RAB-11 Permissively Regulates Spindle Alignment by Modulating Metaphase Microtubule Dynamics in Caenorhabditis elegans Early Embryos

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Alignment of the mitotic spindle along a preformed axis of polarity is crucial for generating cell diversity in many organisms, yet little is known about the role of the endomembrane system in this process. RAB-11 is a small GTPase enriched in recycling endosomes. When we depleted RAB-11 by RNAi in Caenorhabditis elegans, the spindle of the one-cell embryo failed to align along the axis of polarity in metaphase and underwent violent movements in anaphase. The distance between astral microtubules ends and the anterior cortex was significantly increased in rab-11(RNAI) embryos specifically during metaphase, possibly accounting for the observed spindle alignment defects. Additionally, we found that normal ER morphology requires functional RAB-11, particularly during metaphase. We hypothesize that RAB-11, in conjunction with the ER, acts to regulate cell cycle-specific changes in astral microtubule length to ensure proper spindle alignment in Caenorhabditis elegans early embryos.

INTRODUCTION

The first cell division of the Caenorhabditis elegans zygote (P0) is asymmetric, giving rise to two daughter cells with different sizes and fates: the smaller P1 cell inherits germine determinants such as PIE-1 (Mello et al., 1996) and the larger AB cell develops into most of the somatic tissues (Sulston et al., 1983). This asymmetric division requires two well-regulated spindle alignment phases. First, before metaphase, the centrosomal-nuclear complex migrates to the center of the embryo while undergoing a 90° rotation to align the mitotic spindle onto a preformed A-P axis defined by the axially segregated PAR proteins (Cowan and Hyman, 2004a). This rotational alignment depends on the interactions of astral microtubules (MTs) with cortical regulators such as dynein-dynactin (Skop and White, 1998; Gonczy et al., 1999a) and the DEP domain protein LET-99 (Tsou et al., 2002). In the second phase, the redundant G proteins, GOA-1 and GPA-16, transduce the polarity cues of the PAR proteins into forces that displace the spindle posteriorly during anaphase (Gotta and Ahringer, 2001; Tsou et al., 2003). Although Gα distributes uniformly around the cortex (Gotta and Ahringer, 2001), its upstream activator, GPR-1/2, localizes asymmetrically in response to the PARs: it is enriched on the posterior cortex where PAR-2 is localized and reduced on the anterior cortex where PAR-3 is localized (Colombo et al., 2003; Gotta et al., 2003). The activity of Gα is down-regulated by Gβγ and LET-99, possibly by reducing the level of GPR-1/2 on the cortex (Tsou et al., 2003). Although little is known about the downstream effectors of Ga, it has been hypothesized that the minus-end MT motor dynein-dynactin might be the force generator activated by Ga (Grill et al., 2003). Both the rotation of the spindle and the posterior spindle displacement at anaphase are dependent on the cortical PAR polarity (Colombo et al., 2003), together with region-specific cortical interactions with astral MTs. Mutations in genes that result in short astral MTs, such as tbb-2 (tubulin β-subunit; Wright and Hunter, 2003), zyg-8 (a doublecortin-related kinase; Gonczy et al., 2001), and zyg-9 (the XMAP215 ortholog; Matthews et al., 1998), all fail to align the mitotic spindle properly. How MT length is regulated during the cell cycle in order to execute different spindle alignment processes is not well understood.

A factor that might contribute to the regulation of spindle alignment is membrane trafficking. Studies in Fucus (Shaw and Quatrano, 1996) and the EMS cell of C. elegans (Skop et al., 2001) have shown that treating the embryos with the secretion inhibitor brefeldin A (BFA) inhibits rotational alignment of the spindle. In the case of C. elegans, it is not clear whether BFA prevents spindle rotation in the EMS cell by perturbing the P2/EMS signaling (such as Wnt and MES-1/SRC-1 pathways) that act to polarize EMS (Walston and Hardin, 2006) or by affecting the spindle alignment process directly. Furthermore, when either of two C. elegans ER proteins, OOC-3 (a putative transmembrane protein) and OOC-5 (a Torsin-related AAA ATPase), are mutated, the majority of the embryos exhibit P1 spindle rotation defect, caused by either disrupting the polarization of the P1 cell or the organization of actin cytoskeleton at the midbody remnant (Pichler et al., 2000; Basham and Rose, 2001).

To understand further how membrane trafficking may affect spindle alignment, we examined the functions of the Rab family proteins in C. elegans one-cell embryos (P0) in which spindle alignment is cell autonomous (Goldstein,

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Abbreviations used: A-P, anterior-posterior; RNAi, RNA interference; Ga, α subunit of the trimeric G protein; Gβγ, β and γ subunit of the trimeric G protein; MT, microtubule; WT, wild-type; Δ, deletion.
par-2 RNAi, double-strand RNA (dsRNA) was produced using the in vitro T7 transcription kit (Ambion, Austin, TX). 1 mg/ml dsRNA was injected into N2 young adults and analyzed 36 h later. Full-length rab-11 3’ untranscribed regions (UTRs) were amplified and cloned into pGEM T-easy (Promega, Madison, WI) and WH3437 (RAB-11-GFP) strains were fed at the same time for at least 40 h before imaging or counting dead embryos. The zyg-9 feeding vector was from Ahringer’s feeding library (Kamath et al., 2003).

