Cytoplasmic Dynein Is Required for Distinct Aspects of MTOC Positioning, Including Centrosome Separation, in the One Cell Stage C. elegans Embryo

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Abstract. We have investigated the role of cytoplasmic dynein in microtubule organizing center (MTOC) positioning using RNA-mediated interference (RNAi) in C. elegans to deplete the product of the dynein heavy chain gene dhc-1. Analysis with time-lapse differential interference contrast microscopy and indirect immunofluorescence revealed that pronuclear migration and centrosome separation failed in one cell stage dhc-1 (RNAi) embryos. These phenotypes were also observed when the dynactin components p50/dynamitin or p150/Glued were depleted with RNAi. Moreover, in 15% of dhc-1 (RNAi) embryos, centrosomes failed to remain in proximity of the male pronucleus. When dynein heavy chain function was diminished only partially with RNAi, centrosome separation took place, but orientation of the mitotic spindle was defective. Therefore, cytoplasmic dynein is required for multiple aspects of MTOC positioning in the one cell stage C. elegans embryo. In conjunction with our observation of cytoplasmic dynein distribution at the periphery of nuclei, these results lead us to propose a mechanism in which cytoplasmic dynein anchored on the nucleus drives centrosome separation.

Key words: microtubules • minus end-directed motor • mitosis • RNAi • MTOC positioning

Proper positioning of microtubule organizing centers (MTOCs) is central to a number of cell division processes. Correct separation of MTOCs is necessary for bipolar spindle formation, whereas accurate positioning of spindle poles during mitosis dictates proper cleavage furrow placement in animal cells. The mechanisms responsible for MTOC positioning are incompletely understood, but they probably involve microtubule-dependent motors that pull or push MTOCs to appropriate cellular locations.

Cytoplasmic dynein, the major minus end-directed microtubule-dependent motor in eukaryotic cells, has been postulated to play a role in several aspects of MTOC positioning (for reviews see Holzbaur and Vallee, 1994; Vallee and Sheetz, 1996; Hirokawa et al., 1998). This multisubunit mechanochemical enzyme is composed of two heavy chains and several intermediate and light intermediate chains. The heavy chains are each ~500 kD in size, and their ATPase activity generates the force that results in translocation along microtubules. Biochemical and genetic data indicate that cytoplasmic dynein requires the presence of the multisubunit complex dynactin for proper function (for reviews see Ilan, 1994; Schroer et al., 1996). Cytoplasmic dynein is present throughout the cytoplasm in a punctate manner in most interphase cells, and is enriched at kinetochores during prometaphase, as well as on the spindle during metaphase and anaphase (Pfarr et al., 1990; Steuer et al., 1990; Lin and Collins, 1992; Busson et al., 1998). In polarized MDCK cells, cytoplasmic dynein is enriched in addition at the periphery of nuclei and at discrete cortical sites during metaphase and anaphase (Busson et al., 1998).

The actual role of cytoplasmic dynein in MTOC positioning in complex eukaryotes has not been clearly established. Initial evidence for an involvement came from antibody injection experiments in vertebrate cells (Vaisberg et al., 1993). Injection of function-blocking antibodies against the dynein heavy chain resulted in a failure of centrosome separation. However, in subsequent experiments, injection of mAbs against a dynein light intermediate chain, while perturbing spindle assembly and focusing of

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Abbreviations used in this paper: DIC, differential interference contrast; ds, double-stranded; MTOC, microtubule organizing center; NEB, nuclear envelope breakdown; RNAi, RNA-mediated interference; ss, single-stranded.
spindle poles, did not prevent centrosome separation (Gagliò et al., 1997). A similar outcome was observed when the dynactin complex was inactivated by overexpression of the dynactin component p50/dynamin (Echeverri et al., 1996). A complication in interpreting these apparently contradictory results is that neither antibody injections nor disruption of dynactin necessarily faithfully reflect the consequences of a loss of cytoplasmic dynein function.

An alternative experimental approach has been the investigation of cytoplasmic dynein function in genetic systems, where loss-of-function phenotypes can be examined. In Saccharomyces cerevisiae, null mutations in the genes encoding the dynein heavy chain or a dynactin component result in defective positioning of the spindle pole body at the bud neck during mitosis (Eshel et al., 1993; Li et al., 1993; Clark and Meyer, 1994; Mühua et al., 1994). These mutants undergo normal spindle pole body separation. Moreover, they are viable, demonstrating that cytoplasmic dynein is dispensable in S. cerevisiae. In contrast, cytoplasmic dynein is essential in complex eukaryotes such as Drosophila and mice since animals homozygous for mutations in the dynein heavy chain gene die during early development (Gepner et al., 1996; Harada et al., 1998). A analysis of weak alleles in Drosophila revealed a role for cytoplasmic dynein in spindle orientation during oogenesis, whereas that of homoygous mutant blastocysts in mice confirmed a requirement for localizing the Golgi apparatus to the posterior cortex. Third, the centrosome pair rotates with the associated male pronucleus, away from the posterior cortex. This, the centrosome pair rotates to become oriented onto the longitudinal axis, a prerequisite for proper spindle orientation. It has been shown recently using RNAi that the dynactin components p150\(^{Glued}\) (known as dnc-1 in C. elegans) and p50/dynamin (known as dnc-2) are required for the latter form of centrosome positioning, and, thus, for correct spindle orientation (Skop and White, 1998). However, no other defects in MTOC positioning were observed in one cell stage embryos, suggesting that cytoplasmic dynein may not be required for centrosome separation in complex eukaryotes.

In this study, we used RNAi to directly examine the role of the force producing dynein heavy chain in MTOC positioning. Our data demonstrate that the dynein heavy chain is required for pronuclear migration and centrosome separation in the one cell stage embryo. We also find that p150\(^{Glued}\) and p50/dynamin are required for these processes, in contrast to what has been reported recently (Skop and White, 1998). In addition, we observe that cytoplasmic dynein is involved in maintaining a tight association between centrosomes and male pronucleus. In conjunction with the presence of cytoplasmic dynein at the periphery of nuclei, these results lead us to propose a mechanism in which cytoplasmic dynein, anchored on the nucleus, drives centrosome separation.

### Materials and Methods

#### Antidynein Heavy Chain Antibodies

A peptide predicted to be unique among C. elegans proteins and corresponding to the 19 amino-terminal residues from DHC-1 plus a cysteine (MDSGNESSSL[LPNC]) was synthesized, conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce Chemical Co.), mixed with titer max adjuvant (Boehringer Ingelheim Ltd.), and injected into rabbits at the European Molecular Biology Laboratory animal house according to standard procedures. The third bleed was affinity-purified against a column of sulfolink coupling gel (Pierce Chemical Co.) coupled to the peptide. A anti–DHC-1 antibodies were eluted with 100 mM glycine, pH 2.5, dialyzed against PBS, and concentrated to 0.8 mg/ml in 50% glycerol.

