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Report

Molecular Requirements for Kinetochore-Associated Microtubule Formation in Mammalian Cells

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Summary

In centrosome-containing cells, microtubules nucleated at centrosomes are thought to play a major role in spindle assembly [1]. In addition, microtubule formation at kinetochores has also been observed [2–5], most recently under physiological conditions in live cells [6]. The relative contributions of microtubule formation at kinetochores and centrosomes to spindle assembly, and their molecular requirements, remain incompletely understood. Using mammalian cells released from nocodazole-induced disassembly, we observed microtubule formation at centrosomes and at Bub1-positive sites on chromosomes. Kinetochore-associated microtubules rapidly coalesced into pole-like structures in a dynein-dependent manner. Microinjection of excess importin-β or depletion of the Ran-dependent spindle assembly factor, TPX2, blocked kinetochore-associated microtubule formation, enhanced centrosome-associated microtubule formation, but did not prevent chromosome capture by centrosomal microtubules. Depletion of the chromosome passenger protein, survivin, reduced microtubule formation at kinetochores in an MCAK-dependent manner. Microtubule formation in cells depleted of Bub1 or Nuf2 was indistinguishable from that in controls. Our data demonstrate that microtubule assembly at centrosomes and kinetochores is kinetically distinct and differentially regulated. The presence of microtubules at kinetochores provides a mechanism to reconcile the time required for spindle assembly in vivo with that observed in computer simulations of search and capture.

Results and Discussion

Ran-Dependent Kinetochore-Associated Microtubule Formation in Centrosome-Containing Cells

To study spindle assembly in mammalian cells, we developed a technique in which microtubule formation at centrosomes and chromosomes can be examined separately. GFP-tubulin-expressing cells were first treated with nocodazole to completely disassemble microtubules [3]. In these cells, centrosomes could be visualized because GFP-tubulin fluorescence remained associated with centrosomes, presumably as a result of GFP-tubulin in the centriole triplet microtubules (Figure 1). Importantly, microtubule disassembly resulted in many mitotic cells in which centrosomes were well separated from chromosomes (Figure 1).

Complete removal of nocodazole, which requires four washes in drug-free media, resulted in the rapid formation of a bipolar spindle and completion of mitosis. Microtubule assembly was observed at each centrosome and at discrete sites on chromosomes (Figure 1A, and Movie 1 in the Supplemental Data available online). Within several minutes of nocodazole removal, chromosomes and associated microtubules formed small clusters that were captured and drawn toward the centrosomes [7]. Dynamic interactions of centrosomal microtubules with both kinetochores and microtubules associated with kinetochores were observed in these cells (Movie 1). The long centrosomal microtubules that formed following 4× or 2× washouts (Figure S1; Movie 2) interfered with analysis of microtubule formation near chromosomes, so we partially removed nocodazole with a single rinse in drug-free media. Examination of these cells showed that short microtubules formed first at centrosomes, as evidenced by a rapid increase in centrosome-associated GFP-tubulin fluorescence (Figure 1B insets; Movie 3). Subsequently, microtubules formed at discrete sites on chromosomes and in some cases, fiber-like structures could be detected (Figure 1B′). Although chromosomes initiate microtubule formation more slowly than centrosomes, the extent of microtubule-polymer formation was greater at chromosomes, indicating a stabilizing effect on the polymer that is formed (Figure 1D). These chromosomal microtubules rapidly coalesced into pole-like structures with chromosomes arranged at the periphery (Figure 1B). Analysis of cells released from nocodazole showed that microtubule bundles terminated at structures positive for the kinetochore protein Bub1 (Figure S2).

