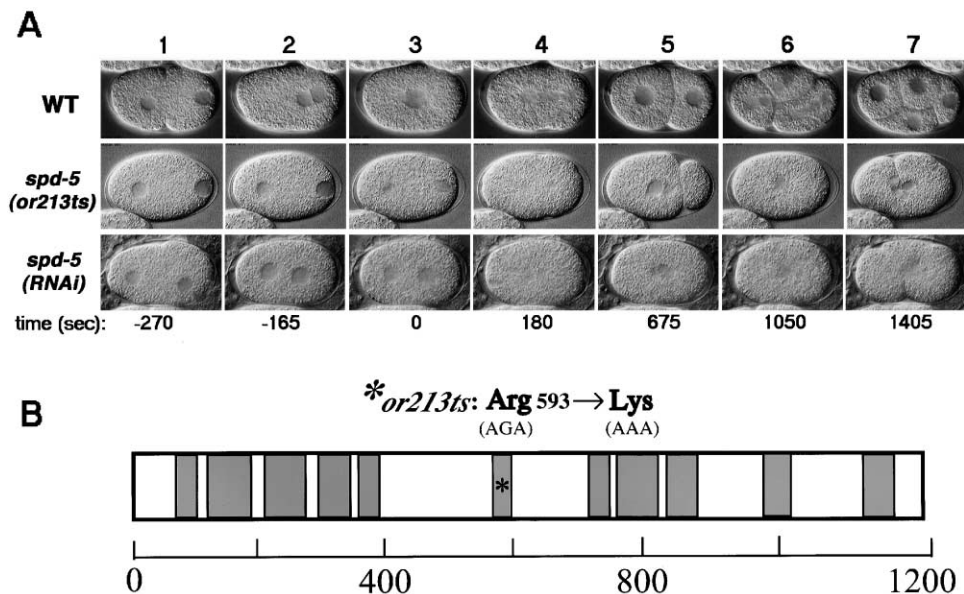
Figure 1. *spd-5* Encodes a Coiled-Coil Protein Required for Pronuclear Migration, Mitotic Spindle Assembly, and Cell Division

(A) Wild-type, *spd-5(or213ts)* mutant, and *spd-5(RNAi)* embryos viewed with Nomarski optics. In this and all figures, anterior is to the left.

(1) The oocyte and sperm pronuclei are visible at opposite ends of the cell.

(2) In contrast to WT, the pronuclei do not meet in *spd-5(−)*.

(3) Nuclear envelope breakdown (NEB).

(4) No mitotic spindle is detectable in *spd-5(−)*.

(5) Nuclear envelopes reform in WT and mutants. In the mutants shown, the two nuclei that reform are not in the same focal plane. Mutant embryos sometimes complete cytokinesis, but the furrow usually regresses.

(6) WT and mutants undergo the second attempt at mitosis at approximately the same time as determined by NEB.

(7) Nuclear envelopes reform after WT and mutants complete second attempts at mitosis.

(B) Predicted coiled-coil domains were predicted using the algorithm of Lupas et al., 1991; the mutation in *spd-5(or213ts)* is present in one predicted coiled coil.

n = 20). The *or213ts* mutation is partially conditional, fully recessive at the restrictive temperature of 25°C, and strictly maternal in its requirements (see Experimental Procedures). Hereafter, we refer to embryos from homozygous *spd-5(or213ts)* hermaphrodites grown at 25°C as *spd-5* mutant embryos or *spd-5* mutants.

Meiosis Appears Normal, But Other MT-Dependent Processes Fail in *spd-5* Mutants

Following fertilization, acentrosomal meiotic spindles extrude maternal DNA into two polar bodies (Albertson, 1984). The remaining chromosomes then decondense to form the oocyte pronucleus. In *spd-5* mutants, meiosis appears normal, as a single maternal pronucleus always formed after extrusion of a second polar body (see Figure 1; n = 20), and we always detected DNA within two polar bodies in fixed, postmeiotic *spd-5* mutants (Figure 2; n = 12). Following meiosis, the maternal and paternal pronuclei usually are positioned at opposite poles of the cell when they first appear. Normally, the maternal pronucleus then moves toward the paternal pronucleus in an MT-dependent manner (Albertson, 1984), eventually meeting near the posterior pole. However, pronuclear migration usually failed in *spd-5* mutants, and nuclear envelope breakdown (NEB) occurred without pronuclei meeting (19/20 embryos).

In wild-type embryos, maturing centrosomes and the mitotic spindle are visible as cytoplasmic clearings, due to their exclusion of lipid droplets. In *spd-5* mutants, NEB occurred at the same time as in wild-type, but no clearings that resembled centrosomal asters appeared, and no spindles were apparent (Figure 1; see movies in Supplemental Data at http://www.developmentalcell.com/cgi/content/full/3/5/673/DC1). After the first attempt at mitosis, two distinct nuclei usually reappeared, without any obvious capture or organization of chromosomes (Figure 1). Membrane invaginations formed late in mitosis, perhaps as aberrant attempts at cytokinesis. In 50% of the mutant embryos scored (10/20), the furrow appeared to completely bisect the zygote but often regressed before the next mitosis (7/10; see Figure 1). Centrosomes were similarly undetectable during the second attempt at mitosis (20/20; see Figure 1).

