The dynactin complex is required for cleavage plane specification in early *Caenorhabditis elegans* embryos

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Background: During metazoan development, cell diversity arises primarily from asymmetric cell divisions which are executed in two phases: segregation of cytoplasmic factors and positioning of the mitotic spindle – and hence the cleavage plane – relative to the axis of segregation. When polarized cells divide, spindle alignment probably occurs through the capture and subsequent shortening of astral microtubules by a site in the cortex.

Results: Here, we report that dynactin, the dynein-activator complex, is localized at cortical microtubule attachment sites and is necessary for mitotic spindle alignment in early *Caenorhabditis elegans* embryos. Using RNA interference techniques, we eliminated expression in early embryos of *dnc-1* (the ortholog of the vertebrate gene for p150^{Glued}) and *dnc-2* (the ortholog of the vertebrate gene for p50/Dynamitin). In both cases, misalignment of mitotic spindles occurred, demonstrating that two components of the dynactin complex, DNC-1 and DNC-2, are necessary to align the spindle.

Conclusions: Dynactin complexes may serve as a tether for dynein at the cortex and allow dynein to produce forces on the astral microtubules required for mitotic spindle alignment.

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Background

In multicellular organisms, the generation of cell diversity often involves asymmetric cell divisions [1–8]. We define an asymmetric division in this context as one that gives rise to daughters that differ in developmental potential. The daughters of an asymmetric division also often differ in size. In animal cells, the mitotic spindle specifies the position of the cleavage furrow; the furrow forms orthogonally to the spindle, midway between the spindle poles thus bisecting the mitotic apparatus [9]. Therefore, the orientation and eccentricity of the mitotic spindle can influence cell size and partitioning of segregated developmental determinants.

The asymmetric and invariant cleavage patterns during embryogenesis in the nematode *Caenorhabditis elegans*, provide an opportunity to study the mechanisms involved in positioning the mitotic spindle [10]. The early cell-division patterns are established by the position of the centrosomes at each division [5,6]. In the zygote, P_0, the egg pronucleus migrates toward the sperm pronucleus at the posterior of the embryo. The two apposed pronuclei then move towards the center of the embryo and, during this time, the centrosome–pronuclear complex rotates such that the spindle is aligned on the anteroposterior (AP) axis. The resulting asymmetric division gives rise to the AB and P_1 blastomeres. At the two-cell stage, there are two different types of division that will occur, proliferative (AB blastomere) and determinative (P_1 blastomere). In both AB and P_1, the centrosomes will duplicate and migrate to opposite poles of the nucleus. In the P_1 blastomere, however, the entire centrosome–nucleus complex rotates 90° on to the longitudinal axis and an asymmetric division occurs. This movement, however, does not occur in the AB blastomere and a symmetric division occurs.

