A model for the proposed roles of different microtubule-based motor proteins in establishing spindle bipolarity

Claire E. Walczak*, Isabelle Vernos†, Timothy J. Mitchison‡, Eric Karsenti† and Rebecca Heald§

Background: In eukaryotes, assembly of the mitotic spindle requires the interaction of chromosomes with microtubules. During this process, several motor proteins that move along microtubules promote formation of a bipolar microtubule array, but the precise mechanism is unclear. In order to examine the roles of different motor proteins in building a bipolar spindle, we have used a simplified system in which spindles assemble around beads coated with plasmid DNA and incubated in extracts from *Xenopus* eggs. Using this system, we can study spindle assembly in the absence of paired cues, such as centrosomes and kinetochores, whose microtubule-organizing properties might mask the action of motor proteins.

Results: We blocked the function of individual motor proteins in the *Xenopus* extracts using specific antibodies. Inhibition of *Xenopus* kinesin-like protein 1 (Xklp1) led either to the dissociation of chromatin beads from microtubule arrays, or to collapsed microtubule bundles on beads. Inhibition of Eg5 resulted in monopolar microtubule arrays emanating from chromatin beads. Addition of antibodies against dynein inhibited the focusing of microtubule ends into spindle poles in a dose-dependent manner. Inhibition of *Xenopus* carboxy-terminal kinesin 2 (XCTK2) affected both pole formation and spindle stability. Co-inhibition of XCTK2 and dynein dramatically increased the severity of spindle pole defects. Inhibition of Xklp2 caused only minor spindle pole defects.

Conclusions: Multiple microtubule-based motor activities are required for the bipolar organization of microtubules around chromatin beads, and we propose a model for the roles of the individual motor proteins in this process.

Addresses: *Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94143, USA. †Cell Biology Program, European Molecular Biology Laboratory, 69117 Heidelberg, Germany. ‡Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA. §Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA.

Correspondence: Rebecca Heald
E-mail: heald@socrates.berkeley.edu

Background

In eukaryotes, the accurate segregation of chromosomes during cell division occurs on a complex apparatus called the spindle, whose assembly requires the interaction of chromosomes with microtubules, which form a bipolar array. The antiparallel organization of microtubules into two poles is essential for the physical separation of chromosomes to two daughter cells during anaphase. The mechanisms and principles behind spindle assembly have begun to be elucidated (reviewed in [1,2]). Upon entry into mitosis, the dynamics of tubulin polymerization are modulated to allow dissolution of the interphase microtubule array and selective stabilization of microtubules around chromosomes (reviewed in [3]). In addition to changes in microtubule dynamics, mechanical forces generated by microtubule-based motor proteins are thought to play an important role in generating the spindle structure. The motor protein cytoplasmic dynein and other motor proteins from at least seven families of kinesin-like proteins (KLPs) have been localized to the mitotic spindle [4–8]. Motor proteins use the energy of ATP hydrolysis to move along microtubules in a unidirectional manner, transporting spindle cargo such as chromosomes or other microtubules toward either the plus or minus end of the microtubule polymer. The proposed functions of motors include driving centrosome separation and chromosome movement, maintaining a force that holds the spindle together, driving poleward microtubule flux, and controlling microtubule dynamics within the spindle. The precise roles of individual motors are poorly understood, however, and it is not clear how multiple motor activities are integrated to form the bipolar structure of the spindle.

Precise interpretation of how motors function in spindle assembly is complicated by the existence of other microtubule-organizing forces. In most cells, microtubules grow from focal nucleation centers, such as centrosomes, which define the polarity of the microtubules and determine the sites of spindle pole formation. In the presence of a single centrosome, or unseparated centrosomes, a monopolar spindle will form even if microtubule motor functions have not been perturbed [9–12]. Centrosomes therefore dominate microtubule organization and make it problematic to distinguish the motor activities required for centrosome

separation from those that are necessary to form a bipolar antiparallel microtubule array.