#### Worm Protein Extract and Western Blotting

Worms from mixed developmental stages were floated off four 9-cm petri dishes with H\(_2\)O, spun for 2 min at 2,000 rpm in a tabletop clinical centrifuge, and resuspended for a wash in 30 ml H\(_2\)O. Worms were spun as above, resuspended in 1.5 ml H\(_2\)O, transferred to an Eppendorf tube, and spun for 2 min in a microfuge, yielding a pellet of ~100 \(\mu\)l. 200 \(\mu\)l modified 2X loading buffer (M2LB: 100 mM Tris, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 1 mM PM SF, 10 \(\mu\)g/ml of each leupeptin, pepstatin, and chomostatin) was added to the pellet. The extract was vortexed for 30 s, boiled for 2 min, supplemented with 100 \(\mu\)l M2LB, vortexed for 30 s, boiled for 1 min, and snap-frozen in liquid nitrogen. Cytoplasmic extracts of unfertilized Xenopus eggs arrested in metaphase of meiosis II were prepared according to standard procedures (Murray, 1991).

20–\(\mu\)l C. elegans extract or 1-\(\mu\)l Xenopus extract was loaded per lane on a 6% SDS–acrylamide gel. Proteins were transferred onto nitrocellulose in SD5 gel running buffer containing 10% methanol. A fluor blotting, the filter was incubated for 90 min at room temperature with primary antibodies (1:200 rabbit anti–DHC-1 or 1:1,000 mouse anti-Xenopus dynein heavy chain, a gift from Sigrid R einsch, NA SA A mes Research Center, Moffet Field, CA). Signal detection was performed with standard enhanced chemiluminescence kit components (A mersham Life Science, Inc.).

#### Quantitation of Anti–DHC-1 Reactivity in Wild-type and dhc-1 (RNAi) Embryos

dhc-1 (RNAi) embryos gave rise to a fully penetrant phenotype recognizable by staining with antibubulin antibodies (see Results). Therefore, levels of anti–DHC-1 reactivity were analyzed in wild-type and dhc-1 (RNAi) embryos 30 h after injection and processed on the same slide to eliminate potential slide-specific differences in staining intensities. Early embryos...
(as judged by the DNA stain, <30 nuclei in wild type, and the approximate equivalent in dhc-1 (RNAi)) were examined for antitubulin reactivity. Embryos with a strong antitubulin signal were deemed to be properly fixed and stained, and were retained for subsequent analysis of anti-DHC-1 reactivity. A n-ti-DHC-1 reactivity was imaged with a 4912 Cohu CCD camera set on manual. M ean pixel intensity was determined for each embryo using A dobe Photoshop 4.0, and expressed as a percentage of the average staining intensity of wild-type embryos on each slide. 5–8 of each wild-type and dhc-1 (RNAi) embryos were examined per slide.

**Generation of Double-stranded RNAs**

D ouble-stranded (ds) RNA corresponding to the dynein heavy chain gene dhc-1 (T21E12 12.4) was generated in the following manner. A xZAP II phage containing a 1.3-kb cDNA insert (yk161f11) was obtained from Y uji Ko-hara (National Institute of Genetics, Mishima, Japan). The insert was PCR-amplified from ~2.4 × 10^6 phage particles using primers corresponding to vector sequences flanking the insert and that contain consen-sus sequences for T3 (forward primer) or T7 (reverse primer) RNA pol-ymerses. The PCR product was purified using the QIAquick PCR purification kit (QIagen). A bout 0.5 μg was used as a template in 20 μl T3 and T7 RNA polymerase reactions to generate sense and antisense single-stranded (ss) RNAs (RiboMAX ™ ; Promega Corp.). A fter treatment for 15 min at 37°C with 0.5 U RQ1 DNAse, the RNAs were extracted with phenol/chloroform and resuspended in 20 μl H2O. A n aliquot was run next to RNA standards on a 1% TBE agarose gel to estimate the quality and quantity of RNA generated. Typically, 20–50 μg (1–2.5 μg/μl) of RNA was produced per reaction. To generate dsRNA, equal volumes of sense and antisense ssRNAs were mixed with 1 vol of 3× injection buffer (20 mM KPO4, pH 7.5, 3 mM potassium citrate, pH 7.5, 2% PEG 6000), incubated 10 min at 68°C and 30 min at 37°C. The resulting dsRNA was ali-quot ed, snap-frozen in liquid nitrogen, and stored at ~70°C.

The same batch of yk161f11 dsRNA was used to quantify all the pheno-typic manifestations reported in the text. However, other batches of yk161f11 dsRNA gave identical phenotypes, as did che-3 (RNAi) dsRNAs (as judged by the DNA stain, <30 nuclei in wild type, and the approxi-mate equivalent in dhc-1 (RNAi)) were examined for antitubulin reactivity. Embryos with a strong antitubulin signal were deemed to be properly fixed and stained, and were retained for subsequent analysis of anti-DHC-1 reactivity. A n-ti-DHC-1 reactivity was imaged with a 4912 Cohu CCD camera set on manual. M ean pixel intensity was determined for each embryo using A dobe Photoshop 4.0, and expressed as a percentage of the average staining intensity of wild-type embryos on each slide. 5–8 of each wild-type and dhc-1 (RNAi) embryos were examined per slide.

**Generation of Antibodies Against the Conventional Cytoplasmic Dynein Heavy Chain DHC-1**

W e sought to determine the function of conventional cyto-plasmic dynein in MTOC positioning in the one cell stage C. elegans embryo. There are two cytoplasmic dynein heavy chain genes in the C. elegans genome, dhc-1 (gene T21E12 12.4; Lye et al., 1995) and che-3 (gene F18C12.12). W e focused our analysis on dhc-1 because the corresponding protein is more similar to the vertebrate conventional cyto-plasmic dynein heavy chain M A P 1 C (57% amino acid identity along the entire protein as opposed to 30% for C HE-3). M oreover, a putative null allele of che-3 is viable and has defects restricted to sensory neuron structure and function (Wicks, S., C. de V ries, and R.H.A. Plasterk, personal communication). A ccordingly, we did not observe a phenotype in early embryos when che-3 expression was sil-enced with RNAi (data not shown).