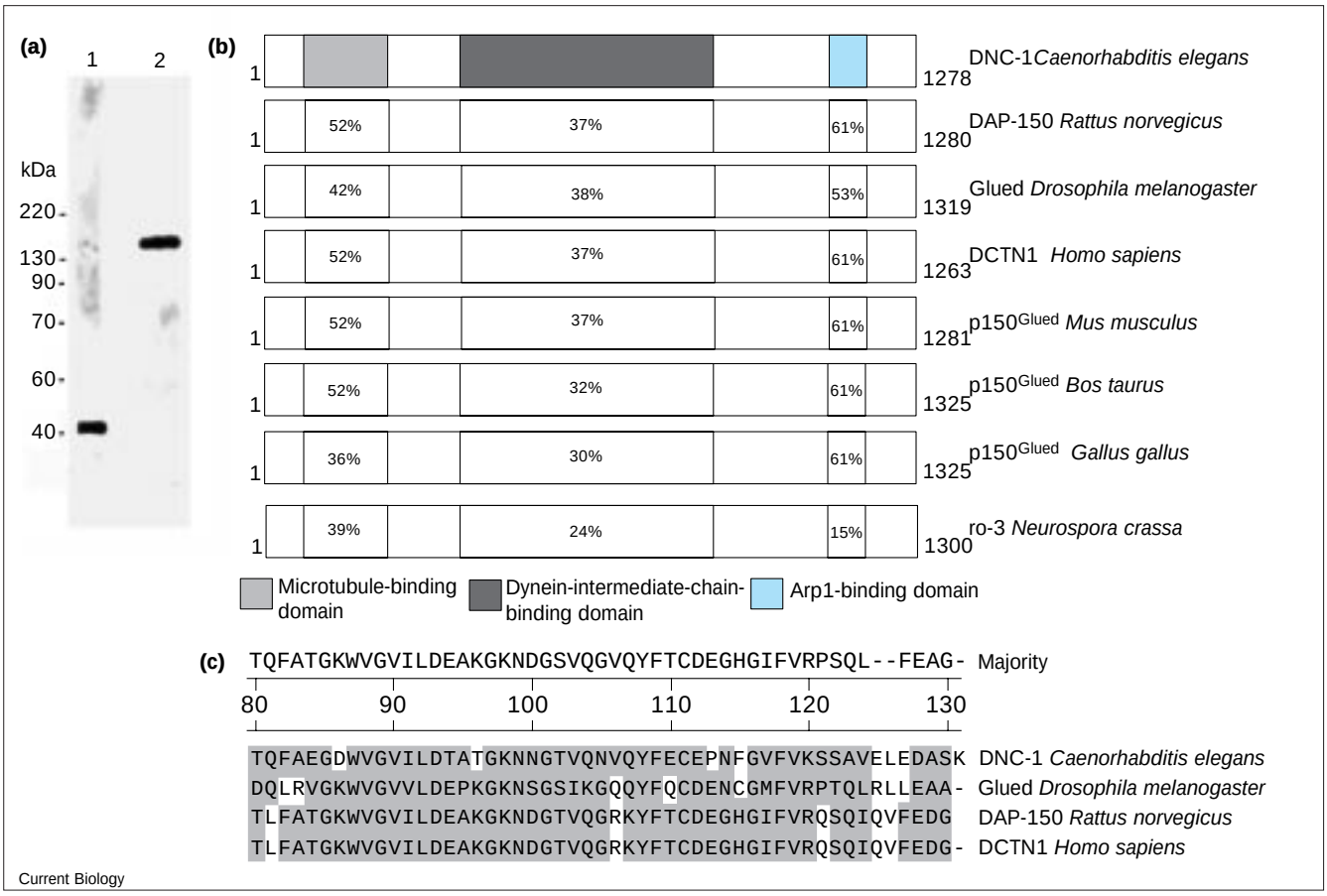
In a variety of organisms, alignment of the spindle probably occurs by the attachment of astral microtubules to a defined site in the cortex [1,4–6,11–13]. In *C. elegans*, previous studies have indicated that both astral microtubules and actin play a role in maintaining proper spindle orientation [5,6]. Laser ablation of regions between centrosomes and a cortical site in the anterior cortex of P_1 prevents the spindle from aligning properly [6]. Microtubule and microfilament inhibitors also disrupt the asymmetric position of the spindle in P_0 and P_1 [5,14]. These experiments suggest that astral microtubules and the actin cytoskeleton both play a role in proper spindle alignment.

Evidence from *Saccharomyces cerevisiae*, *Drosophila*, and *Aspergillus* suggest that dynein and dynactin complexes are required for spindle orientation and nuclear positioning [11,15–18]. Dynein–dynactin complexes might link the cortex to the distal ends of the microtubule and would be responsible for generating the pulling force on astral microtubules. In *C. elegans*, localization of actin and

actin-capping protein, a subunit of the dynactin complex, to putative cortical microtubule attachment sites suggested that dynein–dynactin complexes might be involved in aligning the mitotic spindle in *C. elegans* embryos, specifically in cells that divide asymmetrically [13]. Before this study, the intracellular localization of other dynactin components and their requirement for spindle alignment had not been shown.

Here, we present data demonstrating that dynactin complexes are involved in spindle alignment in polarized cells of early *C. elegans* embryos. We have taken a reverse genetic approach, using RNA interference (RNAi) techniques, to identify proteins that might be involved in the force-producing mechanisms required to orient spindles in polarized cells in the early embryo. Our observations suggest that two components of the dynactin complex, DNC-1 and DNC-2, are involved in spindle alignment in polarized cells in the early *C. elegans* embryo and that DNC-1 co-localizes with late furrows and, ultimately, cell-division remnants. We demonstrate that in *dnc-1* and *dnc-2* RNAi-treated embryos, a complete failure of rotation events in the P_0 and P_1 blastomeres occurs, indicating that the dynactin complex is necessary to align the spindle in the early *C. elegans* embryo.