To avoid the complication of focal microtubule nucleation sites, we decided to study the roles of different motor proteins during spindle assembly around DNA-coated beads in extracts from *Xenopus* eggs. In this system, as in female meiosis of most animal species, bipolar spindles form in the absence of centrosomes by the self-organization of microtubules growing randomly around chromatin [13]. Because this system requires sorting of microtubules according to their polarity, it is highly dependent on the activities of motor proteins to generate an antiparallel bipolar array. To address the general roles of motor proteins in determining the bipolar arrangement of microtubules around chromatin beads, we undertook a comprehensive analysis by inhibiting the functions of motor proteins individually and in combination. To accomplish this, we used specific antibodies to immunodeplete the motor protein, or to inhibit its function by adding the antibody directly to the extract. Except where noted, antibody addition mimicked immunodepletion, indicating that we are blocking the function of the motor protein by both techniques.

Multiple *Xenopus* KLPs that localise to the spindle have been cloned — including Eg5, *Xenopus* kinesin-like proteins 1 and 2 (Xklp1 and Xklp2) and *Xenopus* carboxy-terminal kinesin 2 (XCTK2) — and the functions of these motors have been characterized using centrosome-directed spindle assembly around sperm nuclei [14–21]. In addition, cytoplasmic dynein has been shown to be required for spindle pole formation in *Xenopus* [12,22]. Here, using the chromatin bead spindle assembly assay, we show evidence that both Eg5 and Xklp1 are critical for spindle bipolarity. Eg5 seems to provide a sorting activity that generates an antiparallel array, whereas Xklp1 appears to maintain the interactions between chromatin and microtubules that are required for extending spindle poles. Whereas dynein seems to be the dominant motor that focuses microtubule minus ends into spindle poles, XCTK2 appears to contribute to the organization of spindle poles and to spindle integrity. Xklp2 inhibition did not have a significant effect on the bipolar microtubule organization around chromatin beads, indicating that the main role of this motor is probably in processes that require centrosome separation.

Results

We present here an analysis of the function of different microtubule motor proteins in spindle assembly around chromatin beads. For simplicity, each motor is presented in a separate figure, which includes an indication of the domain structure, the localization pattern on chromatin bead spindles, the different spindle structures seen upon inhibition of the motor, quantification of these structures,

and a proposed model for how the motor functions in spindle assembly.

Xklp1 mediates chromatin–microtubule interactions and contributes to spindle pole extension

Xklp1 was identified as a chromosomally localized motor with an amino-terminal motor domain [18]. Sucrose density gradient sedimentation, gel filtration chromatography and immunoprecipitation experiments revealed that Xklp1 is a dimer in solution and has no associated proteins (data not shown; Figure 1a). The mouse homologue, KIF4, has been shown to be plus-end-directed [23]. Disruption of Xklp1 during spindle assembly around *Xenopus* sperm nuclei by the addition of antibodies leads to a loss of microtubules in the central spindle and to spindle instability [18]. To identify a role for Xklp1 in spindle assembly around chromatin beads, we first determined the localization of the protein by immunofluorescence. Anti-Xklp1 antibodies strongly stained the beads (Figure 1b), indicating that distinct chromosomal sequences are not required for the localization of Xklp1, and that the plasmid DNA on the beads is sufficient to recruit Xklp1 from *Xenopus* egg extracts.