W e began our study by raising polyclonal antibodies against an amino-terminal peptide of DHC-C (see M aterials and M ethods). Afinity-purified anti-DHC-C antibodies recognized two bands on a Western blot of total C. elegans proteins: a very high molecular mass species and a species of ~180 kD (Fig. 1 A ). A ccordingly, we did not observe a phenotype in early embryos when che-3 expression was silenced with RNAi (data not shown).

**Analysis of Embryos by Time-lapse DIC Microscopy and Indirect Immunofluorescence**

W idl-type (N2) adult hermaphrodites were injected bilaterally in the gonads according to standard procedures, and placed at 20°C. A nimals were dissected 24–30 h after injection and their embryos analyzed by time-lapse DIC microscopy (1 frame every 5 s) or indirect immunofluorescence as previously described (Gönczy et al., 1999).

The following primary antibodies were used: 1:100 or 1:200 rabbit anti-DHC-1, 1:100 rabbit anti-ZY G-9 (M athews et al., 1998), 1:5,000 rabbit anti--PGL-1 (K awasaki et al., 1998), and 1:400 mouse antitubulin (clone DM 1 A ; Sigma Chemical Co.). F or peptide blocking experiments, slides were incubated with 0.1 mg/ml DHC-1 peptide, and then in-cubated with anti-DHC-1 antibody in the continued presence of peptide. Secondary antibodies were 1:800 goat anti-mouse A lexa488 (M olecular Probes Inc.) and 1:1,000 donkey anti--rabbit Cy3 (D ianoX). Slides were counterstained with H ochst 33258 (Sigma Chemical Co.) to reveal DNA. A n indirect immunofluorescence data were gathered on a confocal micro-scope (LSM 510; C arl Zeiss). A ll high magnification images are 1.2-μm confocal slices; the stage was refocused slightly between channels in some cases. I mages were processed with A dobe Photoshop 4.0.

**Velocity Measurements of Yolk Granules**

T ime-lapse DIC microscopy was performed at 1 frame every 0.5 s to de-termine the velocity of the fast minus end-directed movements of yolk granules towards the center of asters. In wild type, the focal plane was that of the center of the anterior aster. In dhc-1 (RNAi) embryos, the focal plane included the center of both asters, which are together at the very posterior of these embryos (see Results). The analysis was carried out during the ~ 2 min separating pronuclear envelope breakdown from anaphase in wild-type, when these motility events are most frequent, and the corresponding time interval in embryos in which dhc-1 function was silenced with RNAi (hereafter referred to as dhc-1 (RNAi)).
embryos; see Materials and Methods). As shown in Fig. 1, B and C, 88% of the anti–DHC-1 signal was lost on average in dhc-1 (RNAi) embryos. Residual staining might be due to incomplete silencing of the dhc-1 gene by RNAi. Second, we determined that anti–DHC-1 immunostaining was entirely absent from embryos incubated with anti–DHC-1 antibodies in the presence of 0.1 mg/ml DHC-1 peptide (data not shown). Taken together, these results demonstrate that most, if not all, of the signal detected with anti–DHC-1 antibodies in wild-type embryos is specific for the cytoplasmic dynein heavy chain.

Distribution of Cytoplasmic Dynein in Early C. elegans Embryos

We used anti–DHC-1 antibodies to determine the subcellular distribution of cytoplasmic dynein in early wild-type embryos by immunofluorescence microscopy (Fig. 2). We found that cytoplasmic dynein was present in a punctate manner throughout the cytoplasm at all stages of the cell cycle. In addition, a stronger signal was detected at the periphery of pronuclei in one cell stage embryos (Fig. 2 A, arrow and arrowhead) and of nuclei in later stage embryos (Fig. 2 O, black arrowhead). Moreover, cytoplasmic dynein was present at the cell cortex; this was especially apparent at boundaries between cells, for instance, between the A B and P1 blastomeres of the two cell stage embryo (Fig. 2 O, white arrowheads). The distribution of cytoplasmic dynein changed as cells progressed through mitosis. During prometaphase, cytoplasmic dynein accumulated along both sides of prometaphase chromosomes (Fig. 2, C and D, arrows and arrowhead, respectively). Since chromosomes in C. elegans are holocentric (Albertson and Thomson, 1993), this possibly corresponds to kinetochore staining. During metaphase, cytoplasmic dynein became enriched on the spindle (Fig. 2, G–J). During early anaphase (Fig. 2, K–N), strong spindle signal was still detected, both between segregating chromosomes and spindle poles, as well as centrally (Fig. 2 K, arrow), between the two sets of chromosomes (Fig. 2 L, arrowheads). A similar staining pattern persisted throughout anaphase (Fig. 2 O, arrow). At telophase, cytoplasmic dynein was enriched in two areas of the cytoplasm adjacent to the spindle poles (Fig. 2 O, cell to the left). In addition, a strong signal was detected at the periphery of reforming nuclei (Fig. 2 O, black arrowhead).

A subcellular distribution analogous to the one reported here was observed in C. elegans embryos using polyclonal antibodies raised against purified dynein heavy chain protein (Lye, J., personal communication). This confirms that the distribution described here truly reflects that of dynein heavy chain and not of an unrelated protein.

Minus End–directed Motility of Yolk Granules Is Abolished in dhc-1 (RNAi) Embryos

We wanted to determine if cytoplasmic dynein function is essential in C. elegans. To this end, we specifically silenced the expression of the conventional dynein heavy chain gene dhc-1 using RNAi. Hermaphrodites were injected with dsRNA corresponding to a segment of the dhc-1 gene (see Materials and Methods). Such animals gave rise to 100% dead embryos 20 h or more after injection (n = 268 embryos over three experiments). Thus, dynein heavy chain is essential for C. elegans embryogenesis. In addition, dynein heavy chain is required for fertility, as mature oocytes ceased being produced 35–40 h after injection.

We addressed whether minus end–directed motor activity was indeed abolished in dhc-1 (RNAi) embryos. A manifestation of minus end–directed motility in wild-type one cell stage embryos is the fast movement of yolk granules 0.3–1 μm in diameter towards the center of the asters along linear paths, suggestive of movements along astral microtubules (Fig. 3 A). We determined the average peak velocity of these motility events to be 1.44 μm/s (SD 0.23;
Fig. 3 B), which is in the range of velocities that have been reported for dynein-dependent motility events in other systems (e.g., Paschal et al., 1987). During the ~2 min separating the breakdown of the pronuclear envelopes from anaphase, 10 or more such motility events that lasted at least 2 s could be typically observed in a given focal plane in wild-type embryos.

