# Identification and Characterization of Factors Required for Microtubule Growth and Nucleation in the Early *C. elegans* Embryo

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#### Summary

Microtubules (MTs) are dynamic polymers that undergo cell cycle and position-sensitive regulation of polymerization and depolymerization. Although many different factors that regulate MT dynamics have been described, to date there has been no systematic analysis of genes required for MT dynamics in a single system. Here, we use a transgenic EB1::GFP strain, which labels the growing plus ends of MTs, to analyze the growth rate, nucleation rate, and distribution of growing MTs in the Caenorhabditis elegans embryo. We also present the results from an RNAi screen of 40 genes previously implicated in MT-based processes. Our findings suggest that fast microtubule growth is dependent on the amount of free tubulin and the ZYG-9-TAC-1 complex. Robust MT nucleation by centrosomes requires AIR-1, SPD-2, SPD-5, and  $\gamma$ -tubulin. However, we found that centrosomes do not nucleate MTs to saturation; rather, the depolymerizing kinesin-13 subfamily member KLP-7 is required to limit microtubule outgrowth from centrosomes.

### Introduction

Early embryonic cells undergo rapid and successive mitoses, requiring the assembly and disassembly of MT-based structures such as the mitotic spindle apparatus. Many factors are required for the formation or dissolution of MT polymers within the cell. Regulation occurs at many levels, from the synthesis and posttranslational maturation of both individual tubulin monomers as well as the assembly of the  $\alpha$ - $\beta$  dimer, the primary building block of the tubulin polymer. MTassociated proteins (MAPs) regulate various aspects of MT polymerization behavior but are typically implicated in the formation of stable or long MTs (reviewed in Kinoshita et al. [2002]). Conversely, kinesins of the kinesin-13 family such as XKCM1 (a.k.a. MCAK) cause MTs to depolymerize from both plus and minus ends (reviewed in Howard and Hyman [2003] and Moore and Wordeman [2004]). Katanin enzymes sever MTs and are also implicated in the dissolution of MT polymer and/or the regulation of MT length and postnucleation processing (reviewed in Quarmby [2000]).

Although the factors that affect MT behavior have been studied in many different systems, missing to date is a "system-wide" approach whereby many factors are examined systematically for their affect on a single parameter of MT behavior, such as MT growth rate. Recent genome-wide RNAi screens in *C. elegans* suggest that, given the right assays, it should be possible to identify and study most of the genes required for MT behavior in the early embryo (Kamath et al., 2003; Sönnichsen et al., 2005; Sugimoto, 2004).

The early *C. elegans* embryo contains very dense MT arrays, hindering direct visualization of individual MTs in vivo. However, the development of a fluorescent marker that decorates the growing ends of MTs in *C. elegans* would be an important advance. Although many MT end binding proteins have proven useful to study MT dynamics in other systems (Akhmanova and Hoogenraad, 2005; Schroer, 2001), the *C. elegans* homologs have not been examined. It is not clear how these proteins, collectively referred to as MT plus-end tracking proteins (+TIPs), specifically mark the growing plus ends of MTs (reviewed in Akhmanova and Hoogenraad [2005] and Wittmann and Desai [2005]). However, their presence on plus ends is diagnostic for MT growth behavior.

Herein, we report the application of a C. elegans EB1 homolog to study the growth rate, nucleation rate, and distribution of astral and spindle MTs during mitosis. By using this methodology, we hope to further understand phenotypes in C. elegans that appear similar at the level of differential-interference contrast (DIC) imaging but that nonetheless might have a distinct molecular basis. We examined 40 genes previously implicated in MT-based processes from DIC screens and determined their effect on MT behavior in living cells. Our data show that astral MTs grow at extraordinary speeds (0.7 µm/s) limited by the availability of free tubulin and driven by the ZYG-9-TAC-1 complex. We also report that the number of MTs growing from centrosomes is limited by the kinesin-13-like protein KLP-7 and that, during spindle assembly, RAN-1 is required for a bias in MT outgrowth toward chromatin.

## Results

## EBP-2 Decorates the Plus Ends of Growing MTs

In order to find a suitable marker for measuring MT growth in vivo, we first examined candidate MT end binding proteins via immunostaining. Antibodies generated against VW02B12L.3 (EBP-2; end binding protein), a homolog of human EB1 proteins (Mimori-Kiyosue et al., 2000; Su et al., 1995), produced a staining pattern consistent with its location at the plus-end tips of growing MTs (Figure 1A). We constructed a stable transgenic C. elegans strain expressing EBP-2::GFP. Western blot analysis indicated that the transgene was not overexpressed relative to wild-type (wt) (Figure 1B), and the transgenic worms appeared healthy. ebp-2(RNAi) abrogated all GFP fluorescence in the transgenic worms but did not result in any obvious early embryonic phenotypes or embryonic lethality in our hands (data not shown).



Figure 1. Location of Endogenous C. elegans EBP-2 and EBP-2::GFP at the Growing Plus Ends of Microtubules

(A) Wild-type (wt) one-cell anaphase embryo stained with Hoechst (DNA), anti-α-tubulin antibodies (microtubules [MTs]), and anti-EBP-2 antibodies.

(B) Western blot of wt and TH66 lysate from gravid adult hermaphrodites, probed with anti-EBP-2 antibodies.

(C-G) Selected images (400 msec exp.) from a movie of a single embryo expressing EBP-2::GFP is shown. Arrowheads indicate the position of a single EBP-2::GFP dot over time.

(H) The entire 8 s movie stack represented in (C)–(G) (21 frames) was projected as a single image to show the paths of many EBP-2::GFP dots (which represent the subset of growing MTs) during this interval. Bar is 10  $\mu$ m.

(I–K) Single images from EBP-2::GFP movies of wt and *tubulin(RNAi)* are shown. Two classes of phenotypes were observed with *tubulin(RNAi)* treatment: those with greatly reduced centrosomal EBP-2::GFP fluorescence and short EBP-2::GFP projections that quickly disappeared (weak), and embryos that exhibited no EBP-2::GFP foci in any focal plane (strong).