To test the role of Xklp1 in spindle assembly around chromatin beads, two different polyclonal antibodies raised against non-overlapping domains of Xklp1 were assayed for their effects. The first, Ab65, was raised against the tail domain of the protein. Addition of this antibody to extracts before the initiation of spindle assembly reactions resulted in a much lower proportion of bipolar structures than in control reactions (17% versus 85%; Figure 1e). In the presence of Ab65, monopolar spindles predominated, as well as bent bipolar structures and free monopolar structures lacking chromatin beads (Figure 1c,e). Visualization of the added antibody (secondary staining; Figure 1c) revealed staining on the beads and on microtubules, with an enrichment at the plus ends. These results suggested that Ab65 did not directly interfere with the bipolarity of spindles, but that it inhibited microtubule–chromatin interactions, causing spindle instability and dissociation of the beads from microtubule arrays. Addition of the second antibody, Ab03, which was raised against the neck and stalk region of Xklp1, resulted in a distinctly different effect. In approximately 90% of the structures examined, microtubule bundles formed around chromatin beads, but did not extend poles (Figure 1d,e). In this case, Xklp1 was immunolocalized only to the chromatin beads. A similar effect was observed when Xklp1 was depleted from spindle assembly reactions (data not shown).

Together, the results using anti-Xklp1 antibodies suggest that although both antibodies interfere with Xklp1 function, Ab03 does so more severely as its effect is similar to that of immunodepletion. Because the two antibodies are directed against different regions of Xklp1, it is possible

Figure 1

Xklp1 is involved in chromatin–microtubule interactions and spindle pole extension. (a) The domain structure of Xklp1. (b) Localization of Xklp1. The left-hand panel shows an overlay of a spindle stained for DNA (blue), microtubules (red) and the motor protein (green); the right-hand panel shows staining for the motor protein only. Overlap between the motor protein and the DNA appears aquamarine, and overlap between the motor protein and the microtubules appears yellow. (c,d) Representative images of the spindle structures that formed in the presence of one of two anti-Xklp1 antibodies: (c) Ab65 and (d) Ab03. 'Collapsed' indicates a collapsed microtubule array lacking poles. Staining was for DNA and microtubules only unless secondary staining is indicated, in which case FITC-conjugated secondary antibodies were used, showing that Ab65 decorated microtubule plus ends in a free monopolar spindle, and Ab03 decorated chromatin beads. (e) Quantification of the structures formed in spindle assembly reactions. More than 85% of the spindles were bipolar in the control reaction, which contained control immunoglobulin G (IgG) antibodies (*n* = 607, three separate experiments); addition of Ab65 yielded predominantly monopolar spindles and bent bipolar spindles (*n* = 503, three experiments); addition of Ab03 resulted in collapsed structures lacking spindle poles (*n* = 485, three experiments). (f,g) Two proposed models for Xklp1 function. (f) Because Ab65 caused dissociation of beads from spindle microtubules, Xklp1 is proposed to promote stable microtubule–chromatin interactions. (g) Ab03 addition resulted in microtubule bundles lacking poles, indicating a role for
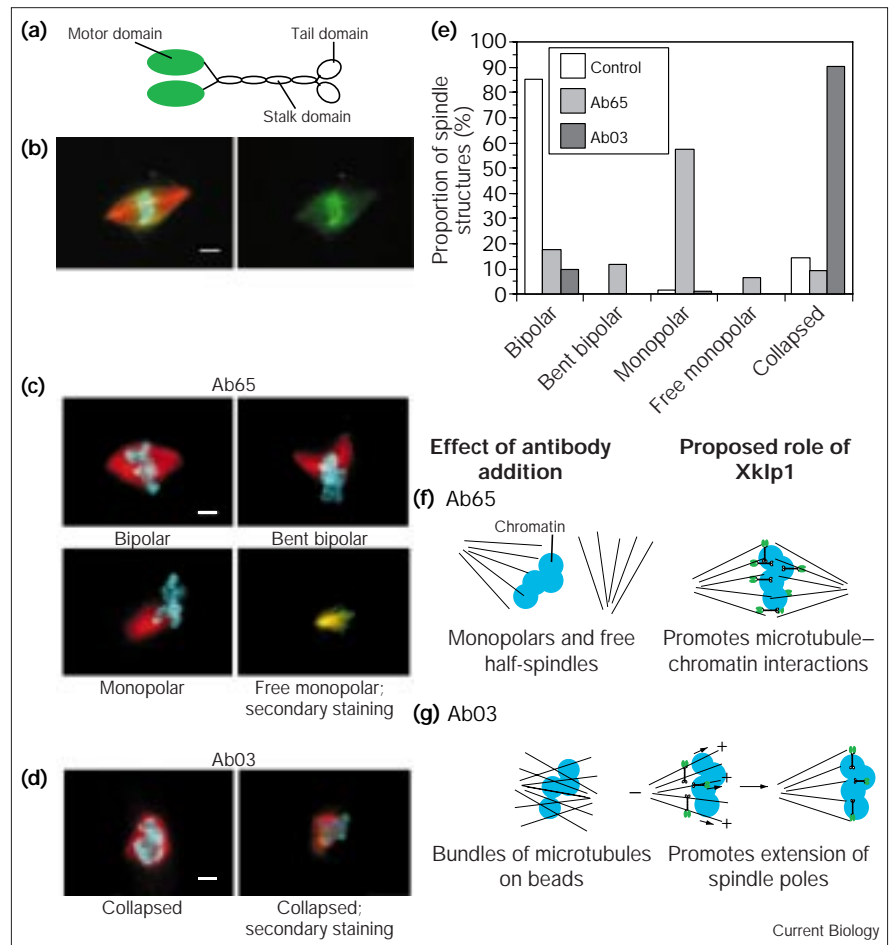
Xklp1 in extending spindle poles. By moving toward microtubule plus ends, Xklp1 would extend minus ends away from the beads. The bars are 10 μm.