We investigated whether these fast minus end-directed motility events were altered in dhc-1 (RNAi) embryos. Of the five dhc-1 (RNAi) embryos examined in detail, three displayed no such movement, whereas the remaining two each had a single instance of fast minus end-directed motility event. In contrast to wild-type, however, these two motility events lasted <2 s. The lack of motility events in dhc-1 (RNAi) embryos was not merely due to an absence of astral microtubules, as asters in dhc-1 (RNAi) embryos were observed both by DIC and immunofluorescence microscopy (see below). Lack of motility events was not due either to a general inability of yolk granule movement because the slower posterior-directed flow of yolk granules that occurs in the cytoplasm of wild-type embryos just before pronuclear migration (Hird and White, 1993) was not affected in dhc-1 (RNAi) embryos (Fig. 4 A). Consistent with this observation, segregation of yolk granules towards the posterior of the embryo, which may be driven by this flow (Strome and Wood, 1983; Hird and White, 1993), was also not affected (32/32 one cell stage dhc-1 (RNAi) embryos examined; Fig. 4 B). Taken together, these findings demonstrate that fast minus end-directed motility of yolk granules is specifically abolished in dhc-1 (RNAi) embryos and suggest that cytoplasmic dynein drives this form of cellular transport.
Cytoplasmic Dynein Is Required for Male and Female Pronuclear Migration

To determine the consequences of the loss of cytoplasmic dynein motor activity on MTOC positioning, we examined dhc-1 (RNAi) one cell stage embryos by time-lapse DIC microscopy. This approach is well-suited to examine MTOC positioning because yolk granules are excluded from areas of high microtubule density, such as the center of asters and the spindle, as well as from pronuclei and nuclei.

Fig. 5, A–D, shows the relevant sequence of events in wild type. After fertilization, the two meiotic divisions are completed in the one cell stage embryo. The resulting female pronucleus lies slightly off the anterior cortex (Fig. 5 A, left arrow), whereas the male pronucleus is tightly opposed to the posterior cortex (Fig. 5 A, right arrow). The sperm contributes the single centrosome of the one cell stage embryo (Albertson, 1984; Hyman and White, 1987). After duplication, the two daughter centrosomes separate, while remaining closely associated with the male pronucleus. The separated centrosomes migrate slightly anteriorly, along with the male pronucleus, whereas the female pronucleus migrates posteriorly towards the centrosomes. As a result, the male and female pronuclei meet at ~70% egg length (Fig. 5 B; 0% anterior-most, 100% posterior-most).

We found that dhc-1 (RNAi) one cell stage embryos displayed several striking phenotypes when examined by time-lapse DIC microscopy (Fig. 5, E–H, and Table I). First, dhc-1 (RNAi) embryos often had multiple female pronuclei (Fig. 5 E, three leftmost arrows) and displayed aberrant polar body formation, both indicative of defects during the female meiotic divisions. The role of cytoplasmic dynein during the meiotic divisions is beyond the scope of this work and will not be discussed further here. Second, migration of the male and female pronuclei never took place in dhc-1 (RNAi) embryos (Fig. 5 F). The nuclear envelope of the male pronucleus broke down 1–2
min before that of the female pronuclei (Fig. 5 G). Such asynchrony is characteristic of mutants defective in pronuclear migration (Gönczy et al., 1999). Third, after breakdown of the pronuclear envelopes, a bipolar spindle was not apparent by DIC microscopy in dhc-1 (RNAi) embryos. While an area devoid of yolk granules did extend towards the anterior of the embryo over time (Fig. 5 G, arrow), consistent with an underlying high density of microtubules, no aster was apparent at the anterior end of this area (Fig. 5 G, arrow). Instead, both asters appeared to be located at the very posterior of dhc-1 (RNAi) embryos (Fig. 5 G, arrowheads). Fourth, proper cell division did not occur in dhc-1 (RNAi) embryos (Fig. 5 H). The absence of cleavage furrow specification was expected given the apparent absence of bipolar spindle. While some furrowing activity did take place towards the anterior of the embryo, this rarely resulted in productive cleavage, like in embryos lacking a spindle after nocodazole treatment (Strome and Wood, 1983). Numerous small nuclei reformed in dhc-1 (RNAi) embryos as the cell returned into interphase (Fig. 5 H, arrows), indicative of failure in chromosome segregation. Quicktime movies of a wild-type and dhc-1 (RNAi) one cell stage embryos, PGL-1 is segregated to the posterior. A rowheads point to anteriorly located polar bodies. The wild-type embryo is in prometaphase (arrow points to chromosomes lining up on the metaphase plate), the dhc-1 (RNAi) embryo later in mitosis (arrow points to chromosomes). Embryos were simultaneously stained with antitubulin antibodies (not shown), which revealed the position of centrosomes and unambiguously identified polarity in dhc-1 (RNAi) embryos (Fig. 6). Bar, 10 μm.

Figure 4. Cytoplasmic flows are not affected in dhc-1 (RNAi) embryos. (A) The average peak velocity of posteriorly directed flow of yolk granules during the pseudocleavage stage is indistinguishable in wild type (5.55 μm/min; n = 12 granules in 5 embryos; SD 1.63) and dhc-1 (RNAi) embryos (5.51 μm/min; n = 15 granules in 5 embryos; SD 1.50). These average velocities are slightly higher than those reported previously in wild type (4.4 μm/min; Hird and White, 1993). (B) Wild-type and dhc-1 (RNAi) embryos stained with anti-PGL-1 antibodies to visualize P granules and counterstained with Hoechst 33258 to reveal DNA. All images are at the same magnification. In both wild-type and dhc-1 (RNAi) one cell stage embryos, PGL-1 is segregated to the posterior. A rowheads point to anteriorly located polar bodies. The wild-type embryo is in prometaphase (arrow points to chromosomes lining up on the metaphase plate), the dhc-1 (RNAi) embryo later in mitosis (arrow points to chromosomes). Embryos were simultaneously stained with antitubulin antibodies (not shown), which revealed the position of centrosomes and unambiguously identified polarity in dhc-1 (RNAi) embryos (Fig. 6). Bar, 10 μm.

Cytoplasmic Dynein Is Required for Centrosome Separation

To test whether centrosome separation was indeed defective in dhc-1 (RNAi) embryos, we determined the position of centrosomes by antitubulin staining; in addition, some of the embryos were simultaneously labeled with antibodies against ZYG-9, a centrosomal marker in C. elegans (Matthews et al., 1998).

