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Report

Making Microtubules and Mitotic Spindles in Cells without Functional Centrosomes

Nicole M. Mahoney,[1] Gohta Goshima,[1] Adam D. Douglass,[1] and Ronald D. Vale[1,*]
[1] The Howard Hughes Medical Institute and Department of Cellular and Molecular Pharmacology University of California, San Francisco San Francisco, California 94107

Summary

Centrosomes are considered to be the major sites of microtubule nucleation in mitotic cells (reviewed in [1–3]), yet mitotic spindles can still form after laser ablation or disruption of centrosome function [4–7]. Although kinetochores have been shown to nucleate microtubules [3], mechanisms for acentrosomal spindle formation remain unclear. Here, we performed live-cell microscopy of GFP-tubulin to examine spindle formation in *Drosophila* S2 cells after RNAi depletion of either γ-tubulin, a microtubule nucleating protein [8], or centrosomin, a protein that recruits γ-tubulin to the centrosome [7, 9]. In these RNAi-treated cells, we show that poorly focused bipolar spindles form through the self-organization of microtubules nucleated from chromosomes (a process involving γ-tubulin), as well as from other potential sites, and through the incorporation of microtubules from the preceding interphase network. By tracking EB1-GFP (a microtubule-plus-end binding protein) in acentrosomal spindles, we also demonstrate that the spindle itself represents a source of new microtubule formation, as suggested by observations of numerous microtubule plus ends growing from acentrosomal poles toward the metaphase plate. We propose that the bipolar spindle propagates its own architecture by stimulating microtubule growth, thereby augmenting the well-described microtubule nucleation pathways that take place at centrosomes and chromosomes.

Results and Discussion

Cells Depleted of Centrosomin Form Spindles by a "Meiotic-like" Process

Drosophila S2 cells depleted of centrosomin (Cnn) (see immunoblot analysis in Figure S1 in the Supplemental Data available online) formed anastral spindles and did not recruit γ-tubulin to spindle poles (Figure 1) [7]. γ-Tubulin, however, was present on spindle microtubules after Cnn depletion, as we also observed for wild-type cells (Figure 1).

To probe the dynamics of spindle formation after Cnn depletion, we performed time-lapse microscopy on GFP-tubulin-expressing cells. In wild-type cells, the majority of the early prophase microtubules originated from the centrosomes immediately after nuclear-envelope breakdown (NEB) (Figure 2A and Movie 1). However, microtubules also clearly formed around the chromosomes, as previously observed (Figure 2A′) [3]. In Cnn-RNAi-treated cells, time-lapse microscopy revealed a very different pathway of spindle formation. Centrosomal microtubule asters did not form at prophase, but robust microtubule nucleation still occurred at chromosomes (Figures 2B and 2B′; Movies 2 and 3). The interphase microtubule array, which only gradually destabilized after NEB, also incorporated into the spindle [10], and in some cases we observed attachment and capture of pre-existing microtubules by chromosomes (Figure S2). After initially collecting around chromatin, microtubules then elongated and became focused to create a bipolar spindle with broad, dynamic poles, as described for meiotic spindle formation [11]. Time-lapse microscopy also revealed that Cnn RNAi cells proceeded into anaphase without any significant delay (Movie 3), a result that is consistent with their normal mitotic index (Table S1). Thus, even when the dominant pathway of microtubule-based search-and-capture of chromosomes by centrosomal microtubules is completely disrupted in Cnn RNAi cells, our live-cell imaging reveals that chromosome-mediated nucleation and incorporation of existing microtubules generate a functional bipolar spindle in a time period comparable to that in wild-type cells.

A Role for γ-Tubulin in Chromatin-Mediated Microtubule Assembly

We next depleted S2 cells of ~90% γ-tubulin by RNAi (Figure S1), although residual γ-tubulin may create a hypomorphic situation rather than a true null. (Note that γ-tubulin refers to the ubiquitously expressed 23C isotype; RNAi of an ovary-specific (37C) isotype did not produce a phenotype in S2 cells [Table S1].) Mitotic S2 cells depleted of γ-tubulin by RNAi still contained microtubules, although the mitotic spindles were virtually all abnormal. The most common morphologies were monopolar spindles (~40%) and anastral bipolar spindles with poorly focused poles (~60%) (Figure 1B; Table S1). The mitotic index was elevated 3.3-fold (Table S1), but anaphase cells were observed in the population. The above results are consistent with prior studies showing that interfering with γ-tubulin function results in severe spindle defects, although microtubules can still form occur and chromosome alignment can occur [12–17].