By using spinning disk confocal microscopy to visualize the EBP-2::GFP fusion protein, we observed small dots of fluorescence moving away from centrosomes, consistent with EBP-2 decoration of growing MT ends (Figures 1C-1H; Movie S1 available in the Supplemental Data with this article online). In addition to the moving fluorescent dots, a strong fluorescence signal was observed throughout the centrosomal region, likely indicating a large population of growing MTs very near the centrosome. To test whether EBP-2 localization to centrosomes was MT dependent, we examined EBP-2::GFP fluorescence after RNAi of  $\alpha$ - and  $\beta$ -tubulin. We observed two classes of embryos: those that contained a very low albeit detectable EBP-2::GFP signal at the centrosome (5/12), and those that had no obvious foci of EBP-2::GFP fluorescence (7/12) (Figures 1J and 1K). In all cases where EBP-2 was detected, however, we

noted the appearance of short EBP-2::GFP tracks that quickly disappeared less than one micron from the centrosome (Movie S2). Therefore, we conclude that the EBP-2::GFP signal is dependent on growing MTs.

# MT Growth Rates and Nucleation Rates in Wt Embryos

In order to track fluorescent EBP-2::GFP dot movements, we used pattern recognition software (LabView, National Instruments; Experimental Procedures and Movie S3). An example of MT growth tracks from one centrosome is shown in Figure 2A. By using this method, we determined MT growth rates throughout the cell cycle (Figure 2B and Movie S4). During pronuclear migration, astral MT growth rates were 0.74  $\pm$  0.07  $\mu$ m/s (n = 36; Figure 2B, column ii). During metaphase, astral MT growth rates were 0.72  $\pm$  0.02  $\mu$ m/s (n = 75;



#### Figure 2. MT Growth Rates in the One-Cell Embryo

(A) Traces of individual astral MTs from a single centrosome. Distance (µm) from the centrosome versus time (s) is plotted.

(B) MT growth rates were measured during the following processes: (i) early pronuclear migration, (ii) late pronuclear migration, (iii) metaphase astral MTs, (iv) metaphase spindle MTs, (v) anaphase, (vi) telophase, and (vii) two cell. Black lines on embryo images indicate the class of MTs measured. Bars represent the average velocity for each stage: (i)  $0.97 \pm 0.10 \mu$ m/s, n = 65; (ii)  $0.74 \pm 0.07 \mu$ m/s, n = 36; (iii)  $0.72 \pm 0.02 \mu$ m/s, n = 75; (iv)  $0.67 \pm 0.03 \mu$ m/s, n = 62; (v)  $0.51 \pm 0.02 \mu$ m/s, n = 83; (vi)  $0.56 \pm 0.02 \mu$ m/s, n = 57; and (vii)  $0.71 \pm 0.03 \mu$ m/s, n = 40. Circles represent average velocity of individual MTs. Left (red) and right (green) clusters represent the anterior and posterior centrosome of origin, respectively, for iii, v, and vi. We found no significant difference between anterior and posterior growth rates: metaphase ( $0.73 \pm 0.03 \mu$ m/s [post.]; p = 0.15, also see Figure S1); anaphase ( $0.52 \pm 0.03 \mu$ m/s [ant.] versus  $0.51 \pm 0.03 \mu$ m/s [post.]; p = 0.26). Note: high EBP-2 velocities during early pronuclear migration (column i) likely result from dynein-dependent acceleration of free microtubules, in addition to polymer growth rate (see text for details).

Figure 2B, column iii). However, after metaphase, the astral MT growth rates dropped to  $\sim 0.51 \pm 0.02 \ \mu$ m/s (n = 83) in anaphase and 0.56  $\pm 0.02 \ \mu$ m/s (n = 57) in telophase (Figure 2B, columns v and vi). Because the MT growth rates in the two-cell embryo were approximately the same as in the metaphase one-cell embryo (0.71  $\pm 0.03 \ \mu$ m/s; n = 40; Figure 2B column vii), the

lower growth rates observed in anaphase and telophase were specific to those stages of the cell cycle.

We also measured the growth rates of anterior and posterior centrosome-nucleated MTs in order to determine if MT growth differences could contribute to asymmetric pulling forces present in the one-cell embryo (Grill et al., 2001; Labbe et al., 2004). We found no dif-



#### Figure 3. Quantification of Wt Astral MT Nucleation Rates In Vivo

(A) A half-circle (9  $\mu$ m radius) was drawn around each centrosome as shown in the EBP-2::GFP movie stack projection (top). Kymographs from metaphase, anaphase, and telophase embryos were generated to count the number of EBP-2::GFP foci intersecting this line over the entire 1 min movie (y axis is time). Examples of two selected dots are shown (red circles).

(B) MT nucleation rates were quantified relative to wt metaphase embryos. SEM at 95% confidence is shown; metaphase, n = 10; anaphase, n = 5; and telophase, n = 6. Only relatively stationary anterior centrosomes were used for anaphase measurements.

ference between anterior and posterior astral MT growth rates during metaphase ([ $0.708 \pm 0.015 \mu$ m/s; n = 158 ant.] versus [ $0.707 \pm 0.017 \mu$ m/s; n = 135 post.]; p = 0.92; Figure S1 and Figure 2B, column iii), anaphase, or telophase embryos (Figure 2B, columns v and vi). Therefore, we conclude that MT growth does not differ between the anterior and posterior centrosomes in the one-cell embryo.

We used EBP-2::GFP to quantify astral MT nucleation rates (Figure 3A and Experimental Procedures). Only MT plus ends reaching a distance of nine microns from the centrosome were scored; thus, very short microtubules near the centrosome or those exiting the focal plane were not detected. We counted an average of 73 ± 8 MTs/half-centrosome/min in wt metaphase embryos (n = 10 centrosomes). Due to the limitations of our optical assay, we are probably detecting less than 50% of all growing MTs. The actual number of MTs growing out of each centrosome during metaphase is likely to be impressively high, perhaps more than 300 MTs/min. We also found wt anaphase and telophase embryos exhibited 25% more astral MT nucleation per minute than metaphase embryos (Figure 3B), similar to results in other systems (Piehl et al., 2004).

# Dynein-Dependent Movement of MTs Near the Nuclear Envelope

During early prophase, prior to significant MT nucleation by the centrosomes, the cytoplasm contained many moving EBP-2::GFP dots, representing individual growing MT ends (Figure 4A). We noticed that many of the fastest EBP-2::GFP dots seemed to derive their speed from brief contact with the pronuclear envelope (Movie S5). By measuring the velocity of these fast EBP-2::GFP dots (n = 29), we found a distribution of speeds consistent with two distinct populations: one at ~1.45  $\mu$ m/s and another at ~2.05  $\mu$ m/s (Figure 4B, gray bars). One possible explanation for these high speeds is the nuclear-envelope-based, minus end directed MT motor dynein DHC-1 (Gonczy et al., 1999). Therefore, we tracked EBP-2::GFP dots that were near to or contacting the maternal pronucleus in dhc-1(RNAi) embryos. Strikingly, we observed no fast-moving EBP-2::GFP particles near the maternal pronucleus, suggesting that dynein was responsible for all fast movements (Figure 4B, white bars). Based on dynein's known minus end-directed activity, its contribution to the EBP-2::GFP velocities observed in the C. elegans embryos was ~0.75  $\mu$ m/s and ~1.35  $\mu$ m/s (Figure 4C).