**Live Imaging**

Because rab-11(RNAi) embryos were sensitive to pressure and osmotic stress (data not shown), embryos were mounted in a hanging-drop blastomere culture medium (Shelton and Bowerman, 1996) for imaging. Worms were cut open in 3 μl blastomere culture medium on the coverslip. A slide with a circle of Vaseline was then pressed onto the coverslip to form a sealed chamber. Four-dimensional Nomarski imaging was performed as described previously (Skop and White, 1998). We used a Nikon Optiphot-2 upright microscope with a Nikon PlanApo 60 × 1.4 NA differential interference contrast (DIC) lens (Melville, NY) and a Hamamatsu C4400 camera (Hamamatsu Photonics, Hamamatsu, Japan). The microscope was equipped with a Sony XC-75 CCD camera (Tokyo, Japan). All GFP images were collected using multiphoton excitation on an optical workstation (Wokosin et al., 2003), which consists of a Nikon Eclipse TE300D inverted microscope with a Nikon Super Fluor 100 × 1.3 NA lens. The excitation source is a Ti:sapphire laser (Spectra Physics, Mountain View, CA) tuned to 890 nm. The detector is a high quantum efficiency Hamamatsu C4400-40 detector. Except for EBP-2-GFP, imaging was collected at 512 × 512-pixel resolution at 4.5-s intervals and analyzed with Image J v. 1.34s (http://rsb.info.nih.gov/ij/). Images for TH66 (EBP-2-GFP) were collected at 256 × 256-pixel resolution at 0.89-s interval with room temperature at 18°C. The posterior part of the embryos was zoomed in for a better visualization.

**Immunohistochemistry**

**rab-11(RNAi)** and WT worms were cut open in blastomere culture medium. Embryos labeled for membrane structures (anti-RAB-11 and anti-HDEL) were prepared as previously described with slight modification (Gonczy et al., 1999b). Fifteen worms were cut open in 15 μl H2O on a subbed slide. An 18-mm coverslip was placed onto the drop and embryos were allowed to settle with 3MM Whatman paper (Clifton, NJ). The slides were frozen on a metal block in a −80°C freezer for 5 min. After removing the coverslips, the slides were fixed in 100% methanol at −20°C for 15 min. Slides were rehydrated in 1× phosphate-buffered saline (PBS) for 5 min and incubated with 50 μl of primary antibody in PBS for 45 min at room temperature. After the incubation, slides were washed for 5 min in PBS (PBS-0.5% Tween 20), 5 min in PBS, and primary antibody was collected for over 45 min with the secondary antibody. Slides were washed twice with PBS for 5 min before mounting in 7 μl mounting media (Vectorshied; Vector Laboratories, Burlingame, CA). Embryos labeled with the anti-ZYG-8 antibody were fixed in 100% methanol at −20°C for 1 min (P. Gonczy, personal communication). All other antibody labeling was performed using other published protocols (Skop and White, 1998). Staining of WT and rab-11(RNAi) embryos was carried out under the same conditions for each antibody. Antibodies were diluted as follows: DM1, mouse anti-α-tubulin, 1:100 (Sigma, St. Louis, MO); rabbit anti-PI-1, 1:100; rabbit anti-GFP-1/2, 1:200; rabbit anti-ZYG-8, 1:200; rabbit anti-PAR-2 (a), 1:5; rabbit anti-PAR-2 (b), 1:5 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); mouse anti-HDEL, 1:20; rabbit anti-RAB-11, 1:200; and rabbit anti-ZYG-9, 1:40. Secondary antibodies were as follows: goat anti-mouse Alexa 568, 1:200 and goat anti-rabbit Alexa 488, 1:200 (Molecular Probes, Eugene, OR). Dilution was 1:500. Molecular Probes, Eugene, OR) and DAPI (1 μg/ml, Vectorshield). Slides were viewed on a Bio-Rad MRC1024 confocal microscope (Hercules, CA); instrument settings were the same for both WT and experimental embryos within each staining procedure. Images were prepared for publication with Adobe Photoshop (version 8.0, San Jose, CA).

**Measure MT Dynamics in rab-11(RNAi) and zyg-9(b235) Embryos**

MT length during metaphase in fixed WT, zyg-9(b235) and rab-11(RNAi) embryos labeled with the anti-α-tubulin antibody was measured as previously described (Gonczy et al., 2000). Namely, the three longest MTs projecting toward the anterior cortex in one optical section from each of five embryos were chosen to measure their length. “MT-cortex gap” refers to the mean distance between the anterior cortex and the plus ends of the longest astral MTs. When the posterior astral MTs were adjacent to the posterior cortex in both WT and rab-11(RNAi) embryos the MT growth rate and nucleation rate in rab-11(RNAi) embryos were calculated as described (Stryczko et al., 2005). Because of the violent movements of both centrosomes during anaphase in Rab-11 RNAi embryos, analysis was performed in metaphase MTs and centrosomes. Tracking of EBP-2-GFP was performed manually in ImageJ v. 1.34s. Because some portion of the astral MTs were attached by extra chromosomes because of the polar body extrusion defect, these MTs are dynamic.
When RAB-11 was depleted by RNAi, the PAR-2 domain et al., and the larger PAR-3 domain anterior (Etemad-Moghadam cortical domains, with the smaller PAR-2 domain posterior rab-11(RNAi) Embryos similar phenotypes. During anaphase (100%, n/H11005 genes (Table S1), known endosome regulators such as Video 2, bottom). The violent spindle movements are spe-
displaced further toward the posterior pole (see Figure 3C; both centrosomes rocked extensively and the spindle was II) in hanging drops containing 150 m/ml nocodazole (Invitrogen, Carlsbad, CA) in egg buffer (Skop and White, 1998). The same dilution of DMSO was used as a control. The coverslip was immediately put on the top of the gasket slide and sealed with petroleum jelly. Embryos undergoing pronuclear migration or centration were imaged either on the multiphoton workstation (β-tubulin::GFP) or with Nomarski optics (N2 embryos). BFA (invitrogen) treatment was carried out by soaking young embryos, before eggshell formation (usually during meiosis II) for at least 2 wk. The rescued worms were examined for GFP expression.

**Supplemental Data**
Supplemental Data including Figures S1 and S2, Tables S1 and S2, and Supplemental Videos are available online.