Although *spd-5* is essential in early development, we have not detected requirements later in embryogenesis or during larval development. When embryos produced at the permissive temperature were shifted to the restrictive temperature at the two-cell (n = 6) or four-cell (n = 3) stages (see Experimental Procedures), we observed defects in chromosome segregation during subsequent divisions, and embryonic lethality. These data suggest that SPD-5 quickly becomes nonfunctional following the upshift (data not shown). However, upshifts at the 24- to 28-cell stage did not substantially increase embryonic
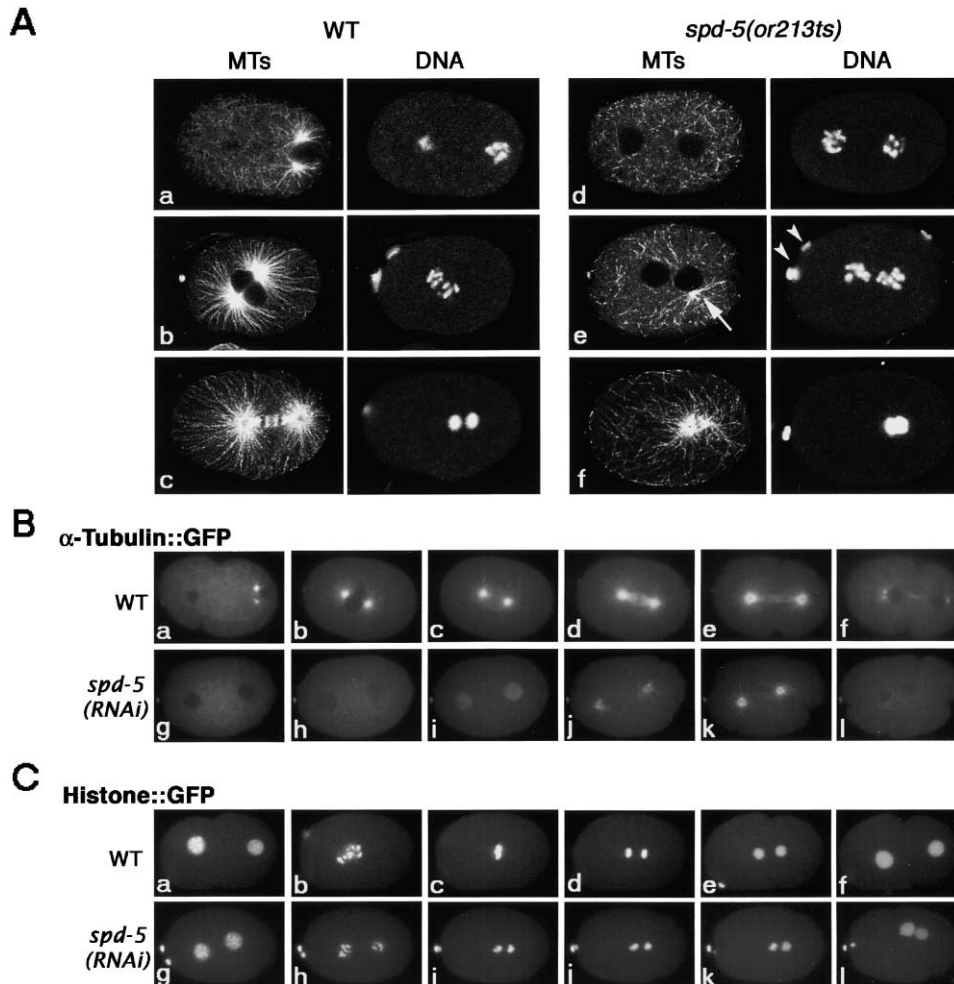
Figure 2. Microtubule Organization Is Severely Disrupted in *spd-5* Mutants

(A) Immunofluorescence microscopy of fixed embryos stained for MTs and DNA in one-cell wild-type (a–c) and *spd-5* mutant (d–f) embryos.
(a) Small microtubule asters associated with the sperm pronucleus.
(b) Large microtubule asters at prometaphase.
(c) Mitotic spindle in anaphase.
(d and e) Pronuclei, which do not meet. Cytoplasmic microtubules are present but no sperm aster is detectable.
(e) A single small microtubule aster is associated with the sperm pronucleus in some mutant embryos (arrow). Polar bodies are indicated by arrowheads.
(f) Microtubules organized around the condensed mitotic DNA.
(B) Images from time-lapse movies of MTs in living embryos expressing an α-tubulin::GFP fusion protein. Timing from left to right: 0 s (a and g), 315 s (b and h), 345 s (c and i), 465 s (d and j), 600 s (e and k), and 900 s (f and l). In wild-type embryos, prominent centrosomal foci of microtubules are visible in premitotic (a–c and f) and mitotic (d and e) cells. In *spd-5(RNAi)* embryos, centrosomally nucleated microtubules are not visible. Rather, microtubules appear to be nucleated around the DNA during mitosis (j and k).
(C) Images from time-lapse movies of embryos expressing a histone::GFP fusion protein. Timing from right to left: 0 s (a and g), 360 s (b and h), 555 s (c and i), 675 s (d and j), 870 s (e and k), and 1320 s (f and l). Chromosome condensation appears normal in *spd-5(RNAi)*, but congression to the metaphase plate and chromosome segregation in anaphase both fail (i–k).

lethality, compared to embryos kept at the permissive temperature. After upshifts at the 24- to 28-cell stage, 30/59 (51%) of the mutant embryos hatched. When embryos were mounted under similar conditions but maintained at the permissive temperature, 93/157 (59%) hatched. Furthermore, larvae raised at the restrictive temperature matured into fertile adults (see Experimental Procedures). We conclude that other genes provide similar functions later in development, or that SPD-5 and centrosomes may be important for bipolar spindle assembly and mitosis in large early embryonic cells, but not in smaller cells later in development (see Discussion).

spd-5 Encodes a Protein with Multiple Short Coiled-Coil Domains

To investigate *spd-5* function at a molecular level, we identified the gene sequence altered in *or213ts* mutants, after mapping *spd-5* to roughly −0.24 map units on chromosome I (see Experimental Procedures). We then

used RNA interference (Fire et al., 1998) to reduce the function of predicted genes in this region of the sequenced genome. Microinjection of dsRNA corresponding to the predicted gene F56A3.4 into the ovaries of wild-type hermaphrodites resulted in a *spd-5*-like mutant phenotype: pronuclei failed to migrate (10/10) and mitotic spindles failed to assemble (10/10; see Figure 1). We then sequenced F56A3.4 in genomic DNA isolated from *spd-5(or213ts)* adults and identified a missense mutation caused by a single base change relative to the wild-type sequence (Figure 1B; see Experimental Procedures). Further confirming the identity of *spd-5* and F56A3.4, SPD-5 protein is not detectable in *spd-5(or213ts)* embryos produced at the restrictive temperature, or in *spd-5(RNAi)* embryos (see below). The similar phenotypes of *spd-5(or213ts)* and *spd-5(RNAi)* mutant embryos suggest that *or213ts* is not an unusual allele and strongly reduces *spd-5* function. The predicted SPD-5 protein is composed of 1198 amino acids, has a calculated molecular mass of 135 kDa, and contains 11 predicted coiled-coil regions that range in size from roughly 30 to 60 amino acids in length (Figure 1B). BLAST searches do not reveal clear orthologs but indicate that SPD-5 is related to many proteins containing extensive or multiple coiled-coil domains (data not shown).

spd-5 Mutants Fail to Organize Microtubules into a Bipolar Mitotic Spindle

We used indirect immunofluorescence and confocal microscopy to compare the organization of microtubules and chromosomes in fixed wild-type and *spd-5* mutant embryos (Figure 2A). In wild-type, short cytoplasmic MTs were dispersed throughout the zygote during meiosis. Two MT asters, associated with the sperm pronucleus, began to form near the completion of meiosis. The number and length of astral MTs increased as zygotes entered mitosis, with many astral MTs contacting the cytoplasmic cortex (Figure 2A, panels a–c). In roughly half (6/11) of *spd-5(or213ts)* mutants examined prior to the full condensation of chromosomal DNA during mitosis, we observed only cytoplasmic MTs and no asters associated with either pronucleus (Figure 2A, panel d). In the remaining embryos (5/11), we observed a small MT aster associated with the sperm pronucleus (Figure 2A, panel e). While we have not determined that these small asters include a centriole, their presence is consistent with the sperm-donated centriole being present but not functional in the absence of SPD-5. After NEB, two foci of condensed chromosomes appeared to nucleate roughly radial arrays of MTs, without forming a spindle-like structure (Figure 2A, panel f). We infer that the two foci of DNA in these fixed embryos were derived from maternal and paternal pronuclei that failed to meet. We found no examples of chromosomes becoming organized or segregated in fixed one-cell stage *spd-5* mutants.