To better understand the mechanism of spindle formation after γ-tubulin depletion, we performed live-cell imaging of GFP-tubulin-expressing cells. In cases where NEB was observed, microtubule formation from the chromosomal region was dramatically reduced or significantly delayed in comparison with wild-type and Cnn-RNAi-treated cells (Movie 4). This result, combined with our observation of spindle localization of γ-tubulin in Cnn-depleted cells, suggests that γ-tubulin functions as a microtubule nucleator in the chromosome-mediated microtubule assembly pathway. This finding

*Correspondence: vale@cmp.ucsf.edu

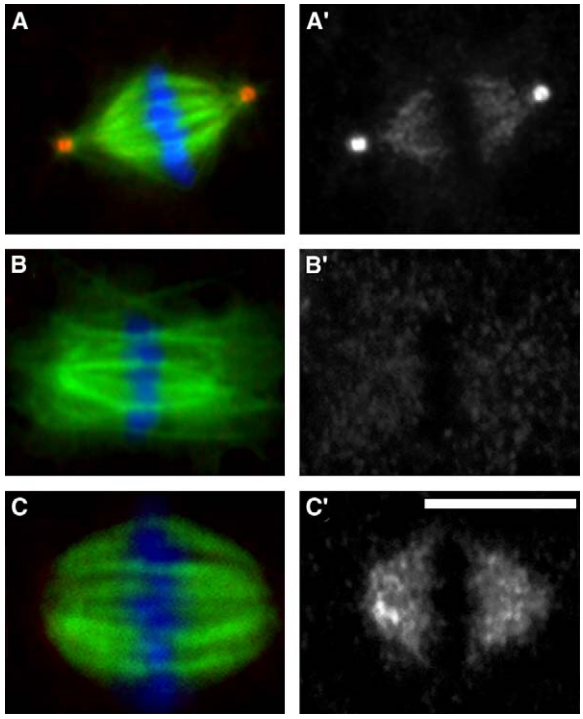

Figure 1. Mitotic-Spindle Phenotypes and γ-Tubulin Localization after RNAi of γ-Tubulin and Centrosomin in *Drosophila* S2 Cells

(A–C) GFP-tubulin (green)-expressing cells were fixed and stained with antibodies against γ-tubulin (red) and DAPI (blue). (A) A typical, untreated S2 cell. (C) RNAi of centrosomin (Cnn) produces well-organized, anastral spindles. (B) Cells treated for 5 days with dsRNA targeted to γ-tubulin 23C display disorganized anastral spindles (an example is shown here) (60%) as well as monopolar spindles (not shown) (~40%). The γ-tubulin signal alone is shown for wild-type (A′), Cnn RNAi (C′), and γ-tubulin RNAi (B′) cells. γ-tubulin localization throughout the body of the spindle is seen in wild-type and Cnn-depleted cells, but not after γ-tubulin RNAi. This results shows that γ-tubulin is targeted to spindles as well as centrosomes. The bar represents 10 μm.

indicates that γ-tubulin need not be anchored at the centrosome to stimulate microtubule nucleation. While this paper was in review, a similar conclusion was reached in mammalian cells on the basis of RNAi of a γ-tubulin-associated subunit [18].

The mechanism of assembly of spindle microtubules in γ-tubulin RNAi cells was difficult to decipher from our time-lapse movies, and it is possible that the microtubules originate from several sources. One such source appears to be pre-existing interphase microtubules, which coalesce into bundles after NEB and can engage chromosomes as described above for Cnn-depleted cells. Fragments of former "interphase" microtubules might also act as nucleating seeds for new microtubule growth. In addition, we also observed "focal points" of microtubule growth, which could represent nucleation from centrioles, as previously reported [17], or from sites on the fragmenting nuclear envelope [19]. After γ-tubulin RNAi, time-lapse imaging showed that cells usually formed a monopolar spindle initially, as seen in fixed cell images, which often converted to a bipolar spindle through the formation of a second pole (Movie 5). Such bipolar spindles, however, are very unstable and exhibited much more splaying and disorganization than Cnn RNAi cells (Movie 4). Bipolar metaphase spindles in γ-tubulin RNAi cells stall for at least twice as long as in wild-type cells, explaining the increased mitotic index, but eventually can complete anaphase and cytokinesis.