In prophase, daughter centrosomes have separated to opposite sides of the male pronucleus in wild type (Fig. 6 A, arrowheads). In contrast, in dhc-1 (RNAi) embryos, daughter centrosomes failed to separate and remained positioned posterior of the male pronucleus (Fig. 6 E, arrowheads). After breakdown of the pronuclear envelopes, the two centrosomes were still in close proximity of one another and located at the very posterior of dhc-1 (RNAi) embryos (Fig. 6 M, arrowheads). In contrast to wild type, a bipolar spindle was never observed in dhc-1 (RNAi) embryos, and chromosomes were never located in the very small space between centrosomes (46/46 dhc-1 (RNAi) embryos examined after breakdown of the male pronucleus). Bundles of microtubules up to 20 μm in length emanated from the posterior where the centrosomes were located and extended anteriorly towards a set of chromosomes (Fig. 6, N and O, arrow). These microtubules most likely correspond to the area devoid of yolk granules that had been observed extending towards the anterior by time-lapse DIC microscopy (Fig. 5 G, arrow). These findings suggest that chromosomes from the male pronucleus are pushed towards the anterior by growing microtubules after breakdown of the pronuclear envelope. Importantly, these results demonstrate that cytoplasmic...
Dynactin Components Are Required for Pronuclear Migration and Centrosome Separation

To confirm that the absence of pronuclear migration and centrosome separation were a result of interfering with cytoplasmic dynein function, we examined the phenotype of embryos depleted of dynactin components by RNAi. Dy

nactin has been shown to be required for proper cytoplasmic dynein function in several systems (for reviews see Al-

lan, 1994; Schröer et al., 1996). Therefore, silencing of dynactin components by RNAi in C. elegans might be ex-

pected to result in a similar phenotype to that observed in dhc-1 (RNAi) embryos. Contrary to this prediction, how-

ever, it has been reported that injection of ssRNA corre-

sponding to the dynactin components p150Glued or p50/dyna-

mitin yield embryos that undergo pronuclear migration and form a bipolar spindle (Skop and White, 1998). How-

ever, these embryos may have had residual p150Glued and p50/dynamitin function since ssRNA is much less potent than dsRNA in silencing gene expression (Fire et al., 1998).

Therefore, we tested whether pronuclear migration and centrosome separation were affected after silencing of p150Glued or p50/dynamitin gene expression with dsRNA. As reported in Table I and shown in Fig. 7, A and D, 12/20 p150Glued (dsRNAi) and 8/20 p50/dynamitin (dsRNAi) embryos had a pronuclear migration phenotype indistinguishable from that of dhc-1 (RNAi) embryos by time-lapse DIC microscopy. Like for dhc-1 (RNAi) embryos, no bi-

polar spindle was apparent after breakdown of the pronu-

cleus in these p150Glued (dsRNAi) and p50/dynamitin (dsRNAi) embryos, and both asters remained in close proximity to one another at the very posterior of the embryos (Fig. 7, A and D, arrowheads). Staining with anti-

thubulin antibodies confirmed that centrosomes were close to one another at the very posterior of 13/34 p150Glued (dsRNAi) and 14/24 p50/dynamitin (dsRNAi) embryos examined after breakdown of the male pronucleus (Fig. 7, B and E, arrowheads). The remainder of p150Glued (dsRNAi) and p50/dynamitin (dsRNAi) embryos had milder phenotypes, resembling in part those obtained after injections of single-stranded material (Skop and White, 1998; Table I). The fact that some p150Glued (dsRNAi) and p50/dynamitin (dsRNAi) embryos underwent pronuclear migration and centrosome separation may be because of incomplete gene silencing, even by dsRNA. Importantly, these results demonstrate that the dynactin components p150Glued and p50/dynamitin are required, at least in part, for pronuclear migration and centrosome separation in the one cell stage C. elegans embryo.

Figure 5. Failure of pronuclear migration in dhc-1 (RNAi) embryos. Time-lapse DIC microscopy recordings of wild-type (A–D) and dhc-1 (RNAi) embryos (E–H). Time elapsed since the beginning of the sequence is displayed in minutes and seconds in each image. All images are at the same magnification. (A and E) In both wild-type and dhc-1 (RNAi) embryo, the male pronucleus is apposed to the posterior cortex (A and E, rightmost arrow). In wild-type, there is a single female pronucleus located slightly off the anterior cortex (A, leftmost arrow). In contrast, there are five female pronuclei in the dhc-1 (RNAi) embryo (E, arrows towards the left point at three that are visible in this focal plane). Note the pseudocleavage furrow in the middle of both wild-type and dhc-1 (RNAi) embryos. Female pronuclei in some dhc-1 (RNAi) embryos were located towards the middle of the embryo (not shown). (B and F) In wild type, after migration of both male and female pronuclei, the pronuclei have met and move along with the centrosome pair (B, arrowheads) towards the center while undergoing a 90° rotation. In contrast, neither male nor female pronuclei migrate in the dhc-1 (RNAi) embryo. (C and G) In wild type, the spindle sets up in the cell center and along the longitudinal axis (C, arrowheads point to spindle poles). In the dhc-1 (RNAi) embryo, no bipolar structure is visible after nuclear envelope breakdown. However, an area devoid of yolk granules extends towards the anterior of the embryo (arrow in G points to anterior of this area). The asters appear to be at the very posterior of the embryo (G, arrowheads). Note that the membranes of the female pronuclei are still intact after the male pronuclear membrane broke down. (D and H) In wild type, the first cleavage generates two unequally sized daughters, each with a centrally located nucleus (D, arrows). In contrast, no proper cell division occurs in the dhc-1 (RNAi) embryo. While some furrowing activity does take place, this is usually re-

stricted to the anterior and does not result in productive cleavage. Numerous small nuclei reform, presumably around nonsegregated chromosomes, as the cell returns into interphase (H, arrows). Bar, 10 μm.
Cytoplasmic Dynein Is Required, in part, for Maintaining Association between Centrosomes and Pronuclei

Our observations of dhc-1 (RNAi) embryos with time-lapse DIC microscopy and indirect immunofluorescence revealed that cytoplasmic dynein is also involved in the mechanisms that maintain centrosome association with nuclei.

In wild-type one cell stage embryos, the separated daughter centrosomes are initially tightly associated with the male pronucleus, and with both pronuclei after pronuclear meeting. This tight association is apparent by DIC microscopy because yolk granules are excluded both from pronuclei and the center of asters (Fig. 8A, arrows and arrowheads, respectively), as well as by staining with antibodies against tubulin or the centrosomal marker ZYG-9 (Fig. 8B, arrowheads) and counterstaining with Hoechst 33258 to visualize DNA (Fig. 8B, arrow).