### RESULTS

**rab-11(RNAi) Embryos Exhibit Spindle Alignment Defects**

It has been previously reported that partial knockdown of RAB-11 by RNAi causes cytokinesis failure (Skop et al., 2001). We found that prolonged RNAi treatment (>44 h) also caused additional abnormalities evident during the first cell cycle, including osmotic sensitivity, failure to extrude polar bodies (52%, n = 23), no or minimal pseudocleavage (83%, n = 12), and failure of the centrosomal-nuclear complex to migrate to the center of the embryos (57%, n = 23). Most strikingly, the P0 spindle failed to rotate to the A-P axis (the angle of the spindle to the A-P axis was 45–90°; 73.9%, n = 23), and the spindle exhibited abnormal displacement during anaphase (100%, n = 23). In WT embryos, the anaphase spindle elongates and moves smoothly toward posterior, with a slight rocking of the posterior centrosome (Figures 1A and 3A; Video 1). By contrast, during the meta-
phase to anaphase transition, we found that the mitotic spindle underwent violent movements in 82.6% of the rab-11(RNAi) embryos (n = 23): the spindle migrated to the posterior pole and then rebounded to the A-P axis (Figures 1B and 3B; Video 2, top). In the remaining embryos (17.4%), both centrosomes rocked extensively and the spindle was displaced further toward the posterior pole (see Figure 3C; Video 2, bottom). The violent spindle movements are spec-
ic to RAB-11 depletion, because disruption of other RAB genes (Table S1), known endosome regulators such as RME-1 (Grant et al., 2001) or other osmotic sensitive mutants such as pod-1 and pod-2 (Tagawa et al., 2001) did not exhibit similar phenotypes.

**Polarity Cues Are Partially Defective in rab-11(RNAi) Embryos**

In WT embryos, PAR-2 and -3 occupy mutually exclusive cortical domains, with the smaller PAR-2 domain posterior and the larger PAR-3 domain anterior (Etemad-Moghadam et al., 1995; Boyd et al., 1996; Figure 2, A, C, E, G, and I). When RAB-11 was depleted by RNAi, the PAR-2 domain was reduced in size, whereas the boundary of PAR-3 expanded further to the posterior (Figure 2, B, D, F, H, and I). In some cases, the PAR-3 domain ectopically localized to the posterior cortex (50%, n = 6). Similar observations were seen with live images of PAR-2::GFP– and PAR-6::GFP– expressing embryos (data not shown). In some embryos the two domains partially overlapped as shown by immunofluorescence staining (Figure 2H). This ectopic localization of PARs resembles that seen in par-4– and -5–deficient embryos, although these do not exhibit violent anaphase spindle displacement (Hung and Kemphues, 1999; Watts et al., 2000; Morton et al., 2002). Although the boundary of PAR-2 and -3 was shifted toward the posterior, the anterior-posterior...
polarity was not totally abolished, as the germline factor PIE-1 still segregated normally to the posterior (Figure 2, J and K).

In WT embryos, GPR-1/2 is enriched at the posterior cortex in response to PAR-2 and -3 localization (Colombo et al., 2003; Gotta et al., 2003; Figure 3J). We found the size of the cortical region of GPR-1/2 was reduced in rab-11(RNAi) embryos between the end of prophase and early anaphase (n = 21; Figure 3K). This defect was probably a result of the posterior shift of the PAR-2 and -3 boundary in rab-11(RNAi) embryos (Figure 2). Nevertheless, the level of GPR-1/2 was not more highly enriched on the posterior cortex than in the WT embryos. This observation suggests that the polarity defect may not cause elevated level of GPR-1/2 on the posterior cortex and therefore is unlikely to explain the violent spindle movements.

**Violent Spindle Movements in rab-11(RNAi) Embryos Are Subjected to Ga/GPR-1/2 Regulation**

It is known that Ga and its upstream activator GPR-1/2 are the major force regulators for anaphase spindle displacement in the one-cell *C. elegans* embryo (Cowan and Hyman, 2004a). Because rab-11(RNAi) embryos exhibit violent spindle movements, we first examined whether Ga/GPR-1/2 regulation was altered in rab-11(RNAi) embryos. When we used RNAi to deplete PAR-3, LET-99, or GPB-1, proteins that function to down-regulate Ga/GPR-1/2 activity (Colombo et al., 2003; Gotta et al., 2003; Tsou et al., 2003), in rab-11(RNAi) embryos the violent spindle movements became more dramatic (Figure 3, D–F). Specifically, in par-3; rab-11(RNAi) embryos, the spindle still underwent violent movements but remained positioned in the center of the embryo instead of being pulled to the posterior (100%, n = 11; Figure 3D; Video 3, top), indicating that strong pulling forces were distributed around the entire cell. In rab-11;let-99(RNAi) and rab-11;gpb-1(RNAi) embryos, the centrosomal-nuclear complex, as well as the mitotic spindle, exhibited even more violent movements than depleting RAB-11 by itself. These violent movements commenced from the time of pronuclear centration until the embryo reached anaphase (rab-11;let-99(RNAi): 36.4%, n = 11; rab-11;gpb-1(RNAi): 18.2%, n = 11; Figure 3, E and F; Video 4). Conversely, the violent spindle movements in rab-11(RNAi) embryos were suppressed when Ga activity was down-regulated, such as in rab-11;par-2(RNAi) embryos (91.7%, n = 12; Figure 3G; Video 3, bottom; Colombo et al., 2003; Gotta et al., 2003), as well as in rab-11;gpr-1/2 (RNAi) embryos (100%, n = 10; Figure 3H; Video 5, top). Because Ga/GPR-1/2 can be both up- and down-regulated, resulting in a concomitant change in the violence of spindle movements, it is unlikely that constitutively active Ga/GPR-1/2 activity is the cause of the excessive spindle movement in rab-11(RNAi) embryos. Furthermore, our immunofluorescence staining with anti-GPR-1/2 antibody also suggests that the Ga activity was not up-regulated in rab-11(RNAi) embryos (Figure 3, J and K). We also found that the violent spindle movements required the activity of dynein-dynactin (n = 16; Figure 3I), suggesting they are the downstream targets of Ga/GPR-1/2. These observations indicate a correlation between Ga/GPR-1/2 activity and the extent of the violent spindle movements and that rab-11(RNAi) did not disrupt the interactions among the regulators of the normal anaphase spindle displacement.