Figure 4. Analysis of Cytoplasmic EBP-2::GFP Movement in Early Embryos

(A) Tracings from EBP-2::GFP foci movements in the cytoplasm of two early embryos. Each trace represents the history of EBP-2::GFP movement over ten frames (3.6 s elapsed time). Occasionally, EBP-2::GFP particles are accelerated upon contact with the maternal pronucleus (M) or elsewhere in the cytoplasm. Bar is 5 μm.

(B) A histogram displaying the frequency of velocities for EBP-2::GFP foci contacting the maternal pronucleus in wt (gray) and *dhc-1(RNAi)* (white) embryos. We found a distribution of *dhc-1*-dependent speeds consistent with two distinct populations, one at ~1.45  $\mu$ m/s and another at ~2.05  $\mu$ m/s (gray bars).

(C) A model to explain the distribution of fast EBP-2::GFP velocities. Astral MT growth was found to be 0.7  $\mu$ m/s (see text for details). For free MTs accelerated upon contact with the maternal pronuclear envelope, one peak velocity was centered on ~1.45  $\mu$ m/s, which may represent a single DHC-1 motor acting on the MT, contributing ~0.75  $\mu$ m/s to the total velocity. The next highest peak (~2.05  $\mu$ m/s) suggests a contribution by DHC-1 of ~1.35  $\mu$ m/s, almost twice that of the lower peak, which may indicate the presence of two DHC-1 motors acting together, with limited interference.

### MT Behavior during Mitotic Spindle Assembly

Spindle MTs penetrate the nuclear envelope during spindle assembly and, despite originating from centrosomes like astral MTs, they grow through an environment that is conceivably distinct from the cytoplasm (reviewed in Karsenti and Vernos [2001]). However, the average growth rate of spindle MTs was  $0.67 \pm 0.03$ 

 $\mu$ m/s (n = 62 MTs), similar to metaphase astral MT growth rate (p = 0.014; Figure 2D, column iv). This suggests that centrosomal MTs grow at a similar velocity, irrespective of their direction of growth (i.e., toward the cortex or toward chromatin during spindle assembly).

We noticed that during early spindle assembly, the density of EBP-2::GFP foci was higher within the spin-



Figure 5. A Bias in MT Outgrowth Is Dependent on RAN-1, but Not Kinetochore Function

Stack projections of EBP-2::GFP movies (top) and kymograph projections (bottom) are shown. Kymographs were made from the circular line shown on each embryo. Each kymograph represents the change in pixel intensities along the line over time (clockwise path around the circle is represented as left-right in the x axis of the kymograph). *zyg-1(b1)* embryos contain only one centrosome as a control for proximity effects of the sister centrosome in wt embryos. *zyg-1(b1)* spindles sometimes form with separated masses of chromatin; hence, three zones of increased MT outgrowth are visible in (B). Kymographs represent 30 s of time.

dle region than elsewhere in the cytoplasm. Unfortunately, the high density of EBP-2::GFP dots in the spindle region precluded an accurate estimate of the number of growing MTs. As an alternative method to compare astral and spindle nucleation rates, we performed radial kymograph image analysis, whereby a nearly complete circle was drawn around the centrosomes, and the history of EBP-2 dots intersecting this line was displayed as a single image. These kymographs also depicted a bias in MT growth toward chromosomes (Figure 5A). Because the kymographs represent the amount of EBP-2::GFP intersecting each point along the curve without discrimination for their direction of movement, it is possible that MTs emanating from the opposite pole are responsible for this result. However, we repeated our assay on monopolar spindles that arise from outcrossing the EBP-2::GFP worms to zyg-1(b1) males (which contribute only one centriole; O'Connell et al., 2001). These embryos also exhibited a bias in MT growth toward chromosomes, suggesting that MTs preferentially grow toward chromatin during spindle assembly (Figure 5B).

# RAN-1, but Not Kinetochores, Contributes to the Bias in MT Outgrowth toward Chromosomes

In other systems, the small GTPase Ran has been implicated in local control of MT behavior (Gruss and Vernos, 2004). In C. elegans, ran-1 is required for nuclear import and spindle assembly (Askjaer et al., 2002; Bamba et al., 2002), but its effect on MT growth and nucleation has not been directly measured. We found a modest reduction in MT growth rate in ran-1(RNAi) embryos; however, this is likely unrelated to the observed spindle assembly defects (Figure 7 and Supplemental Data). Importantly, we observed almost no bias in MT outgrowth during spindle assembly in ran-1(RNAi) embryos (Figures 5C and 5D and Movie S6). This suggests that one of the mechanisms by which RAN promotes spindle assembly is by increasing the frequency of potential MT plus end-kinetochore interactions. We also observed spindle poles drifting apart during metaphase due to a lack of chromosomal-MT attachment, as reported previously (Askjaer et al., 2002). Therefore, loss of kinetochore function could cause decreased MT outgrowth toward chromatin. To test this possibility, we removed kinetochore function via hcp-4(RNAi), which depletes the CENP-C homolog and results in a kinetochore null phenotype (Oegema et al., 2001). In hcp-4(RNAi) embryos, the MT outgrowth bias toward chromosomes was still present, despite spindle poles drifting apart in later stages (Figure 5E). Therefore, we conclude that RAN-1, but not kinetochore function per se, is required for the MT outgrowth bias toward chromatin during spindle assembly.