that they interfere with Xklp1 function in different ways. Ab65 recognizes the tail domain and may interfere with the ability of Xklp1 to bind to chromatin, whereas Ab03 recognizes the neck and stalk region and may interfere with Xklp1 motor activity. A model consistent with these results is that Xklp1 is required for chromatin–microtubule interactions, and that this interaction is required both to extend spindle poles away from the chromatin beads, and to hold the structure together once it has formed (Figure 1f,g).

Eg5 is required for spindle bipolarity

Eg5 is a plus-end-directed motor that is a member of the bipolar kinesin family [14,17]. The *Drosophila* homologue has been shown to be tetrameric, with pairs of motor domains at opposite ends of the molecule (Figure 2a) [24]. Eg5 is also tetrameric in solution (data not shown). The bipolar kinesin family is conserved throughout evolution, and has been shown to play a role in spindle pole formation

and separation [25–31]. In spindle assembly reactions containing *Xenopus* sperm DNA, inhibition of Eg5 causes spindle pole defects and results in 'rosette' structures with unseparated centrosomes in the center and microtubules extending radially to surrounding chromosomes [17].

We wondered whether Eg5 also played a role in spindle assembly in the absence of centrosomes. As in spindles assembled around *Xenopus* sperm nuclei, Eg5 was located throughout microtubules of chromatin bead spindles, showing an enrichment at spindle poles (Figure 2b). Immunodepletion of Eg5 or disruption of Eg5 activity by the addition of specific antibodies gave the same result (Figure 2c,d and data not shown); more than 95% of the spindle structures formed consisted of microtubules packed around chromatin beads, often extending outward in astral arrays. To determine the orientation of microtubules in these radial arrays, we immunostained the structures using antibodies against nuclear/mitotic apparatus