**MT Dynamics during Metaphase Are Altered in rab-11(RNAi) Embryos**

Because MT dynamics are likely to play a crucial role in spindle orientation and movements, we examined the MTs...
shown as the anterior centrosomes were frequently out of the plane and underwent violent movements, only the posterior centrosomes are involved in pronuclear centration (asterisks). In embryos whose spindles were checked for intervals. For the end of anaphase (dots). The dots are not at the same time points. Trajectories describe the path of centrosome movement from prometaphase to metaphase in WT (A, Video 1), rab-11(RNAi) (B and C, Video 2), rab-11; par-3(RNAi) (D, Video 3, top), rab-11; let-99(RNAi) (E, Video 4, top), rab-11; gpb-1(RNAi) (F, Video 4, bottom), rab-11; par-2(RNAi) (G, Video 3, bottom), rab-11; gpr-1/2(RNAi) (H, Video 5, top), and rab-11; dnc-2(RNAi) embryo (I, Video 5, bottom). Each drawing is from a single representative embryo. These trajectories describe the path of centrosome movement from prometaphase to metaphase (dots). The dots are not at the same time points. Trajectories for rab-11; let-99(RNAi) and rab-11; gpb-1(RNAi) embryos (E and F), because the violent movements of the centrosomes begin before that observed in rab-11(RNAi), the traces of movements start from pronuclear centration (asterisks). In embryos with which the spindle undertook violent movements, only the posterior centrosomes are shown as the anterior centrosomes were frequently out of the plane of focus. In the rab-11; par-2(RNAi) (G) and rab-11; dnc-2(RNAi) (I) embryos, the P0 spindle first set up perpendicular to the longitudinal axis. In rab-11; dnc-2(RNAi) embryo, the spindle failed to centrate due to disruption of DNC-2 activity. During anaphase, the spindles elongated and flipped to the longitudinal axis due to the constraints of the eggshell. (J and K) Immunofluorescence staining of GPR-1/2 (red) in WT (J) and rab-11(RNAi) (K) embryos during one-cell metaphase. DNA (blue) is labeled with Topro3. Arrows delineate regions of GPR-1/2 enrichment at posterior cortex. In the rab-11(RNAi) embryo, the P0 spindle did not rotate and the chromosomes did not align properly along the metaphase plate. Immunolabeled rab-11(RNAi) embryos are larger because they are pressure sensitive and flattened more than WT embryos during fixation. Scale bar, 10 μm.

Figure 3. Ga/GPR-1/2 activity may not be up-regulated in rab-11(RNAi) embryos. Schematic representations of the movements of one or both centrosomes in WT (A, Video 1), rab-11(RNAi) (B and C, Video 2), rab-11; par-3(RNAi) (D, Video 3, top), rab-11; let-99(RNAi) (E, Video 4, top), rab-11; gpb-1(RNAi) (F, Video 4, bottom), rab-11; par-2(RNAi) (G, Video 3, bottom), rab-11; gpr-1/2(RNAi) (H, Video 5, top), and rab-11; dnc-2(RNAi) embryo (I, Video 5, bottom). Each drawing is from a single representative embryo. These trajectories describe the path of centrosome movement from prometaphase to metaphase (dots). The dots are not at the same time points. Trajectories for rab-11; let-99(RNAi) and rab-11; gpb-1(RNAi) embryos (E and F), because the violent movements of the centrosomes begin before that observed in rab-11(RNAi), the traces of movements start from pronuclear centration (asterisks). In embryos with which the spindle undertook violent movements, only the posterior centrosomes are shown as the anterior centrosomes were frequently out of the plane of focus. In the rab-11; par-2(RNAi) (G) and rab-11; dnc-2(RNAi) (I) embryos, the P0 spindle first set up perpendicular to the longitudinal axis. In rab-11; dnc-2(RNAi) embryo, the spindle failed to centrate due to disruption of DNC-2 activity. During anaphase, the spindles elongated and flipped to the longitudinal axis due to the constraints of the eggshell. (J and K) Immunofluorescence staining of GPR-1/2 (red) in WT (J) and rab-11(RNAi) (K) embryos during one-cell metaphase. DNA (blue) is labeled with Topro3. Arrows delineate regions of GPR-1/2 enrichment at posterior cortex. In the rab-11(RNAi) embryo, the P0 spindle did not rotate and the chromosomes did not align properly along the metaphase plate. Immunolabeled rab-11(RNAi) embryos are larger because they are pressure sensitive and flattened more than WT embryos during fixation. Scale bar, 10 μm.

In rab-11(RNAi) embryos, Immunofluorescence staining of MTs and imaging of EBP-2::GFP (the worm EB1 homolog that labels the growing MT plus ends) revealed that astral MT organization was altered in rab-11(RNAi) embryos. Although the anaphase MTs were normal in rab-11(RNAi) embryos, during metaphase aspects of MT dynamics, such as the distance between the plus ends of the MTs and the anterior cortex, MT growth, and nucleation rates (Table 1 and Figure 4, B and E), were altered compared with WT. During metaphase, the distance between the MT plus ends and the cortex was significantly greater in rab-11(RNAi) embryos than WT, probably because of the reduced MT length: 14.0 ± 1.6 μm in rab-11(RNAi) embryos compared with 22.1 ± 4.4 μm in WT (p < 0.001), although at anaphase, the MT ends contacted the cortex in both cases (Table 1). In addition, fewer growing astral MTs reached to the cortex during metaphase in rab-11(RNAi) embryos (n = 3/3) than in WT (Figure 4H, Video 6), suggesting the catastrophe rate may also be higher in rab-11(RNAi) embryos, although this rate cannot be measured directly using the EBP-2::GFP marker. This observation indicates that the inability of MT to reach the cortex in metaphase in these rab-11(RNAi) embryos may result from both slow growth and increased MT depolymerization.