In cells lacking centrosomes, microtubule assembly is stimulated near chromatin by a Ran-dependent pathway [8–10]. Direct observations further show that microtubule formation near chromatin in *Xenopus* extracts is regulated by a gradient of the small GTPase Ran [11, 12]. Prior to our 1× washout, we microinjected nocodazole-treated mitotic cells with excess importin-β to block Ran-dependent activation of spindle assembly factors [13–15]. In the injected cells, microtubule formation at chromosomes was severely inhibited, whereas microtubule formation at centrosomes either was not altered or was slightly enhanced, demonstrating that microtubule formation at kinetochores, but not centrosomes, is Ran-dependent (Figure 1C). When centrosomal microtubules were present in importin-β-injected cells, chromosome capture was observed, indicating that perturbation of microtubule formation at kinetochores does not affect capture of chromosomes by astral microtubules (Figure 1C′).

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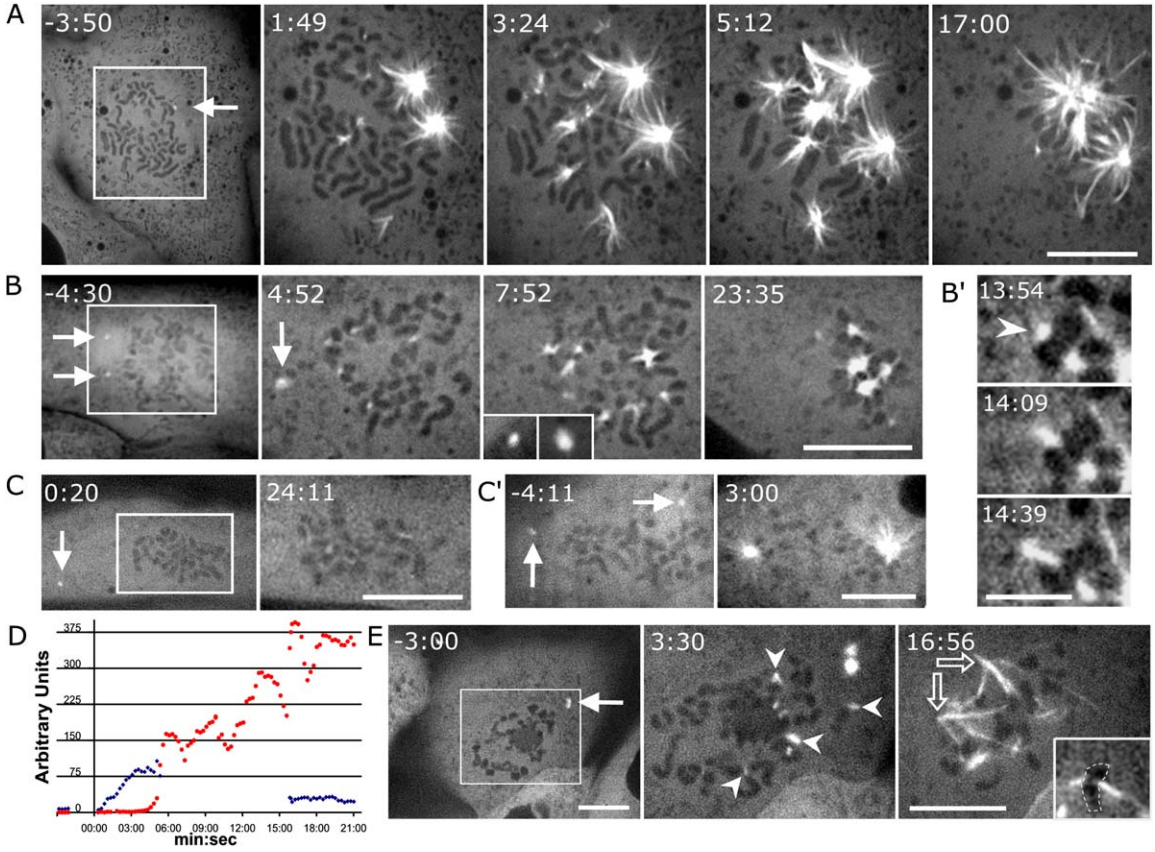

Figure 1. Microtubule Formation in Mitotic LLC-PK1α Cells after Release from Nocodazole