## EBP-2::GFP Accumulates at the Kinetochore/MT Interface Concomitant with Spindle Elongation at Metaphase

After nuclear envelope breakdown (NEBD), centrosome-nucleated MT plus ends establish contact with chromosomes to begin the process of spindle assembly. In PtK1 cells, MT plus ends attached to kinetochores actively polymerize as chromosomes congress toward the metaphase plate (Tirnauer et al., 2002a). We did not observe any obvious increase in EBP-2::GFP fluorescence near chromatin during chromosome congression. However, we consistently observed an accumulation of EBP-2::GFP fluorescence at the kinetochore region later in metaphase, coincident with spindle pole separation (Figures 6A and 6B and Movie S7). Importantly, upon removal of the CENP-C homolog HCP-4 (Oegema et al., 2001), we no longer observed the accumulation of EBP-2::GFP near the chromatin (data not shown). Therefore, this fluorescence pattern depends on kinetochore function and is consistent with the polymerization of MT plus ends at the kinetochore. Based on previous examination of the timing of anaphase relative to NEBD, as well as the inspection of the DIC component of stream-lapse movies (Experimental Procedures and Movie S8), we infer that EBP-2::GFP fluorescence is most prominent at the kinetochore region during early



Figure 6. EBP-2::GFP Accumulates at the Kinetochore/MT Interface during Spindle Elongation at the End of Metaphase (A) A series of stack projections from one interrupted stream-lapse movie is shown. Each single image is a projection of a short stream acquisition movie (ten frames, 4 s total). EBP-2::GFP accumulation at the kinetochore region coincides with spindle elongation during metaphase (arrowheads) and ~100 s before anaphase in this example (as judged by DIC, not shown). Bar is 10 μm.

(B) The distance between centrosomes through the metaphase-anaphase transition was plotted for four embryos (a–d). The appearance of EBP-2::GFP at the kinetochore/MT interface is time = 0. The EBP-2::GFP fluorescence signal becomes more pronounced at the end of metaphase, at which time the poles begin to move apart prior to anaphase.

spindle elongation, preceding sister chromatid separation.

# RNAi Screening with EBP-2::GFP Reveals Classes of Genes Required

## for Different Aspects of MT Behavior

Recent genome-wide RNAi screens have categorized defects in the early embryo via DIC microscopy and thus provided a better understanding of the relative complexity of many biological processes. For example, Sönnichsen et al., (2005) found 661 genes essential for early embryonic development and provided a phenotypic framework for processes such as those likely to be directly dependent on MT function. We searched for genes that affected the following processes: pronuclear migration, spindle assembly, spindle elongation/integrity, asymmetry of division, nuclear envelope, and chromosome segregation. From this set of approximately 200 genes, we selected representatives from each class for further screening with EBP-2::GFP (40 genes

in total; Figure 7 and Table S1). Our strategy was to analyze known key regulators such as ZYG-9 or  $\gamma$ -tubulin as well as representatives of large protein complexes or pathways required for MT behavior. For each gene, we analyzed MT growth and nucleation rate and noted any other unusual behaviors. We identified the following classes of defects: (1) genes whose RNAi decreases MT growth rate, (2) genes whose RNAi decreases nucleation rate, (3) genes whose RNAi increases nucleation rate, and (4) genes whose RNAi increases retrograde movement of MT plus ends back to centrosomes. The data are summarized in Figure 7, and further details are available in Supplemental Data. Below, we describe the phenotypes of selected genes in each class.

## Genes Whose RNAi Decreases MT Growth Rate *Tubulin Folding Factors*

We analyzed genes implicated in tubulin production in the early embryo (Lopez-Fanarraga et al., 2001; Szymanski, 2002). Specifically, we assayed F16D3.4 (tubulin folding



#### Figure 7. MT Growth Rates and Astral MT Nucleation Rates In Vivo

Growth rates of MTs are shown as white bars, scale is on the left; nucleation rates are shown as thin, blue bars, scale is on the right. Red squares (growth) or red circles (nucleation) indicate significantly lower levels compared to individual wt metaphase controls (Student's t test assuming unequal variances with 95% confidence). Yellow circles indicate significantly higher levels than wt metaphase levels. The proximity of both centrosomes to each other in the dynein function class prevented meaningful nucleation rate estimates. SEM at 95% confidence is shown; half-bars were used for clarity.

An asterisk (\*) likely represents a weak phenotype; more severely affected embryos displayed weak or no EBP-2::GFP and were not measured for growth rate.

§ EBP-2::GFP signal was too weak for an estimation of nucleation rate.

Note: tubulin(RNAi) and F54B3.3(RNAi) (data not shown) were not able to be measured due to an extremely faint or absent EBP-2::GFP signal.

factor D), F53F4.3 ( $\alpha$ -tubulin-specific chaperone), C08F8.1 (a prefoldin subunit), F54A3.3 ( $\gamma$  subunit of the CCT chaperonin complex implicated in tubulin and actin folding), and C05D11.3 (phosducin-like protein, family members of which have been postulated to regulate tubulin folding [Lacefield and Solomon, 2003], possibly via interaction with the CCT [Martin-Benito et al., 2004]).

*F16D3.4(RNAi)*, CCTγ(*RNAi*), and C05D11.3(*RNAi*) all resulted in a substantial reduction in the growth rate of MTs (0.39 ± 0.03 μm/s, 0.38 ± 0.08 μm/s, and 0.42 μm/s ± 0.02, respectively). CCTγ(*RNAi*) also resulted in a majority of embryos with no detectable foci of EBP-2:: GFP (data not shown), as observed with *tubulin(RNAi)* (Figure 1C). Therefore, the embryos exhibiting detectable EBP-2::GFP likely represent a weaker phenotype for this gene and suggest a direct relationship between the amount of active CCT and the rate of MT polymerization in the cell. *C05D11.3(RNAi)* resulted in a less severe phenotype than CCTγ(*RNAi*), but its effect on growth rate was nonetheless consistent with a putative role in CCT function.

In our hands, *F53F4.3(RNAi)* and *C08F8.1(RNAi)* did not result in a significant MT growth rate defect (Figure 7). Therefore, it is possible that these factors are redundant with respect to tubulin folding or that their activity is not essential in the early embryo.

One previously uncharacterized gene, F54B3.3, also gave an RNAi phenotype that most closely resembled severe *tubulin(RNAi)* or strong  $CCT_{\gamma}(RNAi)$  phenotypes. *F54B3.3(RNAi)* produced some embryos with no detectable EBP-2::GFP foci and some embryos with a faint signal at the centrosome with extremely short EBP-2::GFP tracks (omitted from Figure 7 because growth rate measurements were not possible). F54B3.3

encodes an ATPase associated with various cellular activities (AAA), and our data suggest that it may be involved in an early stage of tubulin production or assembly.