It has been reported that embryos carrying a mutation in zyg-8, a Doublecortin-related kinase, exhibit similar exaggerated anaphase spindle displacement as observed in rab-11(RNAi) embryos (Gonczy et al., 2001). In zyg-8(b1650) embryos, the length of the metaphase astral MTs is normal, whereas the anaphase MTs are shorter compared with those in WT embryos (Gonczy et al., 2001). Similarly, we found that the distance between the plus ends of the MTs and the cortex in zyg-8(b235) embryos resembles WT, but an anaphase gap remains between the MT ends and the cortex. By contrast, in both WT and rab-11(RNAi) embryos the MT ends touch the cortex at anaphase (Figure 4, D and E, Table 1). In some zyg-8(b235) embryos, the spindle sets up in the posterior, perpendicular to the anterior-posterior axis (Figure 4F). In these embryos, the distance between the MTs and the cortex at anaphase is even more extensive (18.9 ± 1.6 μm). Furthermore, it has been shown that MT nucleation rate at metaphase is 36% higher in zyg-8(b1650) embryos than in WT (Srayko et al., 2005), whereas in rab-11(RNAi) embryos the MT nucleation rate is reduced at metaphase, compared with WT (Table 1). Therefore, the effects of RAB-11 levels on MT length are unlikely to be mediated through ZYG-8 because MT dynamics differ between rab-11(RNAi) and zyg-8 mutant embryos. Additionally, ZYG-8 localization to the spindle and spindle poles was not affected in rab-11(RNAi) embryos (Figure 4, I and J). These observations suggest that the length of the astral MTs is regulated by different mechanisms during the cell cycle. Indeed, it has been shown that, at least for the spindle MTs, the turnover rate is faster during prometaphase than anaphase in C. elegans one-cell embryos (Labbe et al., 2004).

We investigated next whether short metaphase MTs were sufficient to drive the violent spindle movements seen in the rab-11(RNAi) embryos by using the MT-depolymerizing drug nocodazole. The nocodazole dose and time of application were adjusted such that astral MTs in treated embryos were shortened during metaphase, yet the drug effect wore off during anaphase so that the astral MTs resumed elongation. We found that although the spindle movements in control embryos were normal (Figure 5A; Video 7), in embryos treated with nocodazole in this manner, the spindles were shortened during metaphase, yet the drug effect wore off during anaphase so that the astral MTs resumed elongation. We found that although the spindle movements in control embryos were normal (Figure 5A; Video 7), in embryos treated with nocodazole in this manner, the spindles were shortened during metaphase, yet the drug effect wore off during anaphase so that the astral MTs resumed elongation.

Metaphase ER Morphology Is Disrupted in rab-11(RNAi) Embryos
To understand further the role of RAB-11 in spindle alignment, we generated transgenic worms expressing RAB-11::GFP in the embryo under the control of the pie-1 promoter together with its UTRs. RNAi against the rab-11
3’UTR was carried out to determine whether the GFP fusion protein was functional. All embryos lacking the transgene failed cytokinesis at the first division (n = 6), whereas embryos expressing the RAB-11::GFP completed early cell divisions normally (n = 6), although they died at a later stage. The late lethality is probably due to loss of RAB-11::GFP expression when the pie-1 promoter shuts down in older embryos (Mello et al., 1996). The GFP signal localized to cytoplasmic puncta, around centrosomes, and at the periphery of the mitotic spindle (Figure 6, A and B). We also found RAB-11::GFP enriched at the spindle midbody, consistent with observations in mammalian cells (Wilson et al., 2005; data not shown). Indirect immunofluorescence labeling with an antibody raised against C. elegans RAB-11 (Poteryaev et al., 2007) confirmed the GFP localization. Furthermore, these observations revealed a more elaborate cytoplasmic structure not obvious in the GFP strain (Figure 6, C and D). By co-staining with an ER antibody (anti-HDEL), we were surprised to find RAB-11 (i.e., putative recycling endosomes) colocalized extensively with ER, although there were regions unique to each organelle (Figure 6, C–I).

Because of the overlap between RAB-11 and ER structures, we examined ER morphology in rab-11(RNAi) embryos by both immunofluorescence staining with anti-HDEL antibody as well as in live embryos expressing an ER marker, SP12::GFP. We found in both cases that although the periferispindle ER was still normal, the ER formed large aggregates throughout the cytoplasm in metaphase (Figure 7, C and H). During anaphase, the ER was still able to undergo morphological transitions from reticulate to dispersed structures as seen in the WT embryos (Poteryaev et al., 2005), but the large aggregates disappeared at the end of anaphase (Figure 7, D and H). Similarly, we found that the ER formed large aggregates during metaphase in zyg-8(b235) embryos (n = 5/5; Figure 7E). However, unlike in rab-11(RNAi) embryos, these aggregates persisted through anaphase (n = 4/4; Figure 7F), possibly accounting for the observed differences in MT length between these two cases.

Table 1. rab-11(RNAi) and zyg-8(b235) embryos exhibit different defects in MT dynamics

<table>
<thead>
<tr>
<th>MT-cortex gap</th>
<th>Metaphase</th>
<th>Anaphase</th>
<th>MT dynamics</th>
<th>Metaphase MT growth rate</th>
<th>Metaphase MT nucleation rate</th>
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<td>N2</td>
<td>2.8 ± 1.4 μm</td>
<td>Adjacent</td>
<td>0.72 ± 0.04 μm/s</td>
<td>102 ± 6</td>
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<tr>
<td>rab-11(RNAi)</td>
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<tr>
<td>zyg-8(b235)</td>
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<td>8.8 ± 2.9 μm</td>
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Measurements of MT dynamics in rab-11(RNAi) embryos are described in Methods and Materials. “MT-cortex gap” refers to the mean distance between the anterior cortex and the plus ends of the longest astral MTs in a single optical section in embryos in which the spindle is essentially parallel to the anterior-posterior axis.

a Statistically significant difference from WT (Student’s t test with two-tailed unequal variance).

b EBP-2::GFP worms became sterile after 32 h of rab-11 RNAi treatment before the embryos showed the severe spindle alignment defects. This growth rate may be even slower with prolonged RNAi treatment using wild-type N2 embryos (H11022).