Figure 1



(a) Specificity of the anti-rat p150^{Glued} polyclonal antibody in whole *C. elegans* protein extract. Lane 1 was probed with an anti-actin antibody and shows an ~42 kDa band. Lane 2 was probed with the anti-rat p150^{Glued} antibody and shows a single band of ~140–150 kDa. The presence of a single immunoreactive band of 150 kDa is characteristic of p150^{Glued} isolated from brain extracts of various vertebrate species [19,20]. The anti-p150^{Glued} antibody was raised against the first 200 amino acids of rat p150^{Glued}, corresponding to the well-conserved microtubule-binding domain; see (c). In all figure legends, p150^{Glued} staining will be referred to as DNC-1 staining. (b) Comparison of *C. elegans* DNC-1 with other p150^{Glued} protein sequences. The proteins share 19–23% sequence identity throughout, but a higher degree of conservation in the microtubule-binding, dynein-intermediate-chain-binding and the Arp1-binding domains. The percentage of amino-acid identity in the specific regions is indicated. The protein sequences are all drawn uniformly for ease of comparison. There are slight differences in both size and spacing of the domains. Accession numbers for the sequences are as follows: *C. elegans*, 1184606; *R. norvegicus*, 2506256; *D. melanogaster*, 118962; *H. sapiens*, 2493480; *M. musculus*, 2104495; *B. taurus*, 108456; *G. gallus*, 544198; *N. crassa*, 2493479. Sequences were obtained from GenBank and aligned using MegAlign (DNASTAR). (c) Comparison of the microtubule-binding domain in DNC-1 with other p150^{Glued} orthologs. The shading represents homology.

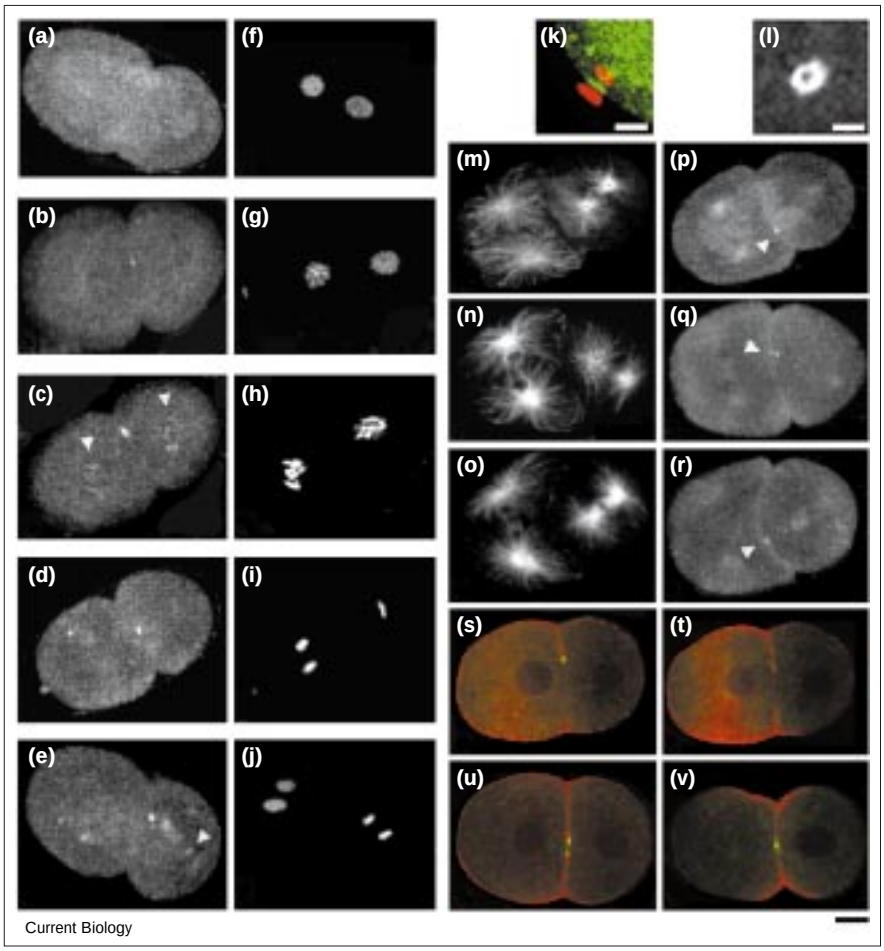
Results and discussion

The specification of the orientation of the cleavage plane is a crucial aspect of cell division as it defines the manner in which cellular components are segregated. In polarized cells of the early embryo, a localized region in the cortex acts to align the centrosome–nucleus complex on to the proper axis, apparently through the capture and subsequent shortening of microtubules [6]. We report here that the dynactin complex localizes to cell-division remnants and is involved in aligning the mitotic spindle in polarized cells in early *C. elegans* embryos.