In conclusion, our live-cell imaging reveals several redundant mechanisms for creating mitotic spindles via (1) centrosome-based nucleation, (2) chromosome-based assembly [4], and (3) recruitment of microtubules created at other sites [10]. Centrosome nucleation of microtubules constitutes the dominant pathway of spindle formation in wild-type cells, but the other processes can generate spindles in the absence of centrosome function (i.e., after Cnn or γ-tubulin depletion).

Microtubule Nucleation during Metaphase as Assayed with EB1-GFP

Time-lapse microscopy of GFP-tubulin was effective for examining the initial events in bipolar spindle formation. However, because of the high density of microtubules in the spindle, it was difficult to visualize sites of microtubule nucleation and growth during metaphase. To gain information on these issues, we performed live-cell imaging of a stable S2 cell line expressing low levels of EB1-GFP, a microtubule-plus-end-tracking protein that localizes to the terminal ~0.5 μm tip of growing microtubules and that has been used to investigate cell-cycle-dependent microtubule nucleation [20, 21].

In wild-type metaphase cells, EB1-GFP punctae emerged in a radial pattern from the centrosome (Movie 6), as has been noted in other studies [20, 21]. In addition, individual EB1-GFP punctae were visible in the microtubule-dense regions of the spindle. A semiautomated program was employed to identify and track EB1-GFP over time and create vectors plots for the growth of individual microtubules (Figure 3A, Supplemental Experimental Procedures). The vector maps of EB1-GFP revealed an overall image that corresponded to the shape of the wild-type mitotic spindle (Figure 3B), and computer-generated vectors were in good agreement with manual tracking and visual inspection of the movies. However, we acknowledge limitations in establishing the precise origin of all EB1-GFP puncta, given that some may have originated out of the plane of focus (most punctae, however, appeared suddenly as expected for nucleation in the focal plane) and that the automated program often terminated tracking when punctae crossed and overlapped in dense regions of the spindle.

For Cnn-RNAi-treated cells in metaphase, the radial distribution seen of vectors at the poles for wild-type cells was not observed. Surprisingly, however, EB1-GFP tracking revealed vectors originating throughout the spindle, including many vectors arising from the acentrosomal poles and traveling toward the chromosomes (Figure 3C; Movie 7; see Figure S4 for analysis of vector angles). This result was not anticipated, given that our imaging of GFP-tubulin showed initial microtubule nucleation around chromosomes after NEB and not from a peripheral nucleating site. Thus, whereas microtubule formation initially relies upon the chromosomes, the spindle itself acquires a mechanism for forming new EB1 punctae distal from the chromosomes.