While the majority of dhc-1 (RNAi) embryos maintained association between the unseparated centrosomes (Fig. 8, C and D, arrowheads) and the male pronucleus (Fig. 8, C and D, arrow), this was not always the case. In ~15% of dhc-1 (RNAi) embryos (7/45 embryos in prophase analyzed by antitubulin antibodies and Hoechst 33258), the centrosomes were not in the immediate vicinity of the male pronucleus (Fig. 8, E and F, arrow), but instead located 3–11 μm away (average 6.1 μm, SD 2.63; Fig. 8, E and F, arrowheads). In addition, we noted that centrosomes remained at the posterior cortex in these embryos (Fig. 8, E and F, arrowheads), even though the male pronucleus was not present anterior to them. This suggests that cytoplasmic dynein is required for movement of centrosomes away from the posterior cortex.

These results indicate that cytoplasmic dynein is required, at least in part, for proper association between centrosomes and the male pronucleus in the one cell stage C. elegans embryo. Cytoplasmic dynein appears to play a role in maintaining this association, rather than in establishing it, because asters initially in close proximity to the male pronucleus can be observed drifting away in time-lapse DIC recordings of dhc-1 (RNAi) embryos (data not shown).

Table I. Summary of Time-lapse DIC Microscopy Analysis

| Embryos with one female pronucleus | Average number of multiple female pronuclei* | Defect in pronuclear migration (no bipolar spindle)** | Defect in pronuclear migration (with bipolar spindle)** | No apparent defect in one cell stage embryo |
|----------------------------------|---------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| N2                               | 20                                          | NA                                           | NA                                           | 20†‡‡                                           |
| dhc-1 (RNAi)                     | 20                                          | 3 (SD, 0.95)                                  | 20**                                          | —                                              |
| p150(dia) (RNAi)                 | 20                                          | 2 (SD, 0)                                    | 6**                                          | —                                              |
| p50/dynamitin (RNAi)             | 19                                          | 2 (SD, 0)                                    | 8**                                          | 1                                              |

*Average number of multiple pronuclei among those embryos with multiple female pronuclei. Multiple female pronuclei can be of different sizes; in particular, some are smaller than normal, indicating that they do not contain a full complement of chromosomes.
†Typical phenotype: both male and female pronuclei fail to migrate. After breakdown of the male pronucleus at the posterior of the embryo, no bipolar spindle is apparent. Consistent with the absence of a functional spindle, there is no cleavage furrow ingress from the posterior of the embryo.
‡Typical phenotype: both male and female pronuclei fail to migrate. After breakdown of the male pronucleus at the posterior of the embryo, a bipolar spindle is assembled, although it is typically less apparent than in wild type. Consistent with the presence of a functional spindle, a cleavage furrow ingresses from the posterior of the embryo.
‡‡In one embryo, rotation did not take place, while it was incomplete in another one. However, centration was normal in both cases.
**In one embryo, the spindle poles became separated by 2–3 microns by the end of mitosis, although no spindle was apparent between them; probably as a consequence of this separation, a cleavage furrow ingressed from the very posterior of the embryo. In two embryos, the centrosomes were located a few microns away from the male pronucleus; in one of these cases, the two centrosomes were separated to some extent.

In two embryos, progress through mitosis seemed affected. In one case, the cell spent about 25 min in mitosis (measured from breakdown of the male pronucleus to the reformation of small nuclei), as opposed to approximately 4 min in wild type. In the other case, the cell stayed in mitosis for at least 23 min, after which the recording was interrupted.

In one embryo, progress through mitosis seemed affected, as the cell stayed in mitosis for at least 12 min, after which the recording was interrupted.

In three embryos, the female pronucleus underwent part of its migration, in the absence of male pronuclear migration. In one embryo, the clustered centrosomes were located a few microns away from the male pronucleus. Another embryo spent about 21 min in mitosis.

In one embryo, rotation took place in the absence of centration.

Cytoplasmic Dynein Is Required for Proper Spindle Orientation in the One Cell Stage Embryo

We wanted to test whether cytoplasmic dynein is required for the positioning of centrosomes onto the longitudinal axis that leads to proper spindle orientation in the one cell stage C. elegans embryo. However, the lack of centrosome separation in dhc-1 (RNAi) embryos precludes addressing this question because of the resulting absence of spindle assembly. Therefore, we sought to generate weaker phenotypes with RNAi to bypass the early requirement for centrosome separation.

Weaker phenotypes were produced by injecting undiluted ssRNA and examining embryos 12–16 h after injection or by injecting 16-fold diluted ssRNA and examining embryos 24–30 h after injection. The resulting dhc-1 (ssRNAi) embryos reproducibly fell into one of three broad phenotypic classes, corresponding to the equivalent of an allelic series. First, embryos that were wild type. In these cases, the RNAi effect was probably too weak to significantly deplete dynein heavy chain. Second, embryos that had phenotypes akin to those obtained after injection of double-stranded material. In these cases, the RNAi effect was probably strong enough to deplete a substantial fraction of dynein heavy chain. Third, embryos that exhibited milder phenotypes that probably resulted from intermediate dim-

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In wild type, the centrosome pair is positioned at 70% egg length and transverse to the longitudinal axis after pronuclear meeting (Fig. 9 A, arrowheads). The centrosome pair and associated pronuclei subsequently move to the embryo center while undergoing a 90° rotation (Fig. 9 B, arrowheads). As a result, after breakdown of the pronuclear envelopes, the spindle is positioned in the cell center and oriented along the longitudinal axis (Fig. 9 C, arrowheads).

We found that the third class of dhc-1 (ssRNAi) embryos underwent pronuclear migration as in wild type (compare Fig. 9, A and D), but failed to undergo subsequent centration and rotation of centrosomes (compare Fig. 9, B and E). As a result, the spindle was set up at ~70% egg length, perpendicular to the longitudinal axis (Fig. 9 E). However, the spindle was typically rescued onto the embryo center while remaining associated with the male pronucleus; ZYG-9 is also present in the cytoplasm and polar bodies (small arrowheads). (B) Astral microtubules emanate from the centrosomes; the mesh of cortical microtubules is also visible. (C) DNA of both male (arrow) and female (out of focus, arrowhead) pronuclei is condensing; small arrowheads point to polar body material. (E–F) dhc-1 (RNAi), prophase. (E) Daughter centrosomes (arrowheads) fail to separate from one another and are posterior of the male pronucleus. (F) Some astral microtubules are fairly long (arrow). (G) DNA of both the male (arrow) and the three female (arrowheads) pronuclei is condensing. (I–L) Wild type, anaphase. (I) The two spindle poles (arrowheads) have moved away from each other during anaphase B. (J) Numerous and long astral microtubules extend from the spindle poles towards the anterior and posterior cortices; spindle microtubules extend centrally. (K) The two sets of chromosomes segregate towards the spindle poles; small arrowheads point to polar body material. (M–P) dhc-1 (RNAi); after NEB. (M) Centrosomes (arrowheads) are still in close proximity of one another. (N) No bipolar spindle is assembled; astral microtubules seem to grow preferentially towards chromosomes or be stabilized in their vicinity (arrow); such microtubules are directed towards chromosomes coming presumably from the male pronucleus in most dhc-1 (RNAi) embryos. (O) Condensed chromosomes coming most likely from the male pronucleus (arrow) and the female pronucleus (arrowhead) are visible. Small arrowhead points to laterally positioned polar body material. In 1/46 dhc-1 (RNAi) embryo after NEB, the two centrosomes were separated from one another; a bipolar spindle was not apparent in this case either. Bar, 10 μm.