Figure 2
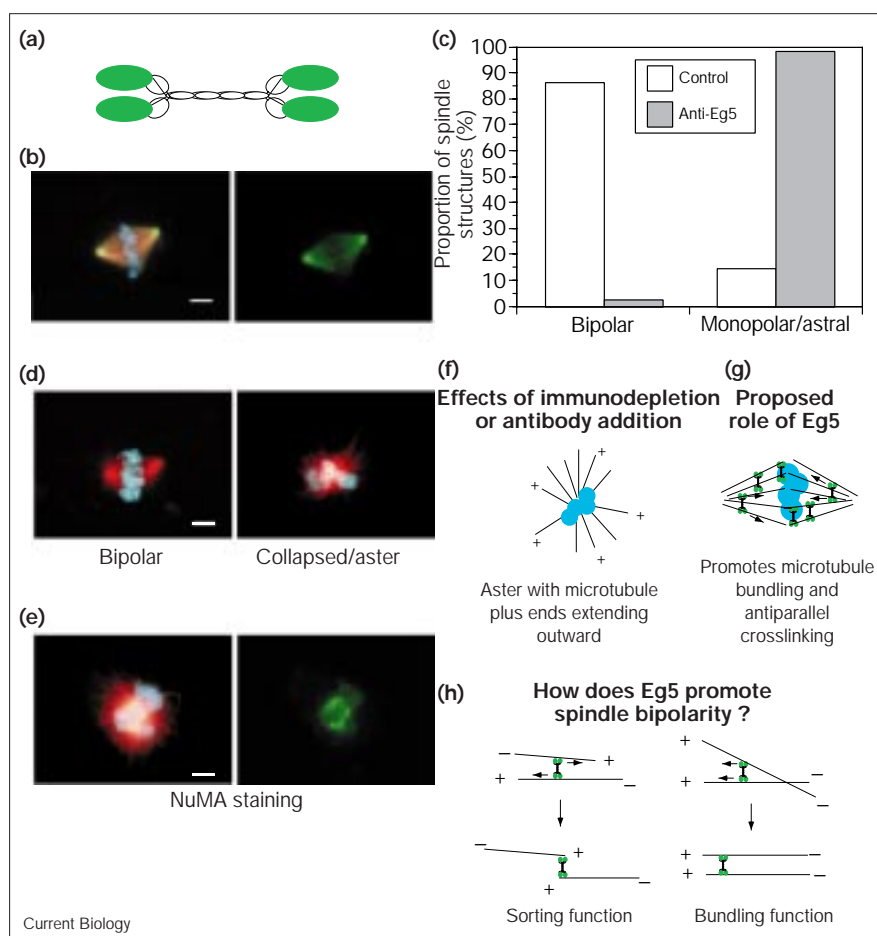
Eg5 is required for forming antiparallel microtubule arrays. Colors and immunofluorescence staining are as for Figure 1. (a) The structure of Eg5. (b) Localization of Eg5 by immunofluorescence. (c) Quantification of the spindle structures formed in the presence of anti-Eg5 antibody showed that there were few bipolar spindles. Instead, collapsed microtubule structures often extending astral microtubules formed on chromatin beads in the presence of anti-Eg5 antibodies ($n = 607$, three experiments for the control and $n = 556$, three experiments for Eg5 inhibition). (d) Representative fluorescence micrographs of a bipolar spindle and a collapsed aster that formed in the presence of anti-Eg5 antibody. Similar structures formed in extracts depleted of Eg5 (data not shown). (e) Immunostaining of the nuclear/mitotic apparatus protein (NuMA) revealed that it is enriched in the center of asters that formed in the presence of anti-Eg5 antibody. (f–h) Proposed model of Eg5 function. (f) In the absence of Eg5 function, asters form with microtubule plus ends extending distally from the chromatin beads. (g) We therefore propose that Eg5 crosslinks microtubules in spindles, bundles them, and sorts them into an antiparallel array. (h) Proposed model for how Eg5 might promote spindle bipolarity for microtubules in the same or in opposite orientations. The bars are 10 μm.