ER Morphology in Embryos Where Spindle Alignment Genes Are Affected

How the ER influences spindle alignment in C. elegans early embryos is not well understood. Representative mutants, gene knockdowns or drug treatments that affect ER morphology, spindle alignment, or both are summarized in Table 2. Disrupting two C. elegans ER proteins OOC-3 and -5 as well as applying BFA to young embryos all led to large ER aggregates during metaphase that persisted until anaphase.
Although the disrupted ER morphologies were similar, their associated spindle alignment defects were quite different: in ooc-3(mn241) and ooc-5(it145) mutant embryos; both P0 and P1 spindles fail to align along the A-P axis (Basham and Rose, 1999; Pichler et al., 2000; Basham and Rose, 2001), whereas in BFA-treated embryos the nuclear-centrosomal complex rocked during pronuclear centration (100%, n = 14; Figure 8, I and J; Video10). None of these conditions affected the length of MTs (Basham and Rose, 1999, 2001; Pichler et al., 2000; Figure 8, C and D; data not shown) as seen in rab-11(RNAi) embryos, suggesting that the ER may influence spindle alignment by multiple mechanisms. On the other hand, some genes that are required for

Figure 5. Nocodazole treatment can phenocopy the violent spindle movements in rab-11(RNAi) embryos. Multiphoton time series of embryos expressing β-tubulin::GFP show the following developmental stages: metaphase, early anaphase, late anaphase, and telophase. (A) β-tubulin::GFP embryos treated with the same dilution of DMSO (Video 7), (B and C) β-tubulin::GFP embryos treated with 50 μg/ml nocodazole during pronuclear migration or centration (Videos 8 and 9). Note that the metaphase MTs were shortened by nocodazole, but they resumed elongation during anaphase. Centrosomal positions that best describe the trace of the movements from metaphase to telophase are shown in the schematic drawings (blue dots, anterior centrosomes; red dots, posterior centrosomes; dots are not at the same time intervals). Scale bar, 10 μm.

Figure 6. RAB-11 colocalizes extensively with the ER. Metaphase (A) and anaphase (B) localization of RAB-11::GFP. One-cell anaphase embryo labeled with anti-RAB-11 (C), anti-HDEL (F), and merged (H). Four-cell embryo with ABa and ABp at anaphase, P2 and EMS at metaphase labeled with anti-RAB-11 (D), anti-HDEL (G), and merged (I). Arrow points to a structure unique to anti-RAB-11 labeling, and arrowhead to a structure unique to anti-HDEL labeling. In rab-11(RNAi) embryo (E), the anti-RAB-11 labeling is greatly reduced. Scale bar, 10 μm.
normal ER morphology, such as car-1, may not be required for spindle alignment because, in car-1 depleted embryos, although violent spindle rocking occurs during anaphase as the spindle midzone breaks, the initial spindle rotation and spindle alignment looks similar to WT (Squirrell et al., 2006; Table 2), indicating that disrupting ER organization does not necessarily lead to spindle alignment defect.

We next examined the ER morphologies of embryos in which the spindle alignment regulators had been suppressed by RNAi. These proteins normally act by either modulating the interactions between the cortex and the astral MTs, such as the trimeric G proteins (Labbe et al., 2003) and LET-99 (Tsou et al., 2002; Tsou et al., 2003) or affecting the MT length, such as ZYG-9 (Matthews et al., 1998; Gonczy et al., 2001). ER morphology was normal in GPB-1–, GPR-1/2–, and LET-99–depleted embryos (Figure 8, E and F; Table 2). Similar results were obtained in ZYG-9–depleted embryos, which exhibit abnormal MT length independently of the cell cycle (Figure 8, G and H; Table 2).

DISCUSSION

Short Astral MTs at Metaphase Contribute to Violent Spindle Movements during Anaphase

Both rab-11(RNAi) and zyg-8 mutant embryos exhibit violent spindle movements during anaphase in the P0 cell. In zyg-8(1650) animals this violent spindle movement is caused by the failure of astral MT elongation during anaphase (Gonczy et al., 2001), whereas in rab-11(RNAi) embryos the lengths of the anaphase astral MTs were normal and the metaphase astral MTs were much shorter compared with those in WT embryos. How do short astral MTs at metaphase cause violent spindle alignment during anaphase?

In WT embryos a posterior pulling force acts on the spindle during late prophase and prometaphase but is balanced by the tethering of astral MTs at the anterior cortex (Labbe et al., 2004; Figure 9A). We propose that the short astral MTs observed during metaphase in rab-11(RNAi) embryos cannot be tethered by the anterior cortex; thus the spindle becomes prematurely subjected to the posterior pulling force. This posterior pulling force may be exerted on the spindle by the few astral MTs that do manage to reach to the posterior cortex and can be captured and shortened by the active force generators (e.g., dynein-dynactin; Figure 9B). Indeed, the number of the active force generators that displace the anaphase spindle in the WT embryo may be as few as 50 throughout the embryo, with more being present on the posterior cortex (Grill et al., 2003). We hypothesize that interactions between cortex and the many anterior astral MTs may function to counter the posterior pulling force in normal situations. Both reduction of the astral MT length and number are necessary to reach a certain threshold in order to destabilize the balance and generate the violent spindle movements that we observed. The observation that nocodazole-treated embryos showed a similar range of defects supports this hypothesis.

A model in which RAB-11 acts to regulate metaphase astral MT length, depicted in Figure 9, can also explain the modulations of the violent spindle movements in rab-11(RNAi) embryos shown in Figure 3. In particular, when there is elevated cortical GPR-1/2 (thereby producing higher G0 activity), the pulling force from the posterior cortex is enhanced, resulting in more violent spindle movements (Figure 9, C and D). In contrast, reduced cortical localization of GPR-1/2 (Figure 9, E and F) or inactivation of the motor dynein-dynactin (Figure 9G) results in reduced pulling force from the posterior, and the spindle movements are suppressed.