A polyclonal antibody raised against the well-conserved microtubule-binding domain (Figure 1c) of rat p150^{Glued} (a gift from Kevin Vaughan and Richard Vallee) reacted with a single band of molecular mass 150 kDa in whole *C. elegans* extracts, as shown by immunoblots (Figure 1a). The presence of a single immunoreactive band of 150 kDa is characteristic of p150^{Glued} isolated from brain extracts of various vertebrate species [19,20]. The antigen detected by the anti-p150^{Glued} antibody in *C. elegans* will be referred to as DNC-1.

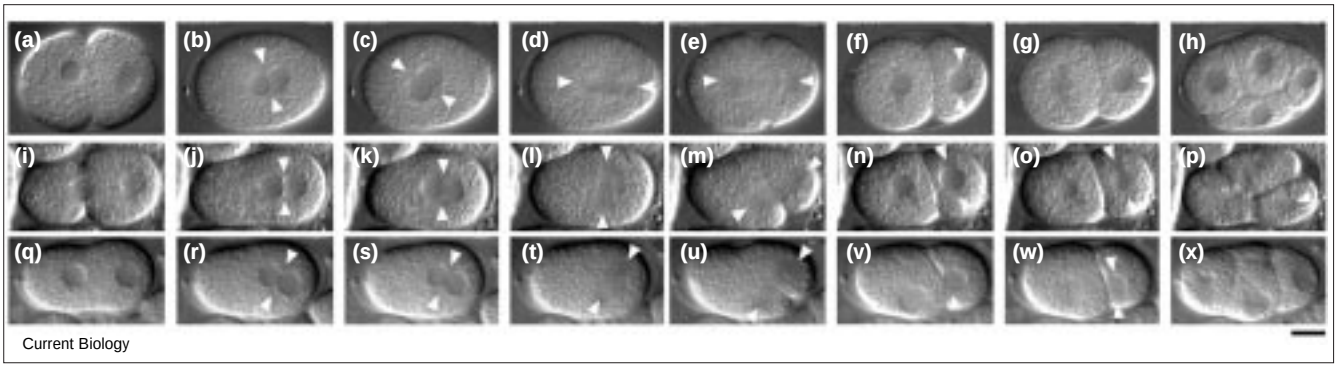
Staining of DNC-1 in one-cell and two-cell embryos revealed a striking accumulation at the cell-division remnant (also known as the residual body or midbody). Centrosomal and kinetochore staining was also seen, which is consistent with the roles of the dynactin complex in spindle and kinetochore function [21]. In addition, cytoplasmic staining was seen. Initially, the accumulation of DNC-1 at cell-division remnants appeared upon completion of the first meiosis (Figure 2k) during polar-body formation. This spot persisted until the end of the first mitosis. DNC-1 appeared at the leading edge of the cleavage furrow

Figure 2



Immunostaining of DNC-1, DNA, tubulin and actin in early *C. elegans* embryos. (a–j) Localization of (a–e) DNC-1 and (f–j) DNA in two-cell embryos. (a,f) Telophase in the first cell cycle (the discrete spot of DNC-1 has not become apparent at this time). (b,g) Early prophase in the second cell cycle. (c,h) Prometaphase (arrowheads in (c) mark kinetochore staining in both blastomeres). (d,i) Anaphase in the AB blastomere and metaphase in the P_1 blastomere (the spot in AB corresponds to the pericentrosomal staining that is sometimes seen). (e,j) Telophase in AB and anaphase in P_1 (arrowhead in P_1 marks the DNC-1 staining of the spindle midzone; centrosomal staining can also be seen at the spindle poles). (k) One-cell embryo at the completion of meiosis I stained for DNC-1 (green) and DNA (red). DNC-1 accumulated at the cell-division remnant. The DNC-1 staining persists through the two-cell stage (data not shown). (l) Ring of DNC-1 formed at late stages of cytokinesis between blastomeres. (m–r) Double labeling of (m–o) tubulin and (p–r) DNC-1 in two-cell embryos. All embryos are at the same age. Notice that in each embryo the spindle in the P_1 blastomere aligns towards the spot of DNC-1 (arrowheads) regardless of the orientation. (s–v) Double labeling of actin (red) and DNC-1 (green) in early embryos. In (s,t), different focal planes of the same embryo are shown. (s) A spot of DNC-1 is seen on the deepest edge of the contracting furrow. (t) No spot of DNC-1 is seen on the shallow edge of the furrow. (u) Later stage than (s,t) showing that, at the late stages of cytokinesis, a ring of DNC-1 forms (possibly due to membrane touching the midzone of the spindle). (v) Later in the cell cycle, the ring collapses into a spot. Scale bar below (v) represents 10 μm and applies to (a–j) and (m–v); scale bars in (k,l) represent 5 μm.