(A and B) Selected images from time-lapse sequences of LLC-PK1α cells washed four times (A) or one time (B) with drug-free media. In (A), microtubules form at each centrosome (arrow) and at discrete locations on chromosomes. In (B), the first panel shows summation of a Z stack prior to release; both centrosomes are visible (arrows), and they remain well separated from the chromosomes throughout the sequence. In subsequent images, one or both centrosomes are in different Z planes and thus not always visible. Insets show enlargements of centrosomes before (left) and after (right) removal of nocodazole. In (B′), an enlarged region of the cell in (B) shows elongation of microtubules associated with a discrete location on a chromosome; arrowhead marks the microtubule bundle.

(C and C′) LLC-PK1α cells were microinjected with excess importin-β and washed one time to remove nocodazole. In some cells, microtubule formation was completely blocked (C), and in other cells, microtubule formation at chromosomes, but not centrosomes, was blocked (C′). Arrows mark centrosomes in (C) and (C′).

(D) Graph of fluorescence (arbitrary units) at centrosomes (blue) and chromosomes (red) as a function of time after nocodozole removal. Fluorescence increases first at centrosomes and subsequently at chromosomes.

(E) Selected images from a time-lapse sequence of an LLC-PK1α cell treated with nocodazole and then microinjected with anti-70.1 antibodies. Microtubules form near chromosomes (arrowheads), elongate, and make transient associations with neighboring fibers (open arrows). Inset shows enlargement of a chromosome (dashed outline) and associated microtubule fibers. Time (min:sec) starts with removal of nocodazole.

The bar represents 10 μm in (A)–(E) and 5 μm in (B′). Movie of cells shown in (A), (B), and (E) can be found in the Supplemental Data.

Regulation of Kinetochore-Associated Microtubule Elongation and Coalescence by Dynein

The rapid coalescence of kinetochore-associated microtubules into pole-like structures is reminiscent of the behavior of newly formed kinetochore-fiber minus ends observed in other systems [6, 16]. We therefore injected nocodazole-treated cells in mitosis with antibody 70.1 to test the possibility that cytoplasmic dynein contributed to coalescence [17–19]. When the injected cells were released from nocodazole, coalescence was severely reduced and kinetochore-associated microtubules were much longer than in uninjected cells (Figure 1E and Movie 4). Elongation of kinetochore-associated microtubules in injected cells is consistent with the observation that dynein-dependent targeting of depolymerizing kinesins to microtubule minus ends limits kinetochore-fiber elongation in *Xenopus* extracts [20]. Microtubule assembly at centrosomes was not detectably altered following inhibition of dynein. These results demonstrate that cytoplasmic dynein regulates kinetochore-fiber dynamics and coalescence into pole-like structures.

TPX2 Is Required for Kinetochore-Associated Microtubule Formation

To determine the molecular requirements for microtubule assembly at kinetochores, we used siRNA to silence target genes. For experiments performed in HeLa cells, microtubule assembly was analyzed following release from nocodazole by fixing the cells and staining for microtubules and γ-tubulin and by counting the number of non-γ-tubulin-positive microtubule foci. For live imaging, siRNA was performed in LLC-PK1 cells