Figure 6. Failure of centrosome separation in dhc-1 (RNAi) embryos. Embryos stained with anti-ZYG-9 and antitubulin (TUB) antibodies and counterstained with Hoechst 33258 to reveal DNA. Images in first two columns: early, before nuclear envelope breakdown (NEB); and prophase. Images in last two columns: late, after NEB. Merged images: ZYG-9, red; TUB, green; and DNA, blue. All images are at the same magnification. (A–D) Wild type, prophase. (A) ZYG-9 labels the two centrosomes (arrowheads), which have separated from one another while remaining associated with the male pronucleus; ZYG-9 is also present in the cytoplasm and polar bodies (small arrowheads). (B) Astral microtubules emanate from the centrosomes; the mesh of cortical microtubules is also visible. (C) DNA of both male (arrow) and female (out of focus, arrowhead) pronuclei is condensing; small arrowheads point to polar body material.
the longitudinal axis by the end of anaphase, presumably because of the physical constraints of the eggshell (Fig. 9 F). An identical phenotype has been reported previously for p150Glued (ssRNAi) and p50/dynamitin (ssRNAi) embryos (Skop and White, 1998), and was observed in this study for some p150Glued (dsRNAi) and p50/dynamitin (dsRNAi) embryos (Table I). These results demonstrate that cytoplasmic dynein, like dynactin components, is required for centration/rotation of centrosomes and, thus, proper spindle orientation, in the one cell stage C. elegans embryo.

Discussion

By using RNAi, we have demonstrated that cytoplasmic dynein is required for all three major aspects of centrosome positioning that occur in the one cell stage C. elegans embryo: centrosome separation, movement of centrosomes away from the posterior cortex accompanying male pronucleus migration, and subsequent positioning of centrosomes onto the longitudinal axis. In addition, we found that cytoplasmic dynein is required for female pronuclear migration and plays a role in maintaining association between centrosomes and male pronucleus.

Using RNAi to Analyze the Function of Cytoplasmic Dynein

The function of cytoplasmic dynein in MTOC positioning in complex eukaryotes has not been unambiguously determined in the past, owing largely to experimental difficulties associated with loss-of-function studies. Both in Drosophila and mice, cells bearing strong mutations in the heavy chain gene fail to proliferate or survive (Gepner et al., 1996; Harada et al., 1998), severely hampering investigation of cytoplasmic dynein function. Such difficulties can be circumvented by using RNAi in C. elegans. Germ cells targeted by RNAi undergo no divisions between the time of injection and fertilization. Therefore, even if cytoplasmic dynein is essential for an aspect of cell division, this alone cannot interfere with analyzing its function in the one cell stage embryo. Cytoplasmic dynein does play a role during the meiotic divisions that take place shortly after fertilization, since dhc-1 (RNAi) one cell stage embryos often possess multiple female pronuclei. However, this does not prevent scoring cell division processes in the remainder of the first cell cycle, and cannot explain the subsequent defects of centrosome positioning. Indeed, the same defects are observed in those dhc-1 (RNAi) embryos that have a single female pronucleus. Conversely, centrosome positioning defects are not apparent in a number of mutant strains with multiple female pronuclei (Gönczy et al., 1999).

One potential limitation of using RNAi resides in the possibility that the component under study is also required to generate mature oocytes, in which case function in the one cell stage embryo may not be assessed. In fact, cytoplasmic dynein does play some role in gametogenesis, since oocyte production ceases 35–40 h after injection of dhc-1 dsRNA. Nonetheless, this has not hampered our analysis, because reproducible phenotypes were observed in one cell stage embryos 24–32 h after injection. Therefore, RNAi in C. elegans offers an excellent opportunity to analyze the in vivo requirements of cytoplasmic dynein in MTOC positioning in a complex eukaryote.
Cytoplasmic Dynein May Be Generally Required for Centrosome Separation in Metazoans

Our results unequivocally establish that cytoplasmic dynein and dynactin are required for centrosome separation in the one-cell stage C. elegans embryo. A similar conclusion had been reached for cytoplasmic dynein from experiments in vertebrate cells that made use of function-blocking antibodies (Vaisberg et al., 1993). However, subsequent studies suggested that dynein and dynactin are not needed for centrosome separation (Echeverri et al., 1996; Gaglio et al., 1997). These apparently conflicting data may be reconciled if the majority of cells in the later studies retained sufficient motor activity to permit centrosome separation and proceed through to subsequent stages of mitosis, where there may be a higher requirement for dynein and dynactin function. Interestingly, cytoplasmic dynein is not required for MTOC separation in S. cerevisiae (Eshel et al., 1993; Li et al., 1993). Therefore, whereas many cell division processes have been conserved throughout evolution of eukaryotic cells, the use of cytoplasmic dynein to separate MTOCs may be specific to metazoans. Perhaps different mechanisms of MTOC separation have been imparted by the fact that spindle pole bodies are embedded in the nuclear envelope, in contrast to centrosomes that are simply associated with nuclei.