Figure 3



Nomarski images showing the progression through the fourth cell division in (a–h) wild-type, (i–p) *dnc-1* RNAi-treated and (q–x) *dnc-2* RNAi-treated embryos. All embryos are aligned with anterior to the left and posterior to the right. The polar body marks the anterior end. Arrowheads mark the centrosomes (regions devoid of yolk granules). (a,i,q) Pronuclei (egg pronucleus is on the left and sperm pronucleus is on the right) meet in the posterior part of the embryo. (b,j,r) Pronuclei migrate to the center of the wild-type embryo (b) but, in *dnc-1* and *dnc-2* RNAi-treated embryos, stay in the posterior part of the embryo (j,r). (c,k,s) In the wild type, pronuclei rotate onto the AP axis (prophase) but, in *dnc-1* and *dnc-2* RNAi-treated embryos, pronuclei never rotate on to the AP axis. In (k) and (s) the timepoint is 1 min later than in (j) and (r), respectively. (d,l,t) At metaphase, the spindle sets up on the AP axis in the wild type (d) but, in *dnc-1* and *dnc-2* RNAi-treated embryos, no rotation events took place and the spindle set up along the dorsoventral axis (l,t). (e,m,u) Late anaphase–early telophase. In the wild type, the furrow is beginning to form (e). In *dnc-1* and *dnc-2* RNAi-treated embryos, during spindle elongation at anaphase B, the spindle skews onto the AP axis because of the constraints of the eggshell; a furrow begins to form on one side of the embryo (m,u). (f,n,v) Two-cell stage embryos. AB is on the left and P_1 is on the right. Arrowheads mark the centrosomes after they have migrated to opposite sides of the nucleus in P_1. In (v), abnormal nuclear movements and placements are seen. (g,o,w) In the wild type (g), in P_1 (the blastomere on the right), the centrosome–nucleus complex has rotated through 90° toward the midbody or cell-division remnant (the anterior centrosome is not visible in the focal plane). In *dnc-1* and *dnc-2* RNAi-treated embryos (o,w), rotation in P_1 does not occur and the spindle sets up along a transverse axis (P_1 is in metaphase). Note that in (w), the spindle morphology is abnormal. (h,p,x) Four-cell embryos. Note that in (p,x), as the orientation of the P_1 spindle was abnormal, division occurred out of the plane of focus; one of the daughter blastomeres is not visible. The scale bar applies to all panels and represents 15 μm.

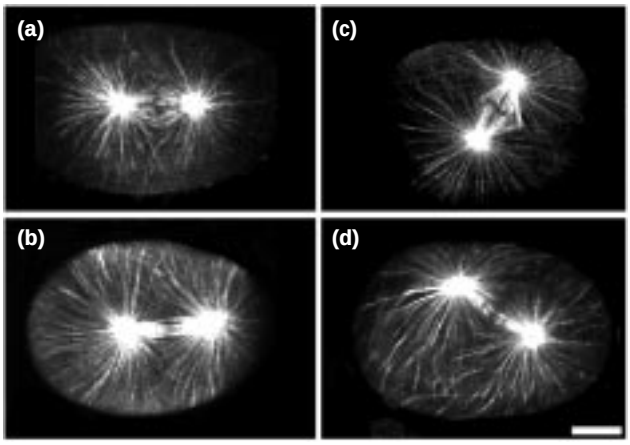
(Figure 2s) of one-cell stage embryos, and persisted at the cell-division remnant until the end of the two-cell stage (Figure 2a–j). The localization to the residual body at the two-cell stage is similar to the localization described for actin and actin-capping protein in *C. elegans* [13].