To examine the mitotic spindle over time in live cells, we used a transgenic strain that expresses a β-tubulin::GFP fusion protein to make time-lapse movies of GFP-labeled MTs during the first two cell divisions (Figure 2B; see Supplemental Data and Experimental Procedures). In wild-type, before pronuclear migration, two
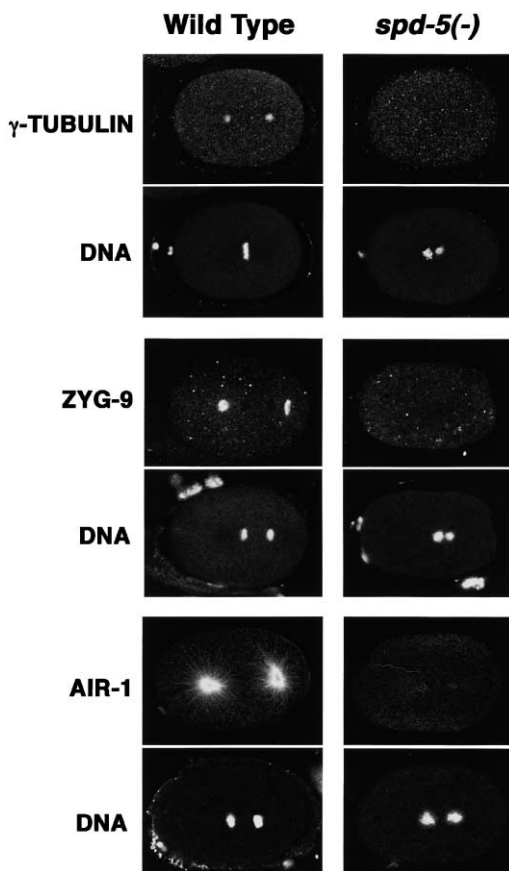

Figure 3. SPD-5 Is Required for Localization of Centrosomal Proteins

Mitotic one-cell stage embryos were fixed and double labeled to visualize DNA and either γ-tubulin, ZYG-9, or AIR-1 proteins. In WT, γ-tubulin, ZYG-9, and AIR-1 localize to centrosomes in one-cell stage embryos (left panel). Localization of these proteins to centrosomes does not occur in *spd-5* mutant embryos (right panel).

MT asters associated with the sperm pronucleus first became detectable shortly after the completion of meiosis, and increased in size until late in mitosis (Figure 2B, panel a). In *spd-5* mutants (Figure 2B, panels g–l), no MT organization was apparent prior to NEB (Figure 2B, panels g and h). After NEB, tubulin::GFP rapidly associated with, and radiated out from, condensing chromosomes (Figure 2B, panels j and k). Upon nuclear envelope breakdown during the second attempt at mitosis, we again observed a rapid and apparently random association of MTs with condensed chromosomal DNA.

We also used time-lapse microscopy to examine chromosome dynamics in live embryos produced by transgenic worms that maternally express a histone::GFP fusion protein (Figure 2C; see Supplemental Data). Pronuclei failed to migrate in *spd-5* mutants (Figure 2C, panel h), and the chromosomes within each pronucleus condensed into separate foci that did not meet (Figure 2C, panel i). The timing of nuclear envelope breakdown, relative to the timing of chromatin condensation, appeared normal, and chromatin decondensed after the failed attempt at mitosis (Figure 2C, panels k and l). During the second attempt at mitosis, we observed reproducible segregation of chromosomes to four discrete
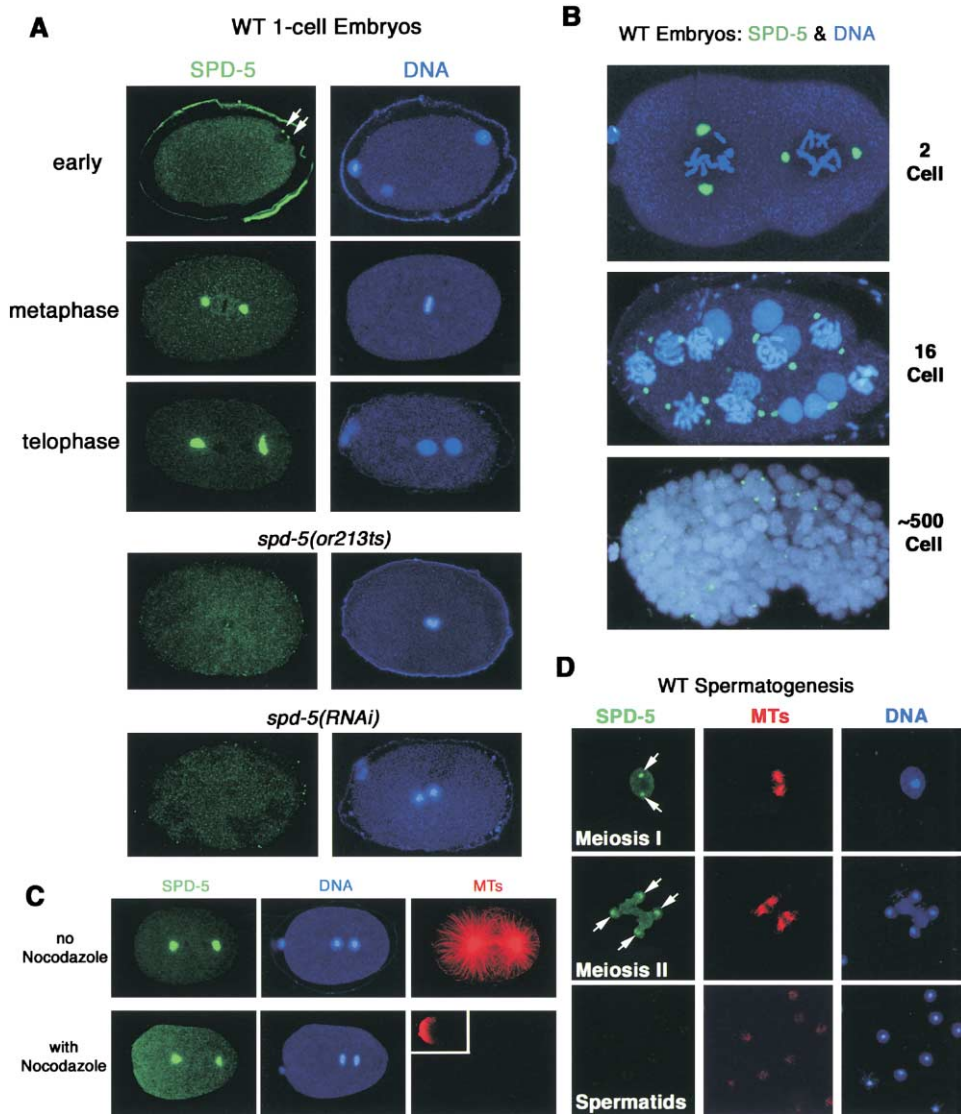
Figure 4. SPD-5 Is a Centrosomal Protein

(A) Embryos were double stained to visualize SPD-5 and DNA. SPD-5 is detected at two spots associated with the sperm pronucleus shortly after fertilization (arrows, top row). SPD-5 accumulates to high levels during mitosis. SPD-5 is not detectable in *spd-5* mutants (*or213ts* or *RNAi*).