#### ZYG-9-TAC-1 and KLP-7

The *C. elegans* XMAP215 ortholog ZYG-9 (encoded by F22B5.7) is required for long astral MTs during pronuclear migration and subsequent mitosis (Bellanger and Gonczy, 2003; Le Bot et al., 2003; Matthews et al., 1998; Srayko et al., 2003). ZYG-9 was found to coimmuno-precipitate with TAC-1 (Srayko et al., 2003), a homolog of the transforming acidic coiled-coil family of proteins (Gergely, 2002), and the protein complex is essential for normal MT function. Manual tracking of EBP-2::GFP (see Experimental Procedures) revealed that both *zyg-9(RNAi)* and *tac-1(RNAi)* resulted in a drastic reduction in the MT growth rate (0.17 ± 0.02  $\mu$ m/s, p = 6.5E–13; and 0.18 ± 0.02  $\mu$ m/s, p = 3.6E–19, respectively; Figure 7; Movie S9).

Previous work in *Xenopus* egg extracts showed that XMAP215 directly opposed the activity of the MT depolymerizing kinesin-13 subfamily member XKCM1 (Kinoshita et al., 2001; Tournebize et al., 2000). This has led to models whereby the relative balance of these opposing activities could regulate MT length in vivo (Holmfeldt et al., 2004; Kinoshita et al., 2002; Kline-Smith and Walczak, 2002). *klp*-7 is the only gene predicted to encode kinesin-13 subfamily members in *C. elegans* (Siddiqui, 2002). By using dsRNA directed against a region common to all predicted splice variants (Chen et al., 2005), we found *klp*-7(*RNAi*) resulted in a decrease in MT growth rate (0.56 ± 0.01  $\mu$ m/s; p = 1.4E–10), perhaps inconsistent with a role in opposing ZYG-9 activity. In order to test whether ZYG-9 counter-

acts the putative depolymerizing activity of KLP-7, we constructed a strain containing both the *zyg-9(b244ts)* mutation and the EBP-2::GFP construct, which we injected with *klp-7* RNA. If ZYG-9 normally protects MTs from KLP-7's MT-depolymerizing activity, one would expect rescue of the Zyg-9(b244ts) phenotype upon removal of KLP-7; however, we found no change in *zyg-9(b244)* MT growth rate after removal of KLP-7 via RNAi (Figure S2; Movie S10; p = 0.51). Therefore, we conclude that KLP-7 and ZYG-9 likely affect MT growth via different pathways.

## ZYG-8

Y79H2A.11 (zyg-8) encodes a doublecortin-like kinase previously reported to affect spindle positioning; in zyg-8 mutants, the mitotic spindle moves prematurely toward the posterior cortex (Gonczy et al., 2001). In vertebrates, loss of doublecortin leads to lissencephaly (smooth brain) and neuronal migration defects, and the family of proteins exhibit MT binding, stabilization, and bundling activity (Edelman et al., 2005; Gleeson et al., 1999; Gonczy et al., 2001; Horesh et al., 1999). In early metaphase zyg-8(RNAi) embryos, we found a slight, albeit significant, reduction in MT growth rate (0.59 ± 0.02  $\mu$ m/s; n = 60; p = 1.3E-10; Figure 7 and Movie S12). Gonczy et al. (2001) reported no obvious MT defects in metaphase stage zyg-8 mutants; however, slightly lower MT growth rate might not manifest as an obvious morphological difference in fixed and immunostained embryos.

### Genes Whose RNAi Decreases MT Nucleation Rates

In our search for factors required for normal MT nucleation rates, we identified components previously implicated in centrosome maturation such as the aurora kinase AIR-1 (Hannak et al., 2001), SPD-2 (Kemp et al., 2004; Pelletier et al., 2004), and SPD-5 (Hamill et al., 2002) (Figure 7, blue bars). After NEBD, these embryos also displayed EBP-2::GFP movements consistent with MT growth from the center of the embryo (Movies S13-S15). As reported in other studies, these MTs are likely nucleated by the chromatin mass (Dammermann et al., 2004; Hamill et al., 2002; Özlü et al., 2005). C25A1.9, a gene that is required for spindle positioning and maintaining spindle length (A.-L. Schlaitz, personal communication: MPI-CBG. Dresden) also displayed a severe MT nucleation deficit (Figure 7, blue bars). Embryos depleted of either  $\gamma$ -tubulin (*tbg-1*) or a member of the γ-tubulin ring complex (gip-1) exhibited intermediately low levels of MT nucleation from centrosomes, supporting previous reports on the existence of an alternate, albeit less efficient, MT nucleation pathway in C. elegans (Hannak et al., 2002; Strome et al., 2001). C08F8.1 (prefoldin) also displayed a similarly intermediate decrease in nucleation levels, consistent with its putative role in  $\gamma$ -tubulin folding (Melki et al., 1993).

We also noticed that centrosomes containing only one centriole throughout the cell-cycle, as in *sas-4(RNAi)* or *zyg-1(RNAi)*, exhibited normal nucleation rates (Figure 7), suggesting that MT nucleation rates are not dependent on the number of centrioles per centrosome.

Genes Whose RNAi Increases MT Nucleation Rates We found that RNAi of the kinesin-13 protein KLP-7 resulted in a  $\sim$ 2-fold increase in astral MT nucleation (195 ± 32% wt; n = 8 cen.; Figure 7, blue bars; Movie S16). The EBP-2::GFP signal at the ends of MTs also appeared brighter in klp-7(RNAi) embryos than in wt; this may indicate an altered plus-end structure or change in EBP-2 affinity. It does not, however, correlate with MT growth rate, because klp-7(RNAi) caused a decrease in MT growth rate (see Results above). Therefore, we conclude that klp-7(RNAi) results in a drastic increase in astral MT nucleation.

We found slightly elevated MT nucleation rates in *zyg-8(RNAi)* embryos ( $135 \pm 12\%$  of wt; n = 8 cen.). This increase, although not as obvious as with *klp-7(RNAi)*, more closely resembled the levels of MT nucleation in anaphase and telophase embryos (Figure 7; blue bars). *zyg-8(RNAi)* also caused a reduction in the intensity of EBP-2::GFP at the ends of MTs, similar to anaphase and telophase embryos but visibly distinct from *klp-7(RNAi)*.