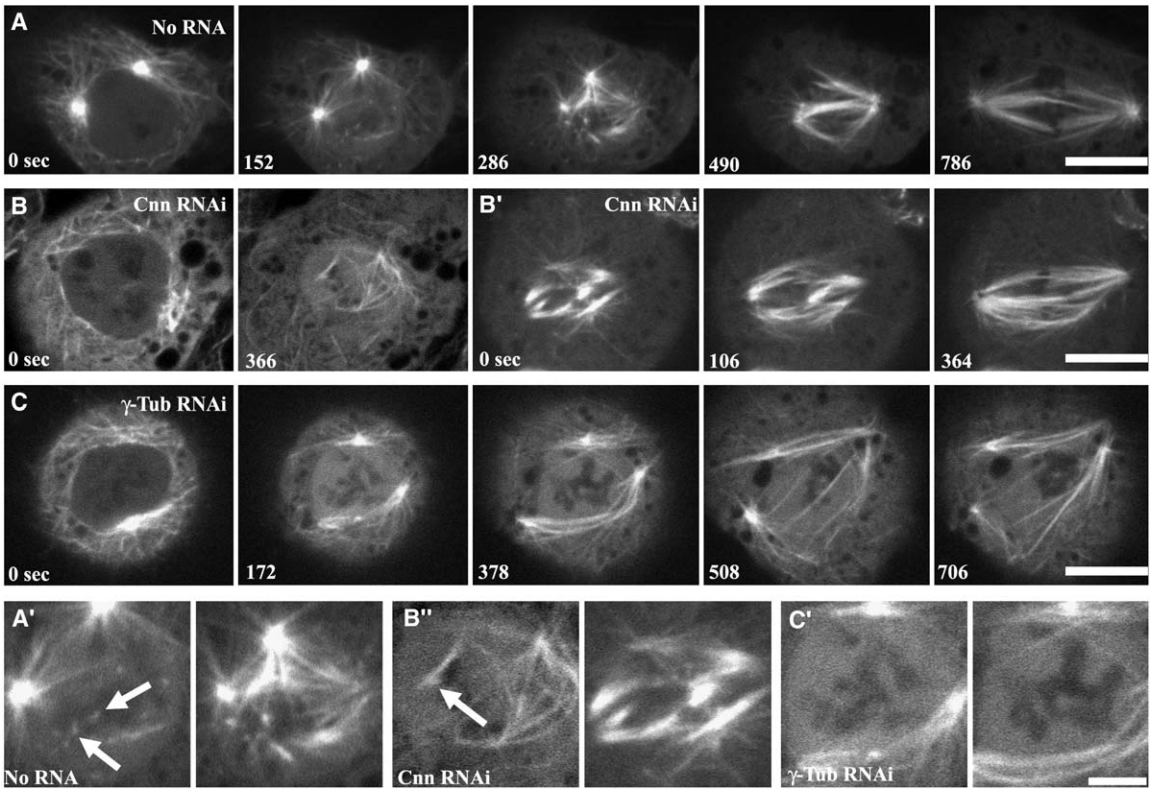

Figure 2. Real-Time Imaging of GFP-Tubulin during Initial Stages of Spindle Formation in Untreated and RNAi Cells

(A) In untreated cells, microtubules are nucleated robustly from the centrosomes shortly after nuclear-envelope breakdown (NEB), and to a lesser degree from the chromosomal region (enlarged images are also presented in A′). See also Movie 1.

(B) After Cnn RNAi treatment, asters do not form. Instead, microtubules form around chromosomes, and the spindle grows outward from the cell center (see [B′] from another cell and enlarged image in [B″]; Movie 2). After γ-tubulin-23C RNAi treatment, microtubule nucleation around the chromosomes does not occur (C and C′). Instead, spindle microtubules form from a variety of sources (discussed in the text) (Movies 3 and 4). It is probable that some residual γ-tubulin remained in this cell, because poorly formed asters were present (which facilitated identification of prophase cells for time-lapse imaging). Nevertheless, after NEB, microtubule and spindle formation differed dramatically from that in untreated cells. Arrows indicate microtubule nucleation sites at chromosomes. The bars represent 5 μm (A–C, B′) and 2 μm (A′, B″, C′).

However, microtubules also probably continue to form around the metaphase chromosomes in Cnn-RNAi-treated cells, because there are many EB1-GFP vectors in the chromosomal region that do not extrapolate back to the spindle poles (not shown). In γ-tubulin RNAi cells that formed anastral bipolar-like spindles (Figures 3B and 3C; Movie 8), the EB1-GFP vector diagrams looked similar to those described for Cnn-RNAi-treated cells, revealing microtubule growth originating throughout the spindle and from the broad polar regions. In contrast to the selective growth of microtubules from acentrosomal poles to chromosomes in the Cnn RNAi cells, however, the orientation of the vectors in the γ-tubulin RNAi spindles tended to be more random (Figure 3C; Figure S4).