(B) SPD-5 accumulates at centrosomes throughout embryogenesis.

(C) SPD-5 localization at centrosomes persists in embryos in which microtubules have been depolymerized with nocodazole. The inset in the last panel shows an overexposed image of the same embryo; no spindle microtubules are detectable.

(D) SPD-5 is present at centrosomes during the first and second meiotic divisions during spermatogenesis (arrowheads), but is not detectable in mature spermatids.

"poles" in *spd-5(or213ts)* embryos (n = 7). However, in *spd-5(RNAi)* mutant embryos, no segregation was detected during either the first or second attempts at mitosis (n = 6; see movies in Supplemental Data).

Centrosome Growth and Maturation Defects in *spd-5* Mutants

The nearly complete absence of sperm aster formation and spindle assembly prompted us to examine centrosomal proteins in *spd-5* mutants. We first assessed the status of γ-tubulin (Hannak et al., 2001; Strome et al., 2001), which nucleates MT assembly at centrosomes

(see Introduction). In wild-type, we always detected two spots of γ-tubulin associated with the sperm pronucleus (n = 9) or the mitotic spindle (n = 12). However, we were unable to detect any discrete foci of γ-tubulin in one-cell stage *spd-5(or213ts)* mutants (n = 32; see Figure 3). We also examined the centrosomal proteins AIR-1 and ZYG-9 (Figure 3). AIR-1 is an Aurora A-like kinase required for γ-tubulin to accumulate to high levels at centrosomes, and for spindle assembly (Hannak et al., 2001; Schumacher et al., 1998). ZYG-9 is a centrosomal MT-stabilizing protein related to XMAP-215 in *Xenopus* (Matthews et al., 1998). In all *spd-5* mutants examined,
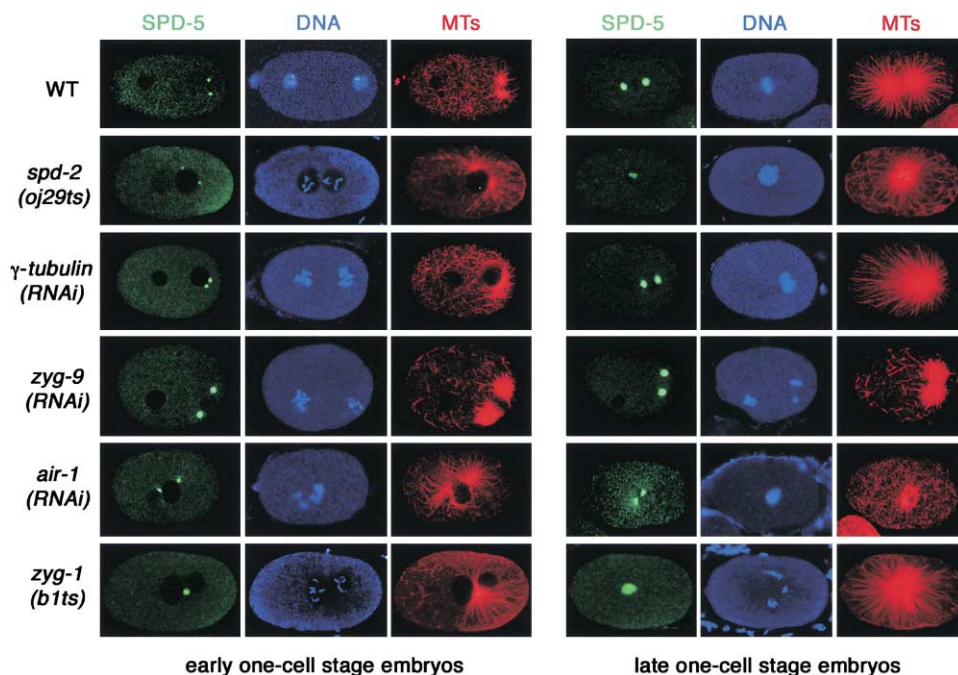
Figure 5. SPD-5 Localizes to Centrosomes in Mutants with Defects in Microtubule Organization

Embryos were triple labeled to visualize SPD-5 (green), DNA (blue), and MTs (red). In early one-cell stage mutant embryos (left panel), two spots of SPD-5 are associated with the sperm pronucleus, as in wild-type. In *zyg-1(b1ts)* embryos, the sperm-donated centrosome fails to duplicate. Consistent with this defect, only one spot of SPD-5 is seen. In late mitotic one-cell stage embryos (right panel), two spots of SPD-5 are detectable in all mutants but *zyg-1*. In *zyg-9(RNAi)* mutants, a small transverse bipolar spindle forms, and SPD-5 is abundant at both poles of the spindle. In the other mutant backgrounds, no bipolar spindles assemble, yet SPD-5 is present in two spots in the center of the foci of microtubules that form.

these proteins were never detected during the first attempt at mitosis (n = 26 for ZYG-9; n = 11 for AIR-1). We conclude that SPD-5 is required for sperm-donated centrioles to grow and mature into functional centrosomes.

SPD-5 Is a Centrosomal Protein
If it recruits other proteins to centrosomes, SPD-5 itself should localize to centrosomes. To test this prediction, we generated polyclonal antibodies specific for SPD-5 to stain fixed embryos, and found that SPD-5 is a centrosomal protein (Figure 4A). We could first detect SPD-5 in early postmeiotic one-cell stage embryos, prior to pronuclear migration but after the sperm-donated centriole had duplicated to produce two detectable foci of SPD-5 staining. These two foci greatly increased in size prior to and during mitosis (Figure 4A). In metaphase, SPD-5 was evenly distributed throughout centrosomes. In late anaphase or telophase, SPD-5 surrounded a central core devoid of both MTs and SPD-5 (Figure 4, telophase, and data not shown). Two different antisera that recognize the same SPD-5 peptide, and one that recognizes a different SPD-5 peptide, gave identical staining patterns (see Experimental Procedures), and no foci of SPD-5 protein were detected in fixed *spd-5(RNAi)* or *spd-5(or213ts)* mutants during the first attempt at mitosis (Figure 4A).

We detected SPD-5 at centrosomes throughout embryogenesis and during postembryonic meiotic divisions that produce sperm in male nematodes (Figures 4B and 4D). In contrast to the acentrosomal meiotic division of maternal chromosomes, meiotic spindles during spermatogenesis are organized by centrosomes (reviewed in Kimble and Ward, 1988). Nevertheless, we were unable to detect any paternal requirement for SPD-5 after raising homozygous *spd-5(or213ts)* males at the restrictive temperature throughout larval development (see Experimental Procedures). SPD-5 was not detectable in mature spermatids (Figure 4D), suggesting that the sperm-donated centrosome is highly reduced in content, and consistent with the strictly maternal requirement for SPD-5.