## Genes Whose RNAi Results in an Increase in the Frequency of Retrograde MT Plus-End Movement

While screening for defects in MT growth, we noticed a few genes whose RNAi phenotype included EBP-2::GFP dots of centrosomal origin exhibiting a sudden switch from anterograde to retrograde movement, often followed by a return to anterograde motion (Figure 8A). This movement was also observed in wt metaphase embryos at a frequency of approximately one to three events/min. However, in zyg-8(RNAi), klp-7(RNAi), and mel-26(RNAi) embryos, the frequency increased to as high as 19 events/min (Figure 8C; Movie S17). We also observed an increase in retrograde EBP-2 movements in tpxl-1(RNAi) embryos, the implications of which are discussed in Özlü et al. (2005). Because EBP-2::GFP decorates the growing plus ends of MTs, its sudden reversal in direction does not indicate depolymerization. The speed of MTs during retrograde movement was estimated at approximately  $-1.4 \pm 0.2 \mu$ m/s, relative to the centrosome (n = 14; Figure 8B). One possible explanation for the increase in retrograde EBP-2 movement is a loss of attachment or stability of MT minus ends at the centrosome (Figure 8D and Discussion). For this analysis, most observations were made on midmetaphase embryos. However, prophase embryos, where the nuclear envelope was still intact, also displayed an increase in rapid EBP-2::GFP movements away from centrosomes, much like the dynein-dependent cytoplasmic MT movements observed near the maternal pronucleus (see Results above). This suggests that the primary defect involves a loss of minus-end anchoring, with the movement of MTs back to (or away from) the centrosome being a secondary consequence and a serendipitous marker of a MT minus-end defect. Consistent with this interpretation, mel-26(RNAi) results in ectopic mitotic katanin activity, which would be expected to sever at least some MTs near their minus ends and contribute to a loss of anchoring.

## Discussion

By screening a large fraction of genes required for MTbased processes, we have obtained a system-wide



Figure 8. Retrograde EBP-2::GFP Movement

(A) Shown are a series of images from a stream-acquisition movie of an EBP-2::GFP dot exhibiting retrograde movement (colored green) and separate dot exhibiting normal anterograde growth movement (colored yellow). Centrosome is down. The EBP-2::GFP particle exhibits a switch from retrograde to anterograde movement (arrow heads).

(B) The average velocity of the retrograde movement was calculated by subtracting the average growth rate of MTs determined independently for each embryo ( $\sim$ 0.7 µm/s) from the observed velocity. The net average rate of MT movement was -1.4 ± 0.2 µm/s relative to the centrosome (n = 14). SEM at 95% confidence is shown.

(C) A histogram showing the frequency of retrograde movements observed per minute in wt, mel-26(RNAi), zyg-8(RNAi), and klp-7(RNAi) embryos.

(D) A model to account for retrograde EBP-2::GFP movement. In wt embryos, the depolymerizing kinesin KLP-7 (lightning bolts) limits the numbers of MTs that grow out from the centrosome (white arrowheads, polymerizing MTs; black arrowheads, depolymerizing MTs). In *klp-*7(RNAi) embryos, a ~2-fold increase in nucleation rate was observed as well as an increase in retrograde EBP-2::GFP movement. We propose that different conditions could cause an increase in the number of MTs that detach from the centrosome, e.g., upon MT severing in *mel-26(RNAi)* embryos or if too many MTs are nucleated as in *zyg-8(RNAi)* or *klp-7(RNAi)* shown here. Unlike the anchored MTs, the detached MTs (i) move freely if MT motor proteins act upon them. Retrograde movement (arrows) may result from a plus-end directed motor(s) fixed to the centrosome (ii, red motor), or in conjunction with minus-end depolymerization of the MT, and/or a minus-end directed motor(s) (iii, yellow motor) acting on adjacent MTs.

viewpoint of the control of MT growth. Our analysis of MT growth behavior in early C. elegans embryos suggests that the polymerization of MTs may be regulated by relatively few proteins and largely dependent on the availability of the individual tubulin subunits. Indeed, in vitro studies have demonstrated that MT polymerization rates are directly dependent on free-tubulin concentration (Mitchison and Kirschner, 1984; Walker et al., 1988). In wt worm embryos, MTs grow at the extraordinary rate of  $\sim\!0.7~\mu\text{m/s}$  (42  $\mu\text{m/min})$  throughout most early stages, compared to polymerization rates of ~0.2  $\mu$ m/s (10–14  $\mu$ m/min) in Xenopus (Belmont et al., 1990; Mimori-Kiyosue et al., 2000; Tirnauer et al., 2002b; Tournebize et al., 2000; Wilde and Zheng, 1999), porcine kidney epithelial cells (Piehl and Cassimeris, 2003), and PtK1 cells (Tirnauer et al., 2002a).

Besides factors implicated in tubulin production, only ZYG-9 (the XMAP215 ortholog) and TAC-1 (transforming acidic coiled-coil protein that interacts with ZYG-9) had a severe impact on MT growth rate. However, we also observed a slight decrease in MT growth rates under conditions where centrosome-based MT nucleation rates increased, such as in anaphase or telophase stages in wt and in *klp-7(RNAi)* and *zyg-8(RNAi)* embryos. Perhaps each of these situations can be explained by the same phenomenon—as more MTs grow from the centrosome, existing free-tubulin stores may be insufficient to maintain high growth rates. Presumably, the rate of tubulin consumption and reuse near the centrosome is extreme; therefore, centrosomes themselves may provide a mechanism to buffer local tubulin concentration. It is intriguing that the centrosomally located proteins ZYG-9 and TAC-1 have a drastic effect on MT plus-end growth; perhaps these proteins function to increase local tubulin concentration or availability.

Other than the exceptions mentioned above, most genes that affected nucleation did not have an effect on MT growth rate, indicating that the control of MT outgrowth can be separated from the polymerization process. Furthermore, our data suggest that centrosomes, normally regarded as strictly promoters of MT nucleation, also limit the numbers of MTs through a kinesin-13-dependent pathway. The MTs that polymerize beyond KLP-7's MT-depolymerizing activity continue to grow toward the cortex.

# KLP-7 and ZYG-9 Regulate MT Behavior through Separate Activities

Kinesin-13 kinesins have been previously implicated in the depolymerization of MTs from both plus and minus ends (Desai et al., 1999). Recently, work in a number of systems has focused on their role in kinetochore/MT attachment (Andrews et al., 2004; Kline-Smith et al., 2004; Lan et al., 2004). However, our data suggest that KLP-7 has a central role in limiting the number of MTs growing from centrosomes. Given the known regulation of the kinesin-13 protein MCAK by Aurora kinases, it is possible that the centrosomal Aurora kinase AIR-1 also regulates the activity of KLP-7 to control MT nucleation rates. It is unclear whether KLP-7's role at the centrosome is a conserved property of the kinesin-13 kinesins; however, they are present at spindle poles in all systems studied.