To determine whether or not this accumulation was consistent with spindle alignment, we double labeled embryos with antibodies against tubulin and $p150^{Glued}$. In each case, the spindle in the P_1 blastomere always was aligned towards the accumulation of DNC-1 at the cell-division remnant (Figure 2m–r).

Double-labeling studies using actin and $p150^{Glued}$ antibodies revealed that the accumulation of DNC-1 initially occurred at the deepest edge of the furrow, which had reached the region of the spindle midzone (Figure 2s). The furrow edge that was not as deep did not have any accumulation of DNC-1 (Figure 2t). At the end of cytokinesis, DNC-1 staining appeared as a ring (Figure 2u) and later, at the time of rotation, as a spot (Figure 2v). The appearance of a ring at the leading edge of an advancing furrow was the first evidence that we have seen as to how the dynactin components may ultimately localize to the cell-division remnant. Because of the role that dynactin may play in kinetochore function [21], it is possible that dynactin is left at the spindle midzone at the metaphase–anaphase transition after being shed by the chromosomes. Contact of the advancing furrow with the spindle midzone may cause dynactin to become localized in the midbody. We have seen some evidence of localization to the spindle midzone in two-cell-stage embryos (Figure 2e; arrowhead), but the localization is faint. This sort of localization pattern is similar to that seen with INCENPs, chromosomal passenger proteins [22], and with CENP-E, a minus-end-directed kinesin required for proper kinetochore function [23]. We propose that when the advancing furrow contacts the midzone microtubules, dynactin localizes to the cell-division remnant and ultimately may play a role in spindle alignment in the next mitotic division. Alternatively, dynactin may also become localized to the vertex of the late furrow through cortical flow, independent of microtubules.

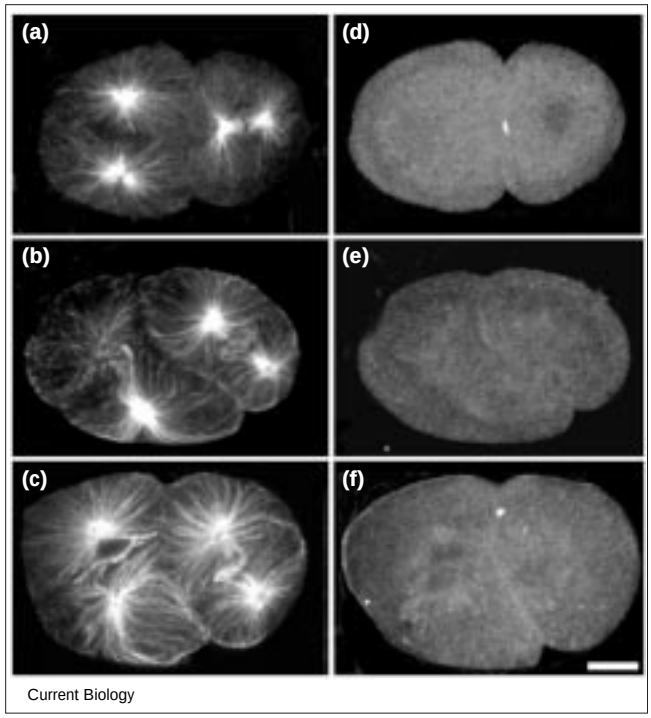
To determine whether DNC-1 and DNC-2 are involved in spindle orientation in early embryonic divisions, we used RNAi techniques to block expression of each gene in the gonad; this allowed us to assess the role of maternally provided DNC-1 and DNC-2 in early embryos [24]. We examined embryos from injected mothers by four-dimensional

Figure 4



Localization of tubulin using anti-tubulin antibody in one-cell-stage (a,b) wild-type and (c,d) *dnc-1* RNAi-treated embryos. (a,c) Metaphase. Spindle is on the AP axis in the wild type (a) but on a transverse axis in the *dnc-1* RNAi-treated embryo (c). (b,d) Anaphase. Compare spindle alignment in the wild type (b) with the *dnc-1* RNAi-treated embryo (d); in (d), the elongating spindle skews onto the AP axis because of the constraints of the eggshell. The scale bar represents 10 μm and applies to (a–d).