We next wished to determine whether EB1-GFP punctae formed within wild-type spindles or whether this phenomenon is a consequence of γ-tubulin depletion/mislocalization. In some wild-type cells, the centrosome and its astral array became transiently disconnected and displaced from the kinetochore fibers, producing a clear spatial separation of the two microtubule networks in the spindle (Figure 3B; Movie 9). In such cells, we still observed EB1 vectors originating from the focused minus-end region of kinetochore fibers; these acentrosomal vectors again were preferentially directed toward the chromosomes (Figure S4). We also performed RNAi of abnormal spindle protein (Asp), and this treatment resulted in centrosome detachment from the main body of the spindle and splaying of kinetochore fibers [22] (Movie 10). In this situation, movies of EB1-GFP also revealed fluorescent punctae originating from acentrosomal regions of splayed kinetochore fibers and moving toward the chromosomes. Thus, a process of spindle-based microtubule formation and growth occurs from acentrosomal foci of kinetochore fibers and within spindles, even in cells that possess functional centrosomes.

In summary, our analysis of both γ-tubulin and Cnn RNAi cells, as well as untreated cells, shows EB1-GFP punctae forming within the spindle and from acentrosomal poles and traveling toward the chromosomes. Figure 4 presents four mechanisms by which EB1 punctae might be generated in the spindle. Although discussed separately, we emphasize that these models are not mutually exclusive, and indeed, it is plausible that multiple mechanisms might be contributing to this phenomenon. One possibility for generating new EB1 punctae is through the rescue of a kinetochore microtubule that underwent catastrophe (Figure 4A). Observation of individual microtubule catastrophe and rescue events in the spindle is not technically possible because of the high microtubule density. Tirnauer et al. [23], however,