RAB-11 Can Colocalize with and Contribute to the Organization of the ER

Rab11 localizes mainly to the peri-centrosomal region in interphase Chinese hamster ovary (CHO) cells with a lower concentration of puncta distributed throughout the cell (Ulrich et al., 1996), whereas in polarized MDCK cells during mitosis, Rab11 forms diffuse puncta in the cytosol during prophase and then becomes clustered near the spindle poles after metaphase; this spindle pole accumulation increases throughout telophase (Hobdy-Henderson et al., 2003). However, we found that C. elegans RAB-11 overlaps with ER extensively and is required for normal ER morphology specifically during metaphase. Although the astral MTs were short during metaphase in rab-11(RNAi) embryos, the perturbation in ER morphology that we observed is probably
not a secondary effect of the MT disruption, as seen in a number of organisms (Voeltz et al., 2002), because disrupting the MT cytoskeleton by nocodazole, tba-2(RNAi) (Poteryaev et al., 2005) or zyg-9(RNAi) (this work) does not affect the ER morphological changes in C. elegans early embryos.

**Why Are Astral MTs Shorter in rab-11(RNAi) Embryos than in WT at Metaphase?**

One possible explanation is that RAB-11 (possibly in conjunction with its RE binding partner FIP3; see Supplementary Data) is required for delivery of proteins that can modulate MT lengths (such as MT-associated proteins [MAPs]) to astral MTs via the REs. When we examined the localizations of several known MAPs in C. elegans early embryos, such as EBP-2, ZYG-8 and -9, and dynactin (DNC-2), none were affected by RAB-11 depletion (Figure 4, H and J, and data not shown); however, some other MAPs may be involved. It is possible that changes in the cortical cytoskeleton contribute to the reduction of the observed MT length. However, we did not observe any significant perturbation in the distribution of cortical myosin before cytokinesis (Supplementary Data).

It may be significant that both astral MT length and ER morphology were affected in rab-11(RNAi) embryos predominantly during metaphase. This observation, together with the extensive colocalization between RAB-11 and ER that was seen, suggests that RAB-11 could mediate the cell-cycle-specific regulation of MT length through its effect on

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**Table 2. ER morphology by depletion of spindle alignment genes or drug treatment**

<table>
<thead>
<tr>
<th>Genes or drug</th>
<th>ER forms large aggregates during metaphase</th>
<th>Large aggregates persist during anaphase</th>
<th>ER cycles</th>
<th>Spindle alignment defect</th>
<th>Astral MT length defect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rab-11 RNAi</td>
<td>Yes, throughout the cytoplasm (n = 16, Figure 7H)</td>
<td>No, the large aggregates dispersed (n = 16, Figure 7H)</td>
<td>Yes</td>
<td>Yes, P0 spd rotation defect and violent anaphase spindle movements</td>
<td>Short metaphase astral MTs (n = 27, Figure 4B)</td>
<td>This study</td>
</tr>
<tr>
<td>zyg-8 (b235)</td>
<td>Yes, throughout the cytoplasm (n = 5, Figure 7E)</td>
<td>Yes, throughout the cytoplasm (n = 4, Figure 7F)</td>
<td>Yes</td>
<td>Yes, P0 spd rotation and anaphase spindle position defect</td>
<td>Short anaphase astral MTs (n = 11, Figure 4F)</td>
<td>Gonczy et al. (2001)</td>
</tr>
<tr>
<td>zyg-9 RNAi</td>
<td>No (n = 4, Figure 8G)</td>
<td>No (n = 5, Figure 8F)</td>
<td>Yes</td>
<td>Yes, P0 spd set up transversely at posterior</td>
<td>Short MTs throughout cell cycle</td>
<td>Matthews et al. (1998)</td>
</tr>
<tr>
<td>let-99 RNAi</td>
<td>No (n = 5/6, Figure 8E)</td>
<td>No (n = 5, Figure 8F)</td>
<td>Yes</td>
<td>Yes, centrosomal-pronuclear complex wobble during centration, P0 and P1 spindle rotation defect</td>
<td>Normal</td>
<td>Rose and Kemphues (1998), Tsou et al. (2002, 2003)</td>
</tr>
<tr>
<td>gpb-1 RNAi</td>
<td>No (n = 3, data not shown)</td>
<td>No (n = 3, data not shown)</td>
<td>Yes</td>
<td>Yes, centrosomal-pronuclear complex wobble during centration, P0 and P1 spindle rotation defect</td>
<td>Normal</td>
<td>Gotta and Ahhringer (2001), Tsou et al. (2003)</td>
</tr>
<tr>
<td>gpr-1/2 RNAi</td>
<td>No (n = 3, data not shown)</td>
<td>No (n = 3, data not shown)</td>
<td>Yes</td>
<td>No anaphase spindle elongation and displacement</td>
<td>Normal</td>
<td>Colombo et al. (2003), Gotta et al. (2003)</td>
</tr>
<tr>
<td>ooc-3/ooc-5</td>
<td>Yes, throughout the cytoplasm (n = 3, Figure 8A)</td>
<td>Yes, throughout the cytoplasm (n = 3, Figure 8B)</td>
<td>Yes</td>
<td>Yes, P0 and P1 spd fail to align along A-P axis</td>
<td>Normal (n = 3, Figure 8, C and D)</td>
<td>Basham and Rose (1999, 2001), Pichler et al. (2000)</td>
</tr>
<tr>
<td>BFA</td>
<td>Yes, throughout the cytoplasm</td>
<td>Yes, throughout the cytoplasm</td>
<td>No</td>
<td>Yes, centrosomal-pronuclear complex rock during centration (Figure 8, I and J)</td>
<td>Normal (n = 3, data not shown)</td>
<td>Poteryaev et al. (2005), and this study</td>
</tr>
<tr>
<td>car-1 RNAi</td>
<td>No, but patchy accumulation or thick strand</td>
<td>patchy accumulation or thick strand persist</td>
<td>Yes</td>
<td>No</td>
<td>Normal</td>
<td>Squirrell et al. 2(006)</td>
</tr>
<tr>
<td>cdc48 RNAi</td>
<td>Yes, throughout the cytoplasm</td>
<td>Yes, throughout the cytoplasm</td>
<td>No</td>
<td>No</td>
<td>ND</td>
<td>Poteryaev et al. (2005), and personal communication</td>
</tr>
<tr>
<td>hsp-4 RNAi</td>
<td>Yes, throughout the cytoplasm</td>
<td>Yes, throughout the cytoplasm</td>
<td>No</td>
<td>No</td>
<td>ND</td>
<td>Poteryaev et al. (2005), and personal communication</td>
</tr>
</tbody>
</table>