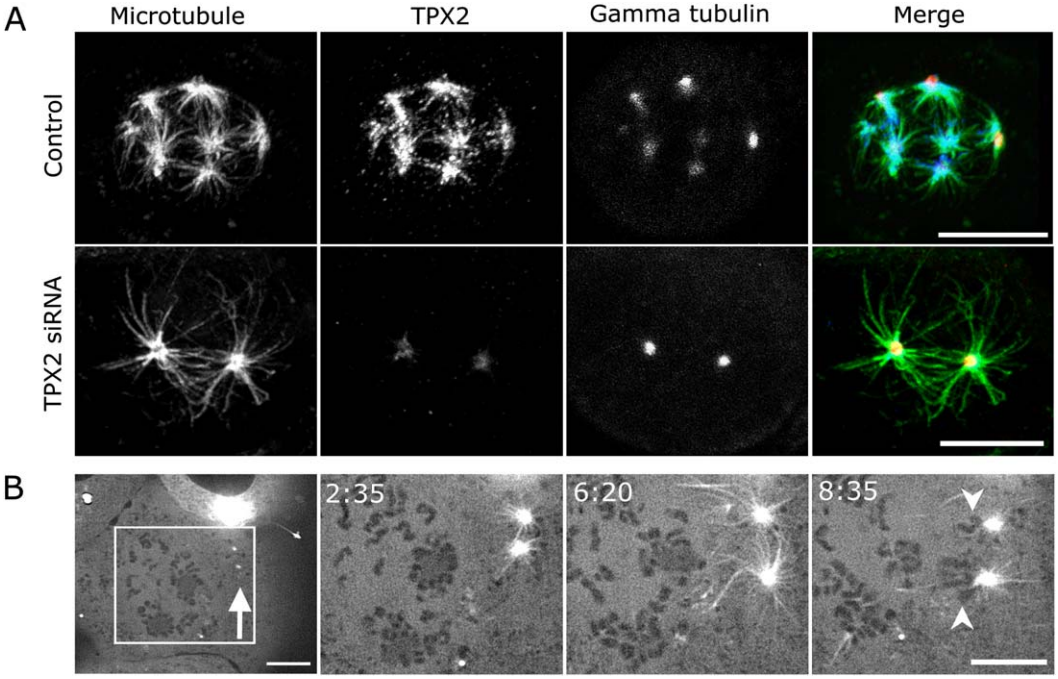

Figure 2. TPX2 Is Required for Microtubule Formation at Kinetochores

(A) Immunolocalization of microtubules (green), TPX2 (blue), and γ-tubulin (red) in control HeLa cells (top) and HeLa cells transfected with TPX2 siRNA (bottom), both of which have been released from nocodazole with a 1× wash. In control cells, microtubules assemble at centrosomes and kinetochores; in TPX2-depleted cells, two large centrosome-associated asters are present.

(B) Chromosome capture in living cells depleted of TPX2. Selected images from movie sequence of an LLC-PK1α cell lacking TPX2 and released from nocodazole (time 0:00) with a 1× wash. Microtubule formation around chromosomes is reduced dramatically, and long astral microtubules grow from centrosomes (arrow in first panel). Chromosomes closer to centrosomes move toward centrosomes and remain attached (arrowheads). Fluorescent area outside of the boxed region in the first panel is a bleed through from the siGLO used to identify transfected cells.

expressing GFP-tubulin (LLC-PK1α; Experimental Procedures).

TPX2, a downstream target of the small GTPase Ran, is required for assembly of morphologically normal bipolar spindles [21]. HeLa cells were treated with siRNA targeting TPX2 [22, 23] for 36 hr, incubated with nocodazole for 3 hr, washed one time to allow microtubule formation, fixed, and stained. The majority of control cells, treated with oligofectamine but not siRNA, contained two centrosomes and associated microtubules and an average of 8.0 foci of kinetochore-associated microtubules (Figure 2A, Table 1). In marked contrast, cells completely lacking TPX2 contained centrosomes that were associated with robust microtubule asters and an average of 0.3 foci of kinetochore-associated microtubules (Figure 2A, Table 1). Cells in which TPX2 was partially depleted showed intermediate numbers of chromosome-associated microtubule foci (Table 1). Microtubule behavior was also examined in living LLC-PK1α cells depleted of TPX2. Consistent with observations of fixed cells, microtubule formation around chromosomes was reduced severely, and long centrosomal microtubules formed (Figure 2B). Following live imaging, cells were fixed and stained to verify TPX2 depletion (Figure S3). Movie sequences of LLC-PK1α cells depleted of TPX2 showed movement of chromosomes

Table 1. Average Number of Microtubule Foci in the Chromosomal Region[a]