Figure 5



Localization of (a–c) tubulin and (d–f) DNC-1 in (a,d) wild-type, (b,e) *dnc-1* RNAi-treated and (c,f) *dnc-2* RNAi-treated embryos. Note that in the wild type, the spindle in P_1 aligns toward the spot of DNC-1 staining (a,d). In *dnc-1* RNAi-treated embryos, the spindle in P_1 does not align on the AP axis and no staining of DNC-1 is seen (b,e). In *dnc-2* RNAi-treated embryos, the spindle in P_1 does not align and DNC-1 staining is localized to the eccentrically placed cell-division remnant (c,f). Spindle midzones in both AB and P_1 blastomeres in *dnc-1* and *dnc-2* RNAi-treated embryos are abnormal. The scale bar represents 10 μm and applies to (a–f).

microscopy [25]. All embryos (30/30 *dnc-1* and 9/9 *dnc-2*) dissected 16–24 hours after injection had defects in spindle alignment (Figure 3i–x). In both *dnc-1* and *dnc-2* RNAi-treated embryos, the sperm and egg pronuclei met in the posterior of the zygote (P_0) as in the wild type (Figure 3a,j,r). Rotation of the centrosome–pronuclear complex onto the AP axis of the embryo did not occur (Figure 3k,s), however, and the spindles set up on a transverse axis (short axis of the cell; Figures 3l,3t,4c). When the spindles elongated during anaphase B, they skewed onto the AP axis because of the constraints of the eggshell (Figures 3m,3u,4d). The division was asymmetric by default as the normal movement of the pronuclei and subsequent eccentric alignment of the spindle did not occur. At the two-cell stage, spindles in the P_1 blastomere misaligned. In *dnc-1* and *dnc-2* RNAi-treated embryos, the rotation of the centrosome–nucleus complex towards the cell-division remnant was abolished in the P_1 blastomere, giving rise to a transversely aligned spindle (Figure 3o,w). The lack of rotation in the P_1 blastomere could be a consequence of the aberrant first cell division, which causes P_1 to acquire a different fate. We suspect, however, that the lack of rotation in the P_1 blastomere is due to the absence of the dynactin complex in these embryos, and not due to the inappropriate segregation of cytoplasmic materials from the defective first division. Cell-cycle times of wild-type and mutant P_1 blastomeres were relatively similar (14.7 minutes for wild-type, 14.1 minutes for *dnc-1* and 14.2 minutes for *dnc-2* embryos; determined as elapsed time from the appearance of one furrow to the next), yet characteristically different from AB blastomere cell-cycle times (11.6 minutes for wild-type, 11.7 minutes for *dnc-1* and 12.4 minutes for *dnc-2* embryos). This suggested to us that the P_1 blastomere had characteristics of wild-type P_1 blastomeres. Spindle orientation was normal in the AB blastomere in both *dnc-1* and *dnc-2* RNAi-treated embryos.

Spindle misalignment was not the only phenotype observed in the *dnc-1* and *dnc-2* RNAi-treated embryos. Embryos also had defects in nuclear movement, spindle morphology (only in two-cell-stage embryos and older) and chromosome segregation, which is consistent with our immunolocalization data and previous studies [21,26].

To determine if DNC-1 was absent and/or mislocalized in RNAi-treated embryos, we examined the distribution of DNC-1 in *dnc-1* and *dnc-2* RNAi-depleted embryos. In contrast to the wild type, in *dnc-1*-depleted embryos DNC-1 staining was not detected at the cell-division remnants or in the cytoplasm (0/17 embryos; Figure 5e). In *dnc-2*-depleted embryos, DNC-1 was localized to a focus

in the cortex corresponding to the cell-division remnant (Figure 5f). As furrows form asymmetrically in *dnc-2* embryos (like *dnc-1* embryos; Figure 3u), the DNC-1 spot at the midbody appears misplaced (8/8 embryos).

Conclusions

We have identified two components of the dynactin complex, DNC-1 and DNC-2, which are necessary for spindle alignment in early *C. elegans* embryos. Immunolocalization and RNAi studies show that the dynactin complex is necessary to specify the orientation of the mitotic spindle in *C. elegans* embryos. Our results, together with other studies [3,7,8,15–18,21], suggest that dynein and the dynactin complex might be widely used as a mechanism to establish the orientation of the mitotic spindle before the division of a polarized cell.