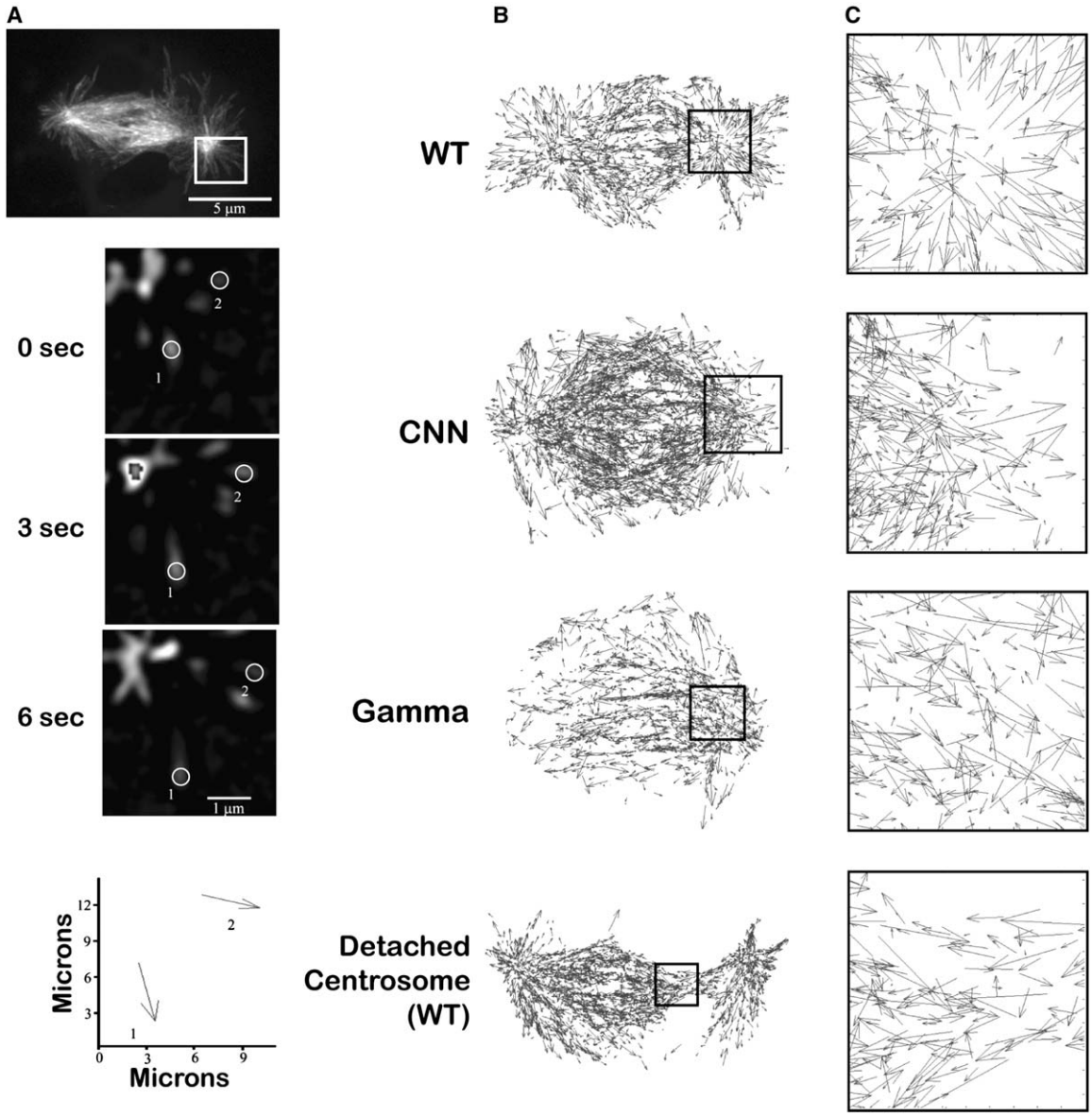

Figure 3. Time-Lapse Imaging and Analysis of EB1-GFP in the Metaphase Spindles of Wild-Type and RNAi-Treated Cells

(A) Example of tracking method from a movie of an untreated, EB1-GFP-expressing cell. Top panel shows a Z stack of a time series of EB1-GFP tracked with a spinning-disk confocal microscope. The boxed region is magnified in the middle panels, and two isolated EB1-GFP spots (arrows) were tracked at 3 s intervals. In the bottom panel, the vectors of these moving EB1-GFP spots are shown (see Supplemental Experimental Procedures).

(B) MATLAB-generated vector diagrams of the EB1-GFP movies for a representative wild-type cell (also Movie 6), a Cnn-RNAi-treated cell (Movie 7), a γ-tubulin-RNAi-treated cell (Movie 8), and a wild-type cell with a detached centrosome (Movie 9).

(C) Higher magnification of the centrosome (wild-type cell) or acentrosomal poles (others). This 4-μm-square box corresponds to the box in (B). Enlargements of boxes are found in Figure S3. Vectors are shown originating near the acentrosomal pole, suggesting a mechanism of microtubule formation that does not rely upon centrosomes or chromosomes. Analysis of the angles of the vectors in (C) is presented in Figure S4.

induced microtubule depolymerization by severing *Xenopus* spindles with a microneedle and concluded that rescue and regrowth was rare, especially near the poles. (This same study also noted EB1-GFP formation throughout *Xenopus* spindles in vitro.) We were able to image single astral microtubules in GFP-tubulin-expressing S2 cells, and our observations also reveal a very low rescue frequency of depolymerizing astral microtubules (only 5% of the shrinking microtubules underwent a clear rescue event [n = 137]; Movie 11). Nevertheless, we cannot exclude the possibility of a novel mechanism that selectively stimulates rescue in spindle versus the astral microtubule population in S2 cells.

EB1 punctae in the spindle could also reflect the generation of additional microtubules, by either microtubule fragmentation or nucleation (Figures 4B–4D). An important clue in considering such models is that the EB1-GFP vectors tend to be constrained within the cone angle of the spindle and grow toward the chromosomes, in