SPD-5 Localization Is Independent of MTs and Other Centrosomal Proteins
To determine where SPD-5 might act in a hierarchy of centrosome maturation, we first determined that it localizes to centrosomes independently of MTs (Figure 4C). We also examined SPD-5 localization in mutant embryos lacking other known centrosomal proteins. Depletion of the Aurora A kinase AIR-1, γ-tubulin, or the XMAP215 homolog ZYG-9 severely disrupted mitotic spindle assembly, but two distinct foci of SPD-5 were always visible, with higher levels detected late in mitosis (Figure 5; at least ten embryos were examined for each mutant). In γ-tubulin and *zyg-9* mutants, SPD-5 stained late mitotic centrosomes brightly, although in *air-1* mutant embryos, somewhat less SPD-5 may accumulate at centrosomes. We detected a single focus of SPD-5 at
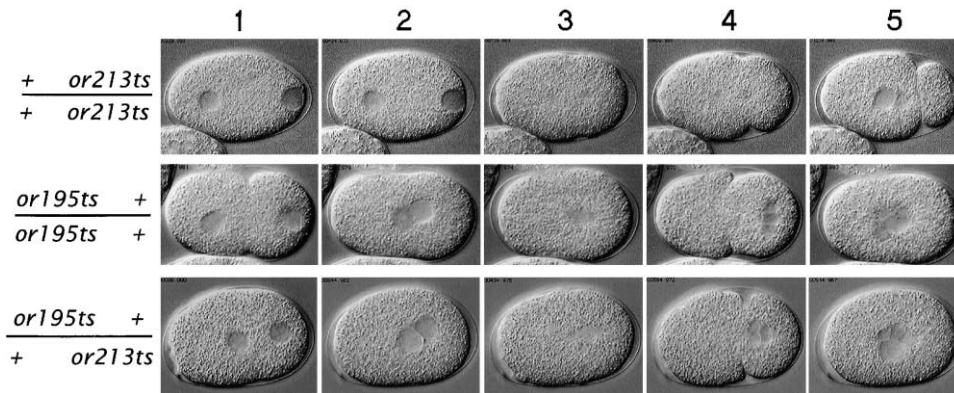
Figure 6. *spd-5* Interacts Genetically with a Dynein Heavy Chain, *dhc-1*

Images from time-lapse digital movies of the first attempt at mitosis in embryos produced by homozygous *spd-5(or213ts)* hermaphrodites (row 1), homozygous *dhc-1(or195ts)* hermaphrodites (row 2), and by hermaphrodites heterozygous for both *spd-5(or213ts)* and *dhc-1(or195ts)* (row 3). The embryos from double heterozygotes resemble embryos from homozygous *dhc-1(or195ts)* hermaphrodites: pronuclei meet posteriorly (1 and 2), a small, posteriorly displaced bipolar spindle assembles (3), and DNA segregation and cytokinesis fail (4 and 5).

monopolar spindles in one-cell stage embryos depleted of ZYG-1 (O'Connell et al., 2001; see Introduction).

Though not entirely absent, we detected only faint SPD-5 foci in 15/17 one-cell stage *spd-2* mutant embryos, with none detected in 2/17. Mitotic spindle assembly is severely compromised in *spd-2* mutants (O'Connell et al., 2000; see Figure 5), but two distinct SPD-5 foci were associated with presumably the sperm pronucleus in all positively staining embryos. We conclude that *spd-2* is required for SPD-5 to accumulate to high levels at centrosomes but may not be required for centriole duplication.

SPD-5 Interacts Genetically with Cytoplasmic Dynein

SPD-5 also interacts with the minus end-directed MT motor protein dynein, which plays a role in spindle function (reviewed in Heald, 2000). In our genetic screens, we identified three conditional alleles of *dhc-1*, which encodes a cytoplasmic dynein heavy chain required for pronuclear migration, centrosome separation, and spindle formation (Göncy et al., 1999). The three conditional alleles we identified (*or195ts*, *or283ts*, and *or352ts*) only partially eliminate DHC-1 function at the restrictive temperature, as pronuclear migration appears normal and a small but bipolar spindle still forms (Figure 6 and data not shown). All three mutations were positioned as expected for *dhc-1* at roughly −1.3 map units on chromosome I, and *or195ts* failed to complement a strong loss-of-function allele of *dhc-1*, called *let-354(h90)* (Howell and Rose, 1990; Mains et al., 1990; D. Schmidt, W. Saxton, and S. Strome, personal communication; see Experimental Procedures). We sequenced the 16 kb *dhc-1* locus in genomic DNA isolated from *or195ts* worms and detected a single base pair change, relative to the wild-type sequence. This mutation results in a histidine-to-leucine change at amino acid position 2157 in the predicted amino acid sequence of DHC-1.

In the process of isolating and mapping these mutations in *spd-5* and *dhc-1*, we performed complementation tests. All three *dhc-1* alleles failed to complement *spd-5(or213ts)*, even though all four mutations are themselves recessive at the restrictive temperature (see Experimental Procedures). For the *dhc-1* alleles *or195ts* and *or283ts*, embryos from hermaphrodites heterozygous for both *dhc-1* and for *spd-5*, when grown at the restrictive temperature of 25°C, had spindle assembly defects indistinguishable from those observed in embryos from homozygous *dhc-1(or195ts)* and *dhc-1(or283ts)* hermaphrodites grown at the restrictive temperature (Figure 6). This example of extragenic noncomplementation appears specific for partial loss-of-function mutations, as the *spd-5(or213ts)* mutation did complement a strong loss-of-function allele of *dhc-1* (see Experimental Procedures).

Anterior-Posterior Body Axis Defects in *spd-5* Mutant Embryos

In *C. elegans*, the position of the paternal pronucleus specifies the posterior pole of the zygote and hence the anterior-posterior (a-p) body axis (Goldstein and Hird, 1996). Furthermore, defects in sperm aster assembly can result in a loss of a-p polarity, suggesting that astral MTs or the centrosome specify this body axis (O'Connell et al., 2000; Rappleye et al., 2002; Wallenfang and Seydoux, 2000). As *spd-5* mutants lack sperm asters, we expected and observed polarity defects (Figure 7). Specifically, cytoplasmic flows normally directed by the sperm pronucleus and its associated centrosomes failed to occur (Figure 7A). In wild-type, these flows move cytoplasmic germline factors called P granules to the posterior pole (Hird et al., 1996; reviewed in Goldstein, 2000). In *spd-5* mutants, P granules were delayed but usually did move to the posterior cortex (Figure 7B). In 23/32 (72%) pronuclear stage embryos in which the DNA was only partially condensed, P granules were found either throughout the embryo or centrally localized. P granules were posteriorly localized in equivalently staged wild-type embryos (9/9). Late in the first mitosis, 81% (21/26) of *spd-5(or213ts)* embryos had posteriorly localized P granules (Figure 7B). Finally, the polarity protein PAR-2 normally is restricted to the cytoplasmic cortex in the posterior half of a wild-type zygote

A

WT   spd-5(or213ts)

B

| DNA | P-granules | DNA | P-granules |

WT

spd-5
(or213ts)

C

| DNA | PAR-2 | DNA | PAR-2 |

WT

spd-5
(or213ts)