protein (NuMA) which has been shown to decorate the minus ends of microtubules at spindle poles and the centers of asters found in cells treated with dimethyl sulfoxide (DMSO) or taxol — two microtubule-stabilizing drugs [12,22,32,33]. NuMA was found associated with the microtubules where they came to a focus on the beads, indicating that microtubule plus ends are distal to the structures (Figure 2e). In addition, stable microtubule seeds, which have been shown previously to accumulate at foci of microtubule minus ends [13], also accumulated at the central focus of microtubules on the beads (data not shown). Eg5 disruption, therefore, appears to prevent the formation of antiparallel, bipolar arrays, yielding instead monopolar, astral arrays with microtubule plus ends extending outward (Figure 2f). This result indicates that in the absence of Eg5 function other motors still function to sort microtubule minus ends into pole-like structures.
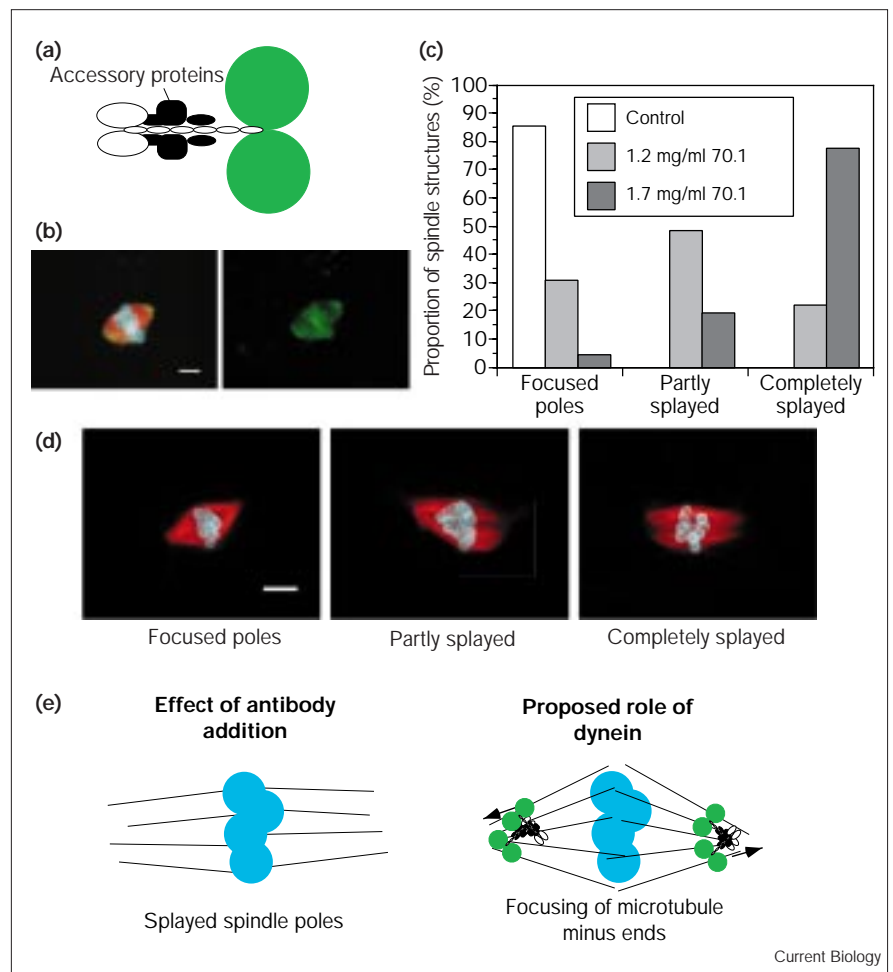
How does Eg5 function in bipolar spindle assembly? It has been shown that microtubules growing around chromatin beads in the early stages of spindle assembly are in random orientations [13]. Because of its tetrameric, bipolar structure, Eg5 could function in two ways to promote spindle formation (Figure 2g,h). First, by crosslinking two microtubules that are in the same orientation and moving toward their plus ends, Eg5 would bundle the microtubules, thereby promoting formation of a spindle axis. Second, microtubules in opposite orientations would be pushed apart by Eg5, and thereby sorted into an antiparallel array.