		Partial Depletion[b]				
siRNA	Control	+++	++	+	Complete Depletion	Number of Cells Counted
TPX2	8.0 ± 1.6	5.6 ± 1.3	4.8 ± 2.3	2.2 ± 1.6	0.3 ± 0.5	n = 66 cells
Survivin	8.1 ± 1.7	—	—	—	2.5 ± 2.7	n = 60 cells
MCAK/Survivin	5.3 ± 1.8	—	—	—	5.2 ± 2.1	n = 40 cells
MCAK	7.1 ± 2.2	—	—	—	7.1 ± 2.8	n = 42 cells
Nuf2	9.0 ± 2.7	—	—	—	8.9 ± 2.9	n = 57 cells
Bub1	4.6 ± 2.3	—	—	—	4.6 ± 1.4	n = 37 cells

Numbers show average ± standard deviation.

[a] The number of non-γ-tubulin-positive foci was determined from cells triple stained for tubulin, γ-tubulin, and the targeted protein. The differences between values in control experiments result from slight variations in the process of nocodazole removal from the cells, replacement with fresh media, and the timing of the incubation to allow microtubule regrowth. Each depletion is compared with control cells on the same slides.

[b] Level of TPX2 (+++ > ++ > +) in each cell was scored according to anti-TPX2 antibody staining.