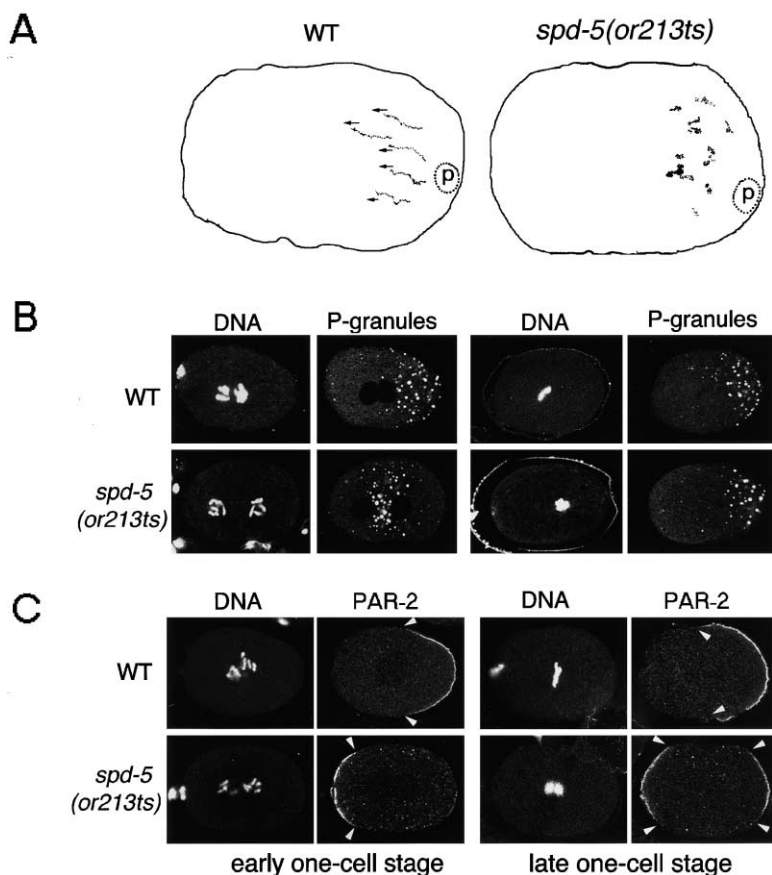early one-cell stage   late one-cell stage

Figure 7. Anterior-Posterior Polarity Is Disrupted in *spd-5* Mutant Embryos

(A) Anteriorly directed cortical flows are absent in *spd-5*. The movements of single yolk granules near the cell surface were documented over time to visualize cortical movements. Arrows indicate the direction of cortical flows in WT. The encircled P shows the position of the sperm pronucleus, projected into the focal plane of the cortical flows.

(B) P granules localize to the posterior prior to the first mitosis in wild-type embryos, but are often found in the center of *spd-5* embryos (see early one-cell stage). P granules often do become restricted to the posterior abnormally late in *spd-5* mutants (see late one-cell stage).

(C) PAR-2 is restricted to the posterior cortex in wild-type, but in *spd-5* it appears to be randomly localized to either or both poles. Arrowheads point to boundaries of PAR-2 staining. In wild-type embryos, PAR-2 extends further anteriorly as the cell cycle proceeds. In *spd-5* mutant embryos, even if PAR-2 is posteriorly localized, it does not extend as far around the cortex as in wild-type. There was no correlation between the different patterns of PAR-2 localization and cell cycle stage in *spd-5* mutants.

(9/9 one-cell embryos with condensed chromosomes; Figure 7C; see Boyd et al., 1996). However, in *spd-5* mutants, PAR-2 was present at either the anterior pole (2/8 one-cell embryos) or the posterior pole (4/8) or both poles (2/8; Figure 7C), much as in *spd-2* mutants (O'Connell et al., 1998). Even when PAR-2 was present at the posterior cortex, the extent of cortical localization was substantially reduced compared to wild-type.

Discussion

SPD-5, a centrosomal protein with multiple predicted coiled-coil domains, is required for centrosome maturation and mitotic spindle assembly during the first division of the *C. elegans* zygote. Although centrosomes dominate MT organization during mitosis in most animal cells, oocyte meiosis in most organisms, including *C. elegans*, occurs in the absence of centrosomes (reviewed in Palazzo et al., 2000). Furthermore, some insect embryos develop mitotically without centrosomes (de Saint Phalle and Sullivan, 1998), and genetic studies in *Drosophila* have shown that most mitotic divisions can occur without detectable centrosomes (Bonaccorsi et al., 2000; Megraw et al., 2001; Vaizel-Ohayon and Schejter, 1999; reviewed in Raff, 2001). In mammalian cell culture studies, laser ablation of one or both centrosomes during prophase does not prevent the assembly of a bipolar mitotic spindle (Khodjakov et al., 2000). Even

DNA-coated beads, when mixed with cell extracts from *Xenopus* embryos, assemble bipolar spindles in the absence of centrosomes (Heald et al., 1996, 1997). These findings have led to the remarkable conclusion that while centrosomes may dominate MT organization when present, they are not necessary for the assembly of functional, bipolar mitotic spindles in many animal cells.

Our analysis of *spd-5* provides genetic evidence that, in at least some contexts, functional centrosomes are required for mitotic spindle assembly and chromosome segregation. However, we cannot rule out the possibility that SPD-5 has other functions responsible for the failure in mitotic spindle assembly. For example, pronuclear migration fails before the first attempt at mitosis. This earlier defect alone probably does not prevent spindle assembly, as we also observed spindle defects after shifting two-cell and four-cell *spd-5(or213ts)* mutant embryos to the restrictive temperature. Furthermore, in mutants such as *zyg-9*, centrosomes mature and a bipolar spindle forms without pronuclear migration (Matthews et al., 1998).

Although *spd-5* is required for spindle assembly during early attempts at mitosis, we have not detected any requirements for SPD-5 beyond the 24-cell stage. Thus, centrosomes in *C. elegans* may be essential for spindle assembly only in larger early embryonic cells and not in smaller cells later in embryogenesis. Alternatively, SPD-5 may function redundantly with other proteins in

late embryos and in larvae. Finally, the *or213ts* mutation may not fully eliminate SPD-5 function, as we consistently observed some chromosome segregation during the second attempt at mitosis in *spd-5(or213ts)* embryos, but not in *spd-5(RNAi)* embryos. Perhaps a complete loss of *spd-5* function in later stage embryos would result in lethality.

Is SPD-5 a Centromatrix Protein?
The multiple coiled-coil domains in SPD-5 are likely to mediate protein-protein interactions. We propose that SPD-5 proteins interact with each other to form a lattice or network, as documented for the vertebrate centrosomal protein pericentrin, which also consists of several small coiled-coil domains (Dictenberg et al., 1998). A SPD-5 lattice could recruit additional proteins through other coiled-coil interactions. Consistent with this model, SPD-5 was required for all centrosomal proteins tested to localize to spindle poles, while SPD-5 localized to centrosomal foci independently of all proteins tested. Recently, it was shown that the *C. elegans* Aurora A kinase AIR-1 is required for the accumulation of γ-tubulin that normally occurs during centrosome maturation. However, γ-tubulin was still readily detectable at centrosomes, though reduced in amount, in *air-1* mutant embryos (Hannak et al., 2001). Thus, SPD-5 appears to be required both for the AIR-1-dependent accumulation of γ tubulin, and for any AIR-1-independent localization of γ-tubulin to centrosomes, as both AIR-1 and γ-tubulin were undetectable in *spd-5* mutants.