Mechanisms of Dynein-dependent Separation of Centrosome

Two conditions must be met for proper centrosome separation to take place in complex eukaryotes. First, centrosomes must move until they are diametrically opposed on the nucleus. Second, separating centrosomes must remain tightly associated with the nucleus. Two types of mechanisms have been invoked to explain centrosome separation. In one, separation results from pushing forces acting on overlapping antiparallel microtubules emanating from the two centrosomes. Plus end-directed motors are expected to generate the force driving separation in this case. The requirement for plus end-directed kinesins like Xklp2 in centrosome separation lends support to this view (Boleti et al., 1996). A minus end-directed motor such as cytoplasmic dynein may still be essential in this scenario by transporting effector molecules like Xklp2 (Wittman et al., 1998). However, this type of mechanism requires the existence of an extranuclear spindle during centrosome separation, which has not been observed in several vertebrate cells or in the C. elegans embryo (Roos, 1973; Rattner and Berns, 1976; Bajer and Ole-Bajer, 1982; Alpertson, 1984; Hyman and White, 1987). Moreover, this type of mechanism predicts that centrosomes move apart in a coordinated fashion, whereas there is evidence to the con-
nuclei fail to migrate in...generate force comes from our discovery that female...astral microtubules can...evidence that interactions between cytoplasmic...how they remain tightly associated with the nucleus. This model is attractive because it provides a single mechanism to explain both how centrosome separate and...work). This model is attractive because it provides a single mechanism involving length-dependent forces. In this scenario, the minus ends of astral microtubules, along with the centrosome, are pulled when they encounter anchored cytoplasmic dynein on the nucleus. Longer astral microtubules encounter more anchored motors and, thus, experience a stronger pulling force than shorter ones. After centrosome duplication, microtubules extending away from the centrosomes along the nucleus are long, whereas those projecting towards the other centrosome are short. Thus, length-dependent forces could ensure that centrosomes move away from each other until such pulling forces are balanced, which occurs when they are diametrically opposed. In this model, the initial position of daughter centrosomes after duplication determines the final position of separated centrosomes.

Why would centrosomes in the model presented in Fig. 10 move apart until they are diametrically opposed to one another? The role of cytoplasmic dynein suggests a possible mechanism involving length-dependent forces. In this scenario, the minus ends of astral microtubules, along with the centrosome, are pulled when they encounter anchored cytoplasmic dynein on the nucleus. Longer astral microtubules encounter more anchored motors and, thus, experience a stronger pulling force than shorter ones. After centrosome duplication, microtubules extending away from the centrosomes along the nucleus are long, whereas those projecting towards the other centrosome are short. Thus, length-dependent forces could ensure that centrosomes move away from each other until such pulling forces are balanced, which occurs when they are diametrically opposed. In this model, the initial position of daughter centrosomes after duplication determines the final position of separated centrosomes.

Such a mechanism for centrosome separation would simultaneously ensure association between separating centrosomes and the nucleus. The nature of the association between centrosomes and nuclei is poorly understood. It has been postulated that organelle-like motility of nuclei along microtubules may serve to maintain this association (Reinsch and Gönczy, 1998). The presence of cytoplasmic dynein on nuclei in MDCK cells and in C. elegans is compatible with this postulate (Busson et al., 1998; this work). Importantly, our finding that the association between centrosomes and male pronucleus is lost in some dhc-1 (RNAi) embryos provides the first evidence that cytoplas-
Cytoplasmic dynein and dynactin are also required for proper orientation of the spindle at the bud neck in S. cerevisiae (Eshel et al., 1993; Li et al., 1993; Clark and Meyer, 1994; Muhua et al., 1994). It has been proposed that dynactin at the bud cortex tethers cytoplasmic dynein, which captures astral microtubules and translocates the associated spindle pole body by minus end–directed motility towards the bud neck (for review see G önczy and Hyman, 1996). An analogous cortical capture mechanism could account for centration/rotation in the one cell stage C. elegans embryo, if cytoplasmic dynein were tethered somewhere in the anterior of the embryo. We have shown that cytoplasmic dynein is present at the cortex of one cell stage embryos, albeit at low levels. Since p150Glued is enriched at the site of polar body extrusion, at the very anterior cortex (Skop and White, 1998), it is formally possible that cytoplasmic dynein at this site mediates centration/rotation. However, we think this is unlikely because this process still occurs in rare embryos in which polar bodies are positioned away from the anterior cortex (Gönczy, P., unpublished observations). Evidence from S. cerevisiae suggests an alternative to the cortical capture model. In this organism, microtubule dynamics are affected in dynein heavy chain mutants, and the average length of microtubules is altered in mutants of other motor proteins that play a role in spindle orientation (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Shaw et al., 1997). While the exact role of microtubule dynamics in S. cerevisiae spindle orientation remains to be determined, these observations raise the possibility that cytoplasmic dynein could similarly influence microtubule dynamics in C. elegans. This, in turn, may be responsible for some of the phenotypic manifestations reported in this work, including improper spindle orientation in the one cell stage embryo.

Spindle Orientation in P1 with Reduced Cytoplasmic Dynein or Dynactin Function

Separated centrosomes in the P1 blastomere of the two cell stage C. elegans embryo also undergo a 90° rotation that aligns them along the longitudinal axis. It has been suggested that P1 rotation also results from a cortical capture mechanism (Hyman and White, 1987). In this case, laser microsurgery experiments identified the requirement for a discrete cortical site, which overlaps with the cell division remnant generated after cleavage of the one cell stage embryo (Hyman, 1989). The dynactin components actin capping protein and p150Glued are enriched at this site, lending support to the hypothesis that cytoplasmic dynein anchored at this site drives P1 rotation (Waddle et al., 1994; Skop and White, 1998). Compatible with this view, we found that cytoplasmic dynein is present all along the cortex in the P1 blastomere, including the cortical site.

However, despite this correlative finding, we could...
not assess the role of cytoplasmic dynein in P1 rotation with certainty. While P1 rotation was defective in some dhc-1 (ssRNAi) embryos (Gönczy, P., S. Pichler, and M. Kirkham, unpublished observations), this may not reflect a direct requirement for cytoplasmic dynein function. Indeed, the first cleavage furrow typically ingresses sooner on one side of dhc-1 (ssRNAi) embryos after rescue of spindle orientation onto the longitudinal axis (Fig. 9 F). A s a result, the cortical site is predicted to be eccentrically located. A similar asynchronous ingestion of the first cleavage furrow has been observed in weak p150
\textsuperscript{Lue} (RNAi) and p50/dynamitin (RNAi) embryos (Skop and White, 1998). In this case, eccentric location of the cortical site has been directly demonstrated by anti-p150
\textsuperscript{Lue} staining in p50/dynamitin (ssRNAi) embryos (Skop and White, 1998). Therefore, we suggest that the failure of P1 rotation with diminished cytoplasmic dynein or dynactin function may result from the eccentric location of the cortical site caused by asynchronous ingestion of the first cleavage furrow. We believe that demonstration of a direct requirement of cytoplasmic dynein and dynactin function in P1 rotation awaits further experiments, including the use of temperature-sensitive alleles or local inactivation of protein function. The development of such experimental approaches will be important to further dissect the requirement of cytoplasmic dynein in C. elegans and other metazoans where RNAi is not available.

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Note Added in Proof. Defects in centrosome separation and in the association between centrosomes and nuclei also have been recently described in Drosophila embryos with reduced cytoplasmic dynein function (Robin-son, J.T., E.J. Wojcik, M.A. Sanders, M. M. Grail, and T.S. Hays. J. Cell Biol. 146:597–608).

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