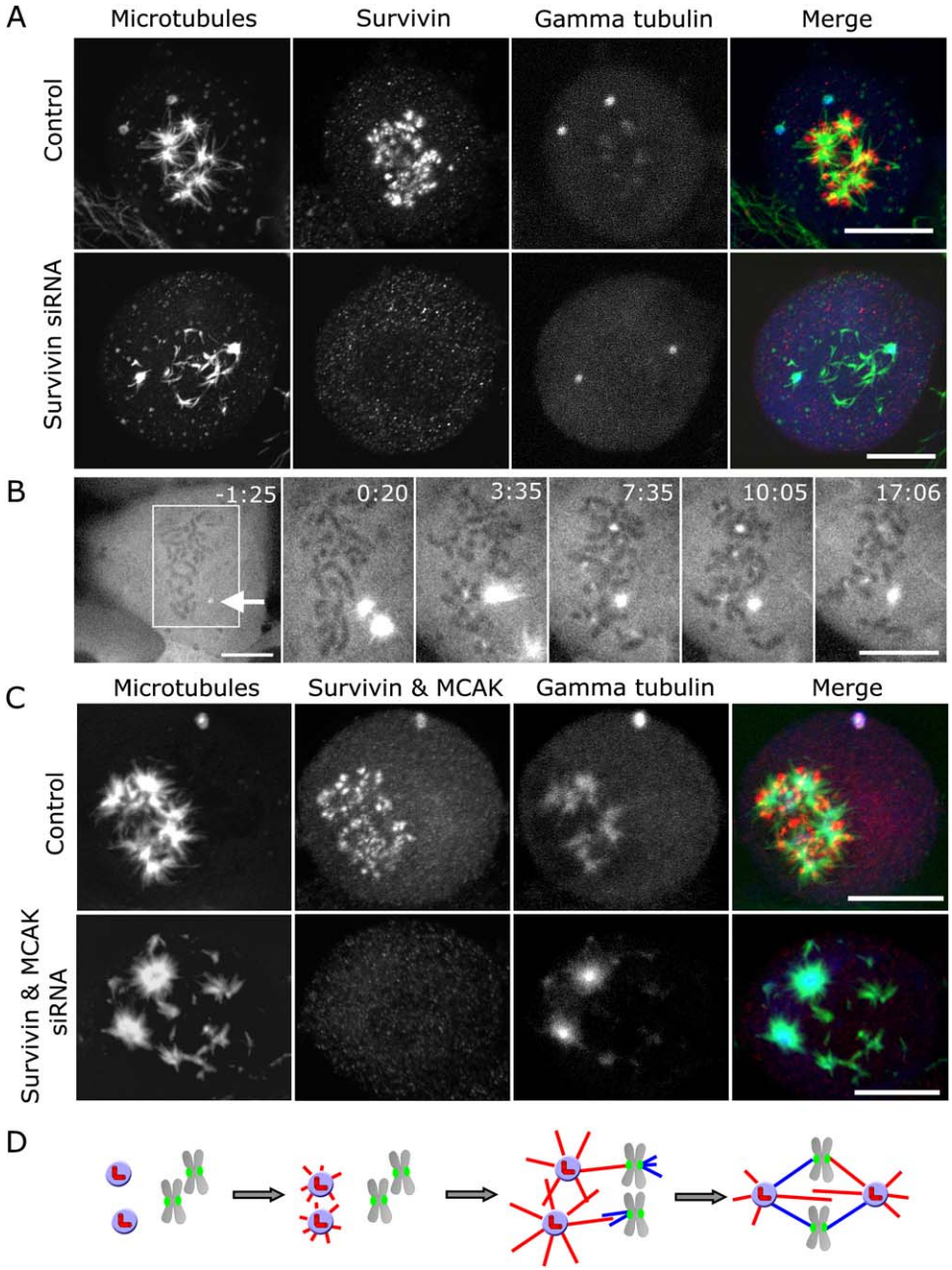

Figure 3. Survivin Contributes to Chromosome-Associated Microtubule Formation in an MCAK-Dependent Manner

(A) Immunolocalization of microtubules (green), survivin (red), and γ-tubulin (blue) in control cells (top) and cells treated with siRNA to deplete survivin (bottom). Several small foci of microtubules near chromosomes are observed in cells lacking survivin.

(B) Microtubules in living LLC-PK1α cells depleted of survivin and released from nocodazole (time 0:00) with a 1× wash. Arrow marks centrosome.

(C) Simultaneous depletion of survivin and MCAK restores microtubule formation near chromosomes. Two centrosomes are on top of each other in control cell (upper panels). Immunolocalization of microtubules (green), MCAK and survivin (red), and γ-tubulin (blue) in control (top) and siRNA-treated cells (bottom). Bars represent 10 μm.

(D) Diagram showing contribution of centrosome- and chromosome-associated microtubules to spindle formation in somatic cells. Following removal of nocodazole, microtubules form first at centrosomes and subsequently at kinetochores, followed by bipolar spindle assembly. Chromosome-associated microtubules are blue; centrosome-associated microtubules are red.

toward an adjacent centrosome (Figure 2B, Movie 5). These chromosomes remained associated with the centrosome without forming an obvious fiber, even in cells washed four times (data not shown), indicating that in the absence of TPX2, chromosomes can be captured, but mature kinetochore fibers are not observed. These results provide evidence that TPX2 is required for chromosome-associated microtubule formation in mammalian cells.

In all TPX2-depleted cells, the centrosome-associated microtubules were longer than observed in control cells (Figure 2). One possible explanation for this

observation is that more tubulin is available for assembly at centrosomes when microtubule formation near kinetochores is reduced or eliminated. Alternatively, TPX2 may negatively regulate some aspect of centrosomal-microtubule formation.

Kinetochore proteins may also contribute to kinetochore-fiber formation and stability. We targeted Bub1, a spindle-checkpoint protein located at kinetochores, and Nuf2, a component of the Ndc80 complex that mediates microtubule attachment to kinetochores (for review, see [24]). In HeLa cells depleted of Bub1, the pattern of microtubule formation was indistinguishable from that in controls, demonstrating that Bub1 is not required for the formation of kinetochore-associated microtubules (Figure S4B, Table 1). Likewise, in HeLa cells depleted of Nuf2, a depletion shown to result in loss of other components of the Ndc80 complex from kinetochores [25], the distribution of microtubules was also indistinguishable from that in controls (Figure S4A; Table 1). One explanation for this phenotype is that the Ndc80 complex contributes to microtubule-kinetochore attachment only when chromosomes are under tension. The distance between sister kinetochores was measured in LLC-PK1 cells expressing GFP-CenpA. In cells washed one time, the interkinetochore distance was similar to that measured in the presence of nocodazole (Figure S5), indicating that the kinetochores are not under tension. However, the distance between sister kinetochores increased with additional washes in drug-free media, suggesting that tension is developed during spindle formation in this system.