While SPD-5 appears to act early in centrosome growth and maturation, it was always reduced and sometimes undetectable in mutant embryos lacking the function of *spd-2*, a maternally expressed locus of unknown molecular identity. Although we analyzed the most severe *spd-2* allele known, we do not know whether it is null (O'Connell et al., 2000). If not, the low levels of SPD-5 we detected in most *spd-2* embryos could be due to residual SPD-2 function. Furthermore, even though *spd-2* and *spd-5* both act early in centrosome growth and maturation, their mutant phenotypes differ considerably. In contrast to *spd-5*, pronuclei meet in *spd-2* mutants (O'Connell et al., 2000). Perhaps this difference is due to the interaction of SPD-5 with DHC-1, which is required for pronuclear migration (Gönczy et al., 1999). In addition, P granules completely fail to localize to the posterior in *spd-2* mutants (O'Connell et al., 2000), while in most *spd-5* mutants, their localization was delayed but not absent. We suggest that SPD-2 and SPD-5 reside near the top of a hierarchy of maternal proteins required for centrosome maturation, but perform at least partially distinct roles.

Are SPD-5 and Pericentrin Functionally Related?
Many centrosomal proteins have predicted coiled-coil domains (Wigge et al., 1998; Andersen, 1999). Nevertheless, SPD-5 and vertebrate pericentrin (Doxsey et al., 1994) have several properties in common. Both are centrosomal proteins required for centrosome maturation, and both are predicted to have small coiled-coil regions distributed throughout their length, 11 in SPD-5 and 13 in *Xenopus* pericentrin. Intriguingly, pericentrin

interacts with the dynein light intermediate chain in vertebrate cells (Purohit et al., 1999; Tynan et al., 2000; Young et al., 2000), and SPD-5 genetically interacts with the dynein heavy chain locus *dhc-1*. Although we were unable to rescue *spd-5* mutants through maternal expression of a human pericentrin cDNA (data not shown), it is possible that both interact with dynein to promote centrosome maturation, and are functionally equivalent. Finally, pericentrin and perhaps SPD-5 probably are not alone in forming a pericentriolar scaffold. Cep135 is another mammalian centrosomal protein with several small coiled-coil domains that localizes around and within centrioles (Ohta et al., 2002). Similar to *spd-5*, reduction of Cep135 by RNAi in CHO cells prevents spindle assembly but not MT polymerization.

While many spindle pole body components in budding yeast have been identified (Wigge et al., 1998), a comparable analysis of animal centrosomes has not been reported. To understand centrosome assembly, maturation, and function, it will be important to identify more comprehensively the molecules required for these processes. Because it is a highly specific and apparently abundant centrosomal protein, and is present at centrosomes throughout mitosis, SPD-5 may prove useful as a tool for more completely defining the proteins present within centrosomes.

Experimental Procedures

Strains and Alleles
C. elegans strains were maintained as described (Brenner, 1974). N2 Bristol was the wild-type strain. The following strains and alleles were used. LGI: *bli-4(e937)*, *dpy-5(e61)*, *let-354(h90)*, *let-354(or195ts)*, *let-354(or283ts)*, *let-354(or352ts)*, *let-359(h94)*, *let-361(h97)*, *let-363(h98)*, *let-364(h104)*, *let-371(h123)*, *spd-2(oj29)*, *spd-5(or213ts)*, *unc-11(e47)*, *unc-13(e450)*, *unc-13(e1091)*, *unc-38(x20)*, *unc-63(x18)*, *unc-73(e936)*; LGII: *rol-6(e187)*, *zyg-1(b1ts)*; LGIII: *unc-32(e189)*; LGIV: *him-8(e1489)*, *unc-5(e53)*; LGV: *dpy-11(e224)*; LGX: *lin-2(e1309)*, *lon-2(e678)*; rearrangements: *sDp2 (I;f)*, *hDf28*. Also used were the GFP lines *AZ244 unc-119(ed3)III; ruIs57[pAZ147:pie-1/GFP/tubulin]* and *AZ212 unc-119(ed3); ruIs32[pAZ132:pie-1/GFP/histoneH2B]* (Praitis et al., 2001).

**Strain Maintenance, Temperature Sensitivity,
and Brood Analysis**
The temperature-sensitive spindle-defective alleles, *or195ts*, *or213ts*, *or283ts*, and *or352ts* described in this study were isolated using EMS chemical mutagenesis (Encalada et al., 2000). All alleles were outcrossed at least four times. Strains were maintained by growing homozygous animals at 15°C. To observe the mutant phenotype, L4 larvae were placed at 25°C overnight and embryos were collected from adults the following day.

At the permissive temperature of 15°C, homozygous *spd-5* mutant hermaphrodites produced 68% viable embryos (1093/1607). At the restrictive temperature of 25°C, no embryos hatched (0/425). The average brood size produced by three homozygous *spd-5(or213ts)* hermaphrodites was 270. For *dhc-1(or195ts)*, 91% of embryos produced at the permissive temperature hatched (480/525), and 0.3% of the embryos hatched when hermaphrodites were grown at 25°C (1/374). By comparison, 99.7% of N2 wild-type embryos hatched at 15°C (396/397) and 97% at 25°C (679/699), with an average brood size of 338 for three hermaphrodites. Temperature upshift experiments with *spd-5(or213ts)* were done as described in Severson et al. (2000).

Genetic Analysis
Linkage group and meiotic mapping were done using standard methods as described in Brenner (1974). Both *or195ts* (formerly called *spd-4*) and *spd-5(or213ts)* map to LGI. Meiotic crossover mapping of *or195ts* gave the following results: *unc-73* (27/76)

or195ts (49/76) *dpy-5*, from which an estimated map position of −1.3 was determined. Meiotic mapping of *or213ts* gave the following results: *unc-73* (65/75) *spd-5* (10/65) dpy-5; *unc-38* (10/20) *spd-5* (10/20) dpy-5; *unc-63* (1/8) *spd-5* (7/8) dpy-5. From these data, a predicted genetic map position of −0.24 was determined.

To determine whether *spd-5(or213ts)* is recessive, *spd-5(or213ts) unc-13(e1091)I* hermaphrodites and *him-8(e1489)IV* males were mated at 15°C. Eight non-Unc F1 L4 larvae were shifted to 25°C. Ninety percent (85/94) of their embryos hatched and grew to adulthood; 25% were Unc and laid all dead embryos with the *spd-5* phenotype. Similar results were obtained with the *dhc-1* alleles *or195ts*, *or283ts*, and *or352ts*.