In Xenopus egg extracts, XMAP215 opposes the activity of a kinesin-13 member to regulate MT length. However, we found that ZYG-9 does not simply oppose the activity of KLP-7, rather, it is required independently for MT growth. Although we were unable to estimate nucleation rates in *zyg-9(RNAi)*, the total amount of MT polymer near the centrosomes in *zyg-9(RNAi)* is similar to wt (Srayko et al., 2003). In light of our analysis of MT nucleation, robust asters seem to form through a sequential process of initial MT polymerization (dependent on centrosomal components such as SPD-5, centrosome maturation factors like AIR-1, and MT nucleators like  $\gamma$ -tubulin) followed by either (1) immediate depolymerization via KLP-7 or (2) continued, rapid polymerization away from the centrosome.

## Spindle Positioning Does Not Involve Centrosome-Specific MT Growth Rates

Upon reaching the cortex, MT plus ends exhibit a period of residence before depolymerizing (Labbe et al., 2003). During their residence, MTs may interact with cortically located MT motors, which could provide the pulling forces that ultimately displace the spindle toward the posterior in anaphase. Although a difference between anterior and posterior MT residence times at the cortex has been reported (Labbe et al., 2003), our data suggest that the basis for this does not arise from differences in MT growth rates. Furthermore, with the exception of *zyg-8*, we found no genes within the "spindle position/asymmetry of division" class to influence MT growth rate, and no RNAi phenotypes from the entire dataset resulted in a de novo difference between anterior and posterior MT growth rates (data not shown).

ZYG-8 is part of the doublecortin family of MT binding proteins, and *zyg-8(RNAi)* results in premature movement of the mitotic spindle toward the posterior cortex (Gonczy et al., 2001). Previous reports suggested that ZYG-8 was required specifically for anaphase MT stability based, in part, on an absence of obvious MT defects in metaphase embryos (Gonczy et al., 2001). Our data revealed a slight but significant decrease in MT growth rate as well as an increase in nucleation rate in metaphase *zyg-8(RNAi)* embryos. Although not necessarily related to the spindle mispositioning defect, it is nevertheless intriguing that metaphase *zyg-8(RNAi)* embryos exhibit many features of anaphase embryos, with respect to the EBP-2::GFP assays described herein. As has been suggested for maturing neuronal cells (Edelman et al., 2005), perhaps doublecortins function to rapidly alter MT dynamics and/or polymer structure in response to changes in the cell cycle or cell fate.

## Retrograde EBP-2::GFP Movement

Predominantly, astral MTs in C. elegans embryos exhibit steady growth of plus ends (EBP-2::GFP dots) away from centrosomes, followed by loss of EBP-2::GFP, usually upon reaching the cell cortex. This presumably corresponds to depolymerization of the MT. However, we also observed movements of EBP-2::GFP that were not consistent with simple growth behavior of wt astral MTs. These retrograde movements could result from a plus-end directed MT motor protein(s) fixed to the centrosome, or a minus-end directed motor(s) traveling toward the centrosome acting on adiacent "free" MTs (Figure 8D). Alternatively, an increase in minus-end depolymerization coupled with some mechanism to maintain connection to the centrosome during the depolymerization may also be responsible for the effect.

In both zyg-8(RNAi) and klp-7(RNAi), we observed an increase in the frequency of retrograde EBP-2::GFP movements. Importantly, KLP-7's putative depolymerizing activity directed against MT minus ends cannot be responsible for this movement. Another phenotype presented by both zyg-8(RNAi) and klp-7(RNAi) is an increase in MT nucleation. Perhaps excessive MT outgrowth saturates the anchoring capacity of the centrosome, which manifests as more retrograde EBP-2 movement (Figure 8D). This interpretation predicts that anchoring and nucleation are distinct processes. In support of this, we did not observe increased retrograde movement in either tbg-1(RNAi) (γ-tubulin) or gip-1(RNAi) (y-tubulin ring complex protein) (Figure 7 and M.S., unpublished data). Also, mel-26(RNAi) led to an increase in EBP-2 retrograde movement, despite normal MT nucleation rates. MEL-26 is a postmeiotic negative regulator of the MT-severing enzyme katanin (Dow and Mains, 1998; Pintard et al., 2003). In mutant scenarios whereby katanin persists into the first mitosis, the proposed molecular consequence is inappropriate MT severing (Srayko et al., 2000). Therefore, if MTs detached from their anchoring sites (as a result of either overnucleation or inappropriate severing), motor proteins could pull free MTs back toward the centrosome where they may reanchor and continue to grow, as evidenced by the recovery of retrograde motion to anterograde motion (Figure 8A).

# Spindle Assembly Involves Biased Nucleation of MTs toward Chromosomes

In meiotic systems, MTs are stabilized around chromosomes by local nucleation and local control of cata-

strophe rate; however, the ways in which MTs are stabilized during mitotic spindle assembly are unclear. Our analysis shows that during mitotic spindle assembly, MT growth rate does not change, consistent with results from PtK1 cells (Tirnauer et al., 2002a). However, we did observe a significant increase in the number of MTs growing toward the chromosomes over time. We also found that ran-1, but not kinetochores, was important for this growth bias. Because RAN-1 has many roles in the cell, including nuclear import/envelope assembly and MT stabilization, it remains an open question as to how the bias in outgrowth is achieved. Our analysis of astral MT nucleation suggests that ran-1 does not influence MT nucleation globally. During early stages of wt spindle assembly in C. elegans, the nuclear envelope remains intact, except for two fenestrae, through which MTs grow toward chromatin (Kirkham et al., 2003). The remnant nuclear envelope could be important for increasing MT turnover, perhaps by stimulating MT depolymerization. In addition, the nuclear membrane may physically restrict the trajectory of MTs emerging from the chromatin side of the centrosome, thereby increasing the relative numbers of MTs that occupy this region of the cell during early spindle assembly. The rapid rate of MT growth in C. elegans suggests that MT plus-end capture by chromosomes could occur within five seconds of entering the nuclear envelope and that any uncaptured MTs would turnover soon after reaching the nuclear envelope.