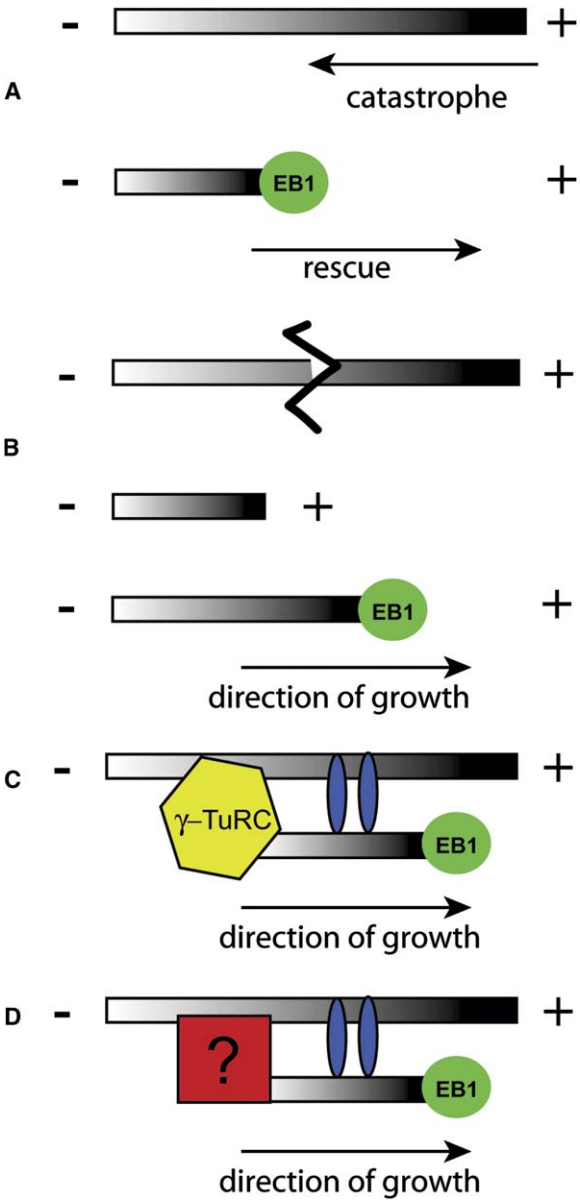

Figure 4. Models for the Observed Growth of EB1-Labeled Microtubules within the Body of the Spindle

(A) Catastrophe of stable microtubules followed by rescue can generate new growth and plus-end labeling by EB1 within the spindle, although our observations of astral-microtubule dynamics show that rescue is rare and thus perhaps unlikely to be the sole source by which EB1-GFP punctae are generated within the spindle.

(B) Microtubule severing of existing microtubules generates new microtubule plus ends that grow and recruit EB1.

(C) γ-tubulin-mediated growth of new microtubules, potentially nucleating from the sides of pre-existing microtubules.

(D) An unidentified protein nucleates new microtubules. See text for more details.

contrast to the radial nucleation/growth that occurs from centrosomes (Figure S4). Microtubule severing followed by regrowth of the newly created plus end could produce such results (Figure 4B). Alternatively, a de novo templating reaction from existing spindle microtubules could occur (Figures 4C and 4D). For example, a nucleator could bind to the side of existing microtubules and template new microtubules at a shallow angle to the mother filament, followed by crosslinking/bundling to pre-existing kinetochore microtubules. Such a mechanism is analogous to the binding and nucleation of new actin filaments by Arp2/3 bound to a pre-existing actin filament. Precedence for this idea comes from recent reports showing that γ-tubulin can nucleate microtubules from pre-existing interphase microtubules in *S. pombe* [24] and in plants [25]. We find that γ-tubulin is present throughout the spindle in S2 cells (Figures 1A′ and 1C′), which might favor such a possibility in mitosis as well (Figure 4C). The fact that γ-tubulin RNAi cells still form EB1-GFP punctae at the broad polar regions cannot necessarily be taken as evidence against γ-tubulin involvement in such a de novo microtubule nucleation mechanism, because residual γ-tubulin remains after RNAi. Moreover, EB1-GFP vectors in γ-tubulin RNAi spindles differ from those in wild-type and Cnn RNAi cells in being less dense and more random in orientation (less selective growth toward the midzone compared with γ-tubulin-containing spindles; Figure S4). Alternatively, a novel microtubule nucleator may be involved (unidentified factor in Figure 4D) or could contribute in addition to γ-tubulin.

Clearly, further studies will be required to identify the molecule(s) responsible for generating microtubule growth at acentrosomal poles and within spindles. However, the present study illustrates that, in addition to the well-described pathways of centrosomal and chromosomal microtubule nucleation, the metaphase spindle possesses a mechanism (or mechanisms) for propagating its own architecture by promoting microtubule assembly.

Supplemental Data

Supplemental Data include four figures, one table, 11 movies, and Supplemental Experimental Procedures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/6/564/DC1/.

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