Male fertility: L1 *spd-5(or213ts)I; him-8(e1489)IV* larvae were raised to adulthood at 25°C. Three young adult *spd-5* males were mated with single *unc-32* hermaphrodites. Seventeen of twenty crosses done at room temperature produced numerous male and non-Unc outcross progeny.

Strict maternal requirement: three to four *him-8(e1489)IV* males were crossed into single *unc-38(x20)or213ts* I hermaphrodites at 15°C. One or two days later, crosses were transferred to plates at 25°C, and transferred again at 25°C after about 6 hr. In crosses that worked, there was no paternal rescue of embryonic lethality at the restrictive temperature.

dhc-1 alleles: *dhc-1(or195ts)* fails to complement a strong allele of *dhc-1* (also called *let-354*; D. Schmidt, W. Saxton, and S. Strome, personal communication). Unc hermaphrodites of genotype *let-354(h90) dpy-5(e61) unc-13(e450)I; sDp2 (I;f)*, were crossed with males of genotype *or195ts dpy-5(e61)I; him-8(e1489)IV* at 15°C. Dpy-non-Unc F1 L4 progeny (*h90/or195ts* worms that did not pick up the duplication; n = 10), were shifted to 25°C. Most embryos failed to hatch and exhibited severe spindle defects during the first embryonic mitosis, when viewed using Nomarski optics (data not shown), but 10% hatched (n = 260). Similarly, when *or195ts* was placed in *trans* to a deficiency *hDf28*, a small fraction of the embryos hatched.

Several crosses demonstrated nonallelic noncomplementation between *spd-5(or213ts)* and *dhc-1/let-354(or195ts)*. *or195ts dpy-5* hermaphrodites were crossed with *spd-5(or213ts); him-8* males at 15°C. L4 F1 non-Dpy hermaphrodites were shifted to 25°C. None of the embryos produced by ten hermaphrodites hatched. The reciprocal cross with *spd-5(or213ts) unc-13(e450)I* hermaphrodites and *or195ts I; him-8(e1489) IV* males was also done. Of the 20 non-Dpy outcross F1s that were singled, 18 produced no viable embryos, and two had mostly dead embryos, with a few hatching. Similar results were obtained with *dhc-1(or283ts)* and *dhc-1(or352ts)*.

Allele and gene specificity of the *spd-5/dhc*-1 interaction: spd-5(or213ts) dpy-5(e61) I; him-8(e1489) IV* males were crossed with *let-354(h90) dpy-5(e61) unc-13(e450) I; sDp2* Unc hermaphrodites. Seven L4 Dpy hermaphrodite outcrosses were shifted to 25°C; most of the embryos laid hatched. Complementation analysis was done between *spd-5(or213ts)* and other lethal mutants that map near it including *let-359*, *let-361*, *let-363*, *let-364*, and *let-371*. In all cases, at least 2/3 of the embryos produced by outcross hermaphrodites hatched.

Generation of SPD-5 Antibodies

Antibodies were raised against SPD-5 peptides by Quality Controlled Biochemicals, as follows: two independent rabbit polyclonal antisera were raised against a peptide sequence at the N terminus of SPD-5 (MEDNSVLNEDSNLC-amide), and two other antisera were raised against a C-terminal peptide (AC-CGKIATDMGGAVKEIRKK-OH). Some of the final bleeds were affinity purified over an agarose column to which the SPD-5 peptide was coupled. The antibodies were eluted from the column with glycine buffer and the pH was neutralized with Tris-HCl (pH 9.5).

Microscopy

Differential interference contrast microscopy with live embryos, and immunofluorescence microscopy with fixed embryos, was done as described previously (Shelton et al., 1999; Severson et al., 2000). Immunostaining of sperm was performed as described in Golden et al. (2000). Cortical flows were determined as in O'Connell et al. (2000). The following primary antibodies and dilutions were used: anti-α-tubulin (clone DM1α; Sigma) diluted 1:250–1:500, anti-SPD-5

diluted 1:1000–1:2000, anti-PAR-2 diluted 1:5, and anti-ZYG-9 diluted 1:50 (kindly provided by Dr. K. Kemphues; Boyd et al., 1996; Matthews et al., 1998), anti-γ-tubulin diluted 1:1000 (kindly provided by Dr. Y. Bobinnec; Bobinnec et al., 2000), anti-PGL-1 P granule antibody diluted 1:1000 (kindly provided by Dr. S. Strome; Kawasaki et al., 1998), and anti-AIR-1 diluted 1:1000 (kindly provided Dr. T. Hyman; Hannak et al., 2001). Most primary antibodies were left on overnight at 4°C, but anti-PAR-2 was incubated for at least 36 hr. SPD-5 and γ-tubulin antibodies were left on for at least 1 hr at room temperature or overnight at 4°C. Fluorescently tagged secondary antibodies were used at 1:200 dilutions: FITC-conjugated goat anti-mouse or -rabbit (Jackson ImmunoResearch Laboratory) and rhodamine-conjugated goat anti-mouse (Molecular Probes). DNA was stained with 0.2 μM TOTO-3 iodide (Molecular Probes). The slides were mounted in Slow Fade (Molecular Probes) before viewing on a Bio-Rad MRC 1024 or a Bio-Rad radiance laser scanning confocal microscope. For time-lapse movies of GFP strains, embryos were mounted on agar pads and observed on a spinning disc confocal microscope (Perkin-Elmer) equipped with a MicroMax cooled CCD camera (Roper Scientific) under the control of MetaMorph imaging software (Universal Imaging). Approximately five focal planes were acquired at 1 μm intervals every 20 s.

To depolymerize MTs, gravid hermaphrodites were cut open in water and early embryos were transferred in a drop of water to a polylysine-coated slide. The water was removed and replaced with freshly prepared nocodazole at 50 μg/ml. Complete microtubule disruption took about 1 min.

Molecular Biology
RNA Interference

To make dsRNA, PCR with T7 and T3 primers was used to amplify cDNA inserts from lambda phage clones kindly provided by Dr. Yuji Kohara. RNA was synthesized using T3 and T7 polymerase (Promega), and purified by phenol/chloroform extraction and EtOH precipitation. Double-stranded RNA was injected into the syncitial gonad of young hermaphrodites by standard methods (Fire et al., 1998). Embryos from injected animals were observed between 24 and 36 hr postinjection. The clones used in this study include: yk53g12 (*dhc-1*), yk80h7 (*tbg-1*), yk28d2 (*zyg-9*), yk274b8 (*air-1*), and yk251e8 (*spd-5*).
DNA Sequencing

Genomic DNA was extracted from *dhc-1/let-354(or195ts)* and *spd-5(or213ts)* mutants, as well as from *lin-2(e1309)X*, the background strain in which the mutations were generated. DNA segments spanning the entire gene (*dhc-1*, Genefinder locus T21E12.4; *spd-5*, Genefinder locus F56A3.4) were amplified by PCR and partially purified using a GeneClean kit (Bio101). Sequencing was done on an ABI Prism automated fluorescent sequencer (Perkin-Elmer) at the University of Oregon DNA sequencing facility.

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