Materials and methods

Examination of embryos

Embryos were examined by light microscopy using Nomarski differential interference contrast microscopy. Embryos were placed on a 2% agarose pad in M9 medium and covered with a coverslip and sealed with vaseline. Development was recorded using a Hamamatsu model 2400 video camera and four-dimensional videomicroscopy [25].

Immunoblotting

Total SDS-soluble nematode extracts were run on a 7% polyacrylamide gel, blotted, and then probed with 1:400 dilution of anti-actin antibody (mAbC4; ICN) as a positive control and a 1:200 dilution of anti-p150^{Glued} antibody (gift of Kevin Vaughan and Richard Vallee). The anti-p150^{Glued} antibody was raised against the first 200 amino acids of rat p150^{Glued} which correspond to the well-conserved microtubule-binding domain (see Figure 1c). Procedures for electrophoresis, immunoblotting, and the preparation of total SDS-soluble nematode extracts were carried out as described previously [13].

Fixation and immunofluorescence microscopy of wild-type and RNAi-treated embryos.

Antibody staining in wild-type and RNAi embryos was performed by techniques previously described [13], with the following modifications. The bleach step was omitted and instead 20–40 hermaphrodites were placed on 0.1% poly-lysine, 0.02% gelatin, 0.002% chrome alum, 1 mM sodium azide subbed slides in M9. The worms were cut open on the subbed slide with a scalpel to remove embryos, an 18 mm × 18 mm coverslip was placed on top and excess M9 was wicked away. Gently tapping on the coverslip with a diamond pen also helped remove embryos from carcasses. The slides were then placed on a dry ice block for 15 min and freeze-cracked and fixed by Method II [13]. Embryos were double stained with anti-p150^{Glued} (rat polyclonal), anti-β-tubulin (N357; Amersham), anti-actin (mAbC4; ICN), and anti-DS-DNA (mAb030; Chemicon) antibodies. After fixation, the embryos were rehydrated with PBSBT (1× PBS, 1% BSA, 0.5% Tween) for 30 min. Primary antibodies were diluted in PBSBT and the slides were incubated overnight at 4°C (anti-tubulin antibody: 1:100; anti-actin antibody: 1:400; anti-DS-DNA antibody: 1:100; anti-p150^{Glued} antibody: 1:200). The unbound primary antibodies were washed off with three rinses of PBST (PBS, 0.25% Tween). The secondary antibodies were diluted 1:200 in PBSBT and added to the slide for a 1 h incubation at room temperature (20–25°C). The unbound secondary antibodies were washed off with four rinses of PBST. The fixed and stained embryos were mounted in 8 μl of ProLong Antifade (Molecular Probes). Confocal imaging was done on a Bio-Rad MRC1024 confocal microscope. Images were processed using NIH Image 1.61 b7 and then Adobe Photoshop 4.0 (Adobe Systems).

RNAi injections

Antisense mRNA from the relevant *C. elegans* cDNA clone was synthesized using the MEGAscript *in vitro* transcription kit (Ambion). Antisense RNA was resuspended in DEPC-treated water at a concentration of 3.5 mg/ml. Injection into the gonads of wild-type hermaphrodites was performed as previously described [27]. The following *C. elegans* mRNAs were injected into hermaphrodite gonads: *dnc-1* cDNA (ZK593.5/yk24f5, yk134f1) and *dnc-2* cDNA (C28H8.12/yk393f9). Water and *Xenopus* elongation factor antisense mRNA (pTRI-xef cDNA, Ambion) were used as controls (no spindle-alignment defects were observed). The phenotypes of the F1 embryos were examined using Nomarski four-dimensional videomicroscopy [12] and immunofluorescence techniques.

Sequence data for dnc-1 *and* dnc-2

The *C. elegans dnc-1* and *dnc-2* sequences were obtained from the *C. elegans* Genome Project. The *dnc-1* gene comes from the ZK593 cosmid on chromosome IV and *dnc-2* sequence comes from the C28H8 cosmid on chromosome III. All p150^{Glued} protein sequences were obtained from GenBank.

Supplementary material

An animation of the embryos in Figure 3 is published with this paper on the internet.

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Supplementary material

The dynactin complex is required for cleavage plane specification in early *Caenorhabditis elegans* embryos

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Animation

Embryos from Figure 3 are shown. The first animation sequence shows rotation of the centrosome–nucleus complex in a wild-type embryo in both P_0 and P_1 blastomeres. The second and third sequences show that rotation does not take place in embryos treated with either *dnc-1* or *dnc-2* RNAi.