ND, not determined.
the ER. One possibility is that proteins regulating metaphase MT length could be processed and transported via the ER, whose normal morphology requires functional RAB-11 (this study). Our failure to demonstrate any mislocalization of MAPs in RAB-11 depleted embryos (see above) makes this possibility somewhat less likely.

It has been shown that ER-regulated calcium levels can alter MT dynamics (Facanha et al., 2002). Thus, as the ER cycles from its reticulate organization during metaphase to dispersed structures during anaphase (Poteryaev et al., 2005), it could provide cell-cycle–specific regulation of free calcium levels, which would affect MT growth or stability. In

Figure 8. Microtubule and ER morphologies for gene disruptions and BFA treatment listed in Table 2. (A and B) Immunofluorescence staining of the ER (anti-HDEL, red) and Topro3 staining of DNA (blue) in ooc-3(mn241) mutant embryos during metaphase (A) and anaphase (B). Although previous work indicated that the ER structure was less affected in ooc-5(it145) mutant embryos than in ooc-3(mn241) embryos (Basham and Rose, 2001), we observed a similar defect of ER morphology in these embryos (data not shown). Arrows mark the position of the mitotic spindle poles. Arrowheads show one of the ER clumps. (C and D) Immunofluorescence staining of the microtubules (red) and Topro3 staining of DNA (blue) in ooc-3(mn241) mutant embryos during one-cell metaphase (C) and anaphase (D). ooc-5(it145) mutant embryos showed similar phenotype (data not shown). ooc-3(mn241) and ooc-5(it145) mutant embryos are often smaller than the WT embryos (Basham and Rose, 1999). Scale bar, 10 μm. (E and F) SP12::GFP embryos treated with let-99 RNAi showed that ER morphology was not affected during either metaphase (E) or anaphase (F). (G and H) SP12::GFP embryos treated with zyg-9 RNAi showed that ER morphology was similar to that observed with nocodazole or the-2(RNAi) treatment (Poteryaev et al., 2005). The accumulation of ER structure at the anterior during anaphase may be due to the exaggerated cytoplasmic flow resulting from short microtubules. (I and J) BFA treatment caused nuclear-centrosomal complex rocking during pronuclear centration. Nomarski images show the centrosome movements during pronuclear centration and the final stage of the first division (the last time point). (I, Video 10, top) WT meiosis II embryos treated with same dilution of DMSO as control (n = 15). (J, Video 10, bottom) WT meiosis II embryos treated with 150 μg/ml BFA (n = 14). Arrows mark the positions of the two centrosomes. Schematic representations of the movements of the posterior centrosome during pronuclear centration are drawn. Scale bar, 10 μm.
this scheme, RAB-11 could act directly in calcium regulation (such as interacting with a Ca²⁺ channel; van de Graaf et al., 2006) or indirectly (by affecting ER morphology, thereby regulating calcium levels in microdomains) to stabilize metaphase astral MTs.

When the ER disperses at the metaphase-to-anaphase transition, MTs become subject to different forms of cell-cycle-dependent regulation, such as by ZYG-8. Interestingly, we found that the ER also forms large aggregates during metaphase in zyg-8(b235) embryos. However, unlike in rab-11(RNAi) embryos, these aggregates persisted through anaphase. If the ER is regulating MT length, this may explain why zyg-8 mutant embryos exhibit defects in anaphase MT assembly (Gonczy et al., 2001), as well as a slight reduction in the MT growth during metaphase (Srayko et al., 2005). Possibly the doublecortin ZYG-8 may change MT dynamics in correspondence to the ER cycle. Although it is not known whether ZYG-8 is associated with the ER, it has been found that the chicken ortholog of ZYG-8 is associated with membrane structures (Capes-Davis et al., 2005). In genes that affect MT length independently of the cell cycle, such as zyg-9 (Matthews et al., 1998), the ER morphology was normal when these genes were depleted (Figure 8, G and H).

Our observations indicate that the length of astral MTs during metaphase is at least partially determined by RAB-11. The stage-specific perturbation of ER structure seen upon RAB-11 depletion suggests that the ER may in some way determine the length of astral MTs at metaphase. However, the depletion of RAB-11 must perturb the ER in a specific way because depletion of other proteins, such as CAR-1, can produce superficially similar disruptions of ER morphology without causing the shortened astral MTs or spindle alignment defects observed with RAB-11 depletion (Squirrell et al., 2006). Furthermore, depletion of the ER proteins OOC-3 and -5 as well as BFA treatment can cause perturbations of ER structure and spindle alignment defects without any concomitant shortening of MTs during metaphase. We should also mention that a normal ER organization alone is not sufficient to permit proper spindle alignment. For genes required for spindle alignment that regulate the interactions between the astral MTs and cortex, such as let-99 (Tsou et al., 2003; Gotta et al., 2003) and the trimeric G proteins (Labbe et al., 2003), the ER morphology was normal when these genes were depleted. However, given that nocodazole-mediated shortening of MTs during metaphase is sufficient to phenocopy the spindle alignment phenotype of RAB-11 depletion, we speculate that RAB-11 acts permissively (possibly via the ER) to specify an appropriate length for astral MTs at metaphase to allow the cortical interactions that mediate the alignment of the mitotic spindle.

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