Chromosome Passenger Complex Regulates the Stability of Kinetochore-Associated Microtubules

Recent work has demonstrated that the chromosomal passenger proteins contribute to microtubule stability near chromatin in a MCAK-dependent manner [26]. We used siRNA to deplete cells of survivin, a member of the chromosome passenger protein complex (for review, see [27]), a depletion that resulted in loss of other members of the complex from centromeres in mammalian cells [28, 29]. The average number of kinetochore-associated microtubule foci per cell was reduced from 8.1 in controls to 2.5 in survivin-depleted HeLa cells (Figure 3A and Table 1). Depletion of survivin from living LLC-PK1α cells (Figure 3B) also demonstrated a reduction, but not elimination, of chromosome-associated microtubules (Movie 6). Increases in centrosome-associated, and in some cases peripheral, microtubules were observed in the survivin-depleted cells (Figure 3B).

The presence of microtubules in the chromosome region of survivin-depleted cells suggests that microtubule stability, but not nucleation, was altered in these cells. To determine whether MCAK was required for the survivin-depletion phenotype, we used siRNA to deplete MCAK alone [30, 31] and MCAK and survivin simultaneously. In cells depleted of MCAK alone or in combination with survivin, chromosome-associated microtubules were similar to controls (MCAK alone: control, 7.1 ± 2.2; siRNA, 7.1 ± 2.8. MCAK/Survivin: control, 5.3 ± 1.8; siRNA, 5.2 ± 2.1), demonstrating that MCAK is required for destabilization of chromosome-associated microtubules in cells lacking survivin (Table 1) [26]. Centrosomes in MCAK- (Figure S6) or MCAK- and survivin-depleted cells (Figure 3C) developed robust microtubule asters that were almost always located adjacent to the chromosomal region, in contrast to control cells, which lacked robust astral microtubule arrays and in which centrosomes were usually distant from chromosomes. These results demonstrate that MCAK regulates the formation of centrosomal microtubules, as well as kinetochore-associated microtubules, in mitotic cells.

Our data demonstrate that microtubule assembly at kinetochores and centrosomes is kinetically distinct and differentially regulated (Figure 3D). The Ran pathway and TPX2 are required for microtubule formation near chromosomes; the chromosome passenger complex regulates microtubule stability near chromosomes. Microtubule assembly at centrosomes is regulated by MCAK and the concentration of assembly competent tubulin. In addition, our data demonstrate that microtubule assembly at kinetochores and centrosomes is interdependent, presumably as a result of the limited supply of tubulin dimers. If microtubule assembly at chromosomes is compromised, larger astral arrays are observed. Conversely, factors that promote microtubule formation near kinetochores may limit the elongation of centrosomal microtubules, as observed in *Drosophila* S2 cells, which have a robust kinetochore-mediated pathway, but contain few centrosomal microtubules [6].

Our results provide a mechanism to reconcile the difference between the time required for spindle formation in vivo with the time based on computer simulations of search and capture [32]: Dynamically unstable centrosomal microtubules are given a much larger target area with the presence of microtubules at kinetochores. In addition, chromosomes with associated microtubules tend to coalesce and thus further increase target size.

Although microtubule assembly at kinetochores has the potential to greatly increase the efficiency of search and capture, chromosome capture is observed in cells lacking TPX2. However, mature kinetochore fibers were not detected. We conclude that the chromosome-mediated pathway plays a fundamental role in spindle assembly in centrosome-containing cells, a role that is masked by the presence of kinetically dominant centrosomes.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and six figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/5/536/DC1/.

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