Although the MT density was too high in the spindle to accurately determine the fate of MTs contacting the chromosomes, we did observe significant incorporation of EBP-2::GFP at kinetochores concomitant with the slight elongation of the spindle at the end of metaphase. Similarly, EB1 was observed in PtK1 cells specifically on polymerizing MT ends at kinetochores, for instance, during antipoleward movement of chromosomes (Tirnauer et al., 2002a). Perhaps EBP-2::GFP accumulates via polymerization of MT ends simply in order to maintain spindle integrity during this preanaphase stretch. In addition, the presence of EBP-2::GFP at the kinetochore at this time may be an important mechanism for the spindle to send a "test for chromosome attachment" signal to a spindle assembly checkpoint/delay pathway prior to the onset of anaphase (Encalada et al., 2005). The lack of an obvious EBP-2::GFP signal at the kinetochores prior to spindle pole separation in metaphase is consistent with the recent suggestion that very little minus-end treadmilling (MT flux) occurs in C. elegans (Labbe et al., 2004).

Taken together, our analysis of spindle assembly in *C. elegans* suggests a simple strategy of increasing MT turnover within the nuclear environment, with attachment of MTs to kinetochores resulting in their stabilization until the spindle is stretched just prior to anaphase. Thus, *C. elegans* mitotic spindle assembly seems, in essence, to recapitulate the search and capture model originally proposed by Mitchison and Kirschner (1985).

#### **Experimental Procedures**

### Worm Strains and Imaging

All worm strains were maintained as described (Brenner, 1974). The following strains were used: N2 (wt), DH1 *zyg-1(b1)*, DH244 *zyg-9(b244)*, and TH66 (EBP-2::GFP). C-terminal EBP-2::GFP is ex-

pressed from the pie-1 promoter, and transgenic worms were created by microparticle bombardment, as described (Praitis et al., 2001). TH66 was maintained at 25°C. Embryos were dissected and mounted with 0.1 M NaCl 4% sucrose or egg buffer (Edgar, 1995) on 2% agarose pads. We observed a direct relationship between temperature and growth rate (Figure S3). Therefore, temperature was maintained at 20  $\pm$  0.5°C by using a temperature-controlled objective collar and measuring the temperature of the immersion oil between the objective and the coverslip immediately after each recording. EBP-2::GFP movies were acquired with a Hamamatsu Orca ER 12 bit digital camera mounted on a spinning disk confocal microscope (Zeiss Axioplan using a 63x 1.4 NA PlanApochromat objective and Yokogawa disk head); illumination was via 488 nm Argon ion laser (Melles Griot). Image processing was done with MetaView Software (images acquired at 400 msec intervals [2 × 2 binning], with the integration time for each acquisition 389-399 msec). Each movie was 150 frames = 1 min.

Stream-lapse movies were a live GFP acquisition of ten frames (4 s, total exposure) interrupted by a short DIC time-lapse movie (~11.5 s total time). This allowed visualization of the intracellular distribution of EBP-2::GFP fluorescence during metaphase and anaphase (Figure 4 and Movie S12).

### Data Analysis and EBP-2 Tracking

Tracking of EBP-2::GFP was performed with a user-designed interface (Michael Volkmer, Scionics Computer Innovation GmBH) for National Instruments LabView pattern recognition software. For pattern recognition, we used the IMAQ Vision module for image processing and machine vision. For MT ends, we used a pattern size of 7 × 7 pixels limited to 100 possible matches and a minimum score of 350–400, within a nine pixel search radius of the last pattern match, on a 2x zoomed TIFF stack. The "update pattern" algorithm was not used.

EBP-2::GFP dots were manually selected for tracking. Roughly ten MTs were analyzed per centrosome, from at least three embryos. We chose dots that remained in the focal plane for long distances (up to  $\sim 10 \ \mu$ m) whenever possible. This reduced errors intrinsic to estimating the velocities of objects in three-dimensional space with a single plane imaging system. By subtracting the centrosome position (origin) from the EBP-2::GFP dot position at each time interval, we determined MT growth over time (Figure 2A). The growth rates of individual MTs were calculated by estimating the slope of each line over the longest linear component (Microsoft Excel). For every RNAi experiment, at least one wt control embryo was recorded. For measuring growth rates of cytoplasmic MTs, we were unable to reliably track the movements by using the tracking program. Therefore, we chose EBP-2::GFP dots that could be observed for 10 frames, determined their start and stop positions within this period, and traced their paths using Metamorph software (Universal Imaging) to estimate their velocity. If EBP-2::GFP was too faint to be reliably tracked automatically (e.g., zyg-9[RNAi]) or the density of MTs was too high to avoid cross pattern matching (e.g., telophase embryos or spindle MTs), a manual tracking interface was used, and the average velocity over the full path was calculated. Other data analysis and graphing of results was performed with Excel (Microsoft) and Canvas.

#### MT Nucleation Assay

For nucleation assays, a half-circle (29–30  $\mu$ m) was drawn around the centrosome, positioned 9  $\mu$ m from the centrosome, and a kymograph of the entire movie (150 frames, 1 min) was generated (Metamorph, Universal Imaging). EBP-2::GFP dots on the kymograph were manually counted. For anaphase measurements, we used anterior centrosomes (which exhibit less rocking during anaphase). Early telophase embryos exhibit relatively stable centrosome positioning, despite their increasing dismorphology and disintegration.

#### Antibodies and Immunofluorescence

Antibodies were generated against nearly full-length VW02B12L.3 (aa 1–297) cloned into BgIII/EcoRI sites of the pRSET-A expression vector (Invitrogen). Primers used for PCR amplification and subsequent cloning were 5'-CGCGCGAGATCTATGGTCGTCAACGTGTT

CAT-3' (forward) and 5'-GCGCGCGAATTCTCACTCCGTATCGTC CAGATTTG-3' (reverse), which produced a PCR product size of 890 bp. The HIS-tagged fusion protein was 32.8 kDa. Fixed and immunostained images in Figure 1 are three-dimensional wide-field data sets computationally deconvolved and projected by using SoftWorx (Applied Precision) software.

#### RNAi

RNA injections were performed as in Sönnichsen et al. (2005). Worms were grown for 22–25 hr (according to the individual dsRNA) at 25°C after injection. RNAi of  $\alpha$ - and  $\beta$ -tubulin was performed as in Cowan and Hyman (2004). Primer sequences for the RNAi experiments described herein can be found online at http:// www.worm.mpi-cbg.de/phenobank2.

#### Supplemental Data

Supplemental Data include Supplemental Results, three figures, one table, and 17 movies and are available with this article online at http://www.developmentalcell.com/cgi/content/full/9/2/223/DC1/.

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