Thus both Xklp1 and Eg5 are required for spindle formation around chromatin beads. These plus-end-directed motors have been proposed to function as sorting devices in establishing a bipolar array [6,34]. Our results indicate that neither motor is sufficient — Eg5 is required to form antiparallel microtubule arrays, but perhaps Xklp1-mediated interactions between microtubule plus ends and chromatin are required to form a spindle. This could explain why asters that form in the presence of DMSO or taxol are monopolar, despite the presence of Eg5 in the cytoplasm. Why is Xklp1 function insufficient to extend the single spindle pole formed when Eg5 is inhibited? We propose

Figure 3

Dyneln is required to focus spindle poles. Colors and immunofluorescence staining are as for Figure 1. **(a)** The structure of dynein and **(b)** its localization by immunofluoresence. Dynein is a large, multimeric minus-end-directed motor localized diffusely along spindle microtubules with an enrichment at spindle poles. The staining of the beads is due to autofluorescence of the beads which occurs at the long exposure time necessary to visualize the dynein staining. **(c)** Quantification of spindle pole structures formed in the presence of different amounts of antibody against the intermediate chain of dynein shows that 1.7 mg/ml antibody leads to completely splayed poles in 75% of the structures formed, whereas 1.2 mg/ml antibody has a weaker effect ($n = 607$, three experiments for control antibody addition; $n = 279$ for 1.2 mg/ml antibody and $n = 375$ for 1.7 mg/ml antibody, two experiments). **(d)** Representative micrographs of a normal bipolar spindle with focused poles, and partly and completely splayed spindle poles. **(e)** Proposed model for dynein function. In the absence of dynein activity, poles are loose and splayed. By crosslinking microtubules and moving toward their minus ends, dynein would function to focus spindle poles. The bars are 10 μm.

(a) Accessory proteins

(b)

(c)

(d) Focused poles | Partly splayed | Completely splayed

(e) **Effect of antibody addition** — Splayed spindle poles | **Proposed role of dynein** — Focusing of microtubule minus ends

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that the microtubule-bundling activity of Eg5 is required to form a bipolar axis, without which microtubules cannot effectively be sorted apart into two arrays. Alternatively, minus-end-directed motors such as dynein might dominate over Xklp1 activity in the absence of Eg5 function.

Dynein is essential for spindle pole formation

Cytoplasmic dynein is required to focus spindle poles, both in the presence and absence of centrosomes [12,13]. Dynein function in pole formation appears to depend on its interaction with the dynactin complex and the spindle pole protein NuMA [22,31,35] (T. Wittmann, H. Boleti, C. Antony, E.K. and I.V., unpublished observations). On the spindles assembled around chromatin beads, dynein is localized to the spindle poles (Figure 3b). As shown previously, in the presence of an antibody (70.1) against the intermediate chain of dynein, microtubule arrays formed that were centered around chromatin beads, but had frayed ends (Figure 3c,d) [13]. We show here that the severity of the defect depends on the amount of antibody added, with the maximum effect at about 1.7 mg/ml anti-

body. At this concentration, more than 75% of spindles had poles that were completely splayed. Addition of 1.2 mg/ml antibody also disrupted spindles, but approximately 50% of the structures contained poles that were only partially splayed and only 21% had poles that were completely splayed (Figure 3c,d). Despite the defect in the spindle poles that occurs when dynein function is blocked, the microtubules of these spindles are still organized into an antiparallel array [12]. These observations suggest both that Eg5 and Xklp1 still function to sort microtubules in the absence of dynein activity so that the minus ends of the microtubules are distal to the beads, and that the primary function of dynein is to focus microtubule minus ends into spindle poles (Figure 3e).

XCTK2 contributes to spindle integrity and pole formation

XCTK2 belongs to the minus-end-directed KinC family of kinesins and exists in a large complex with other non-motor subunits [20] (Figure 4a). Mutations in the *Drosophila* homologue, Ncd, result in spindle instability and spindle pole defects [36–39]. In *Saccharomyces*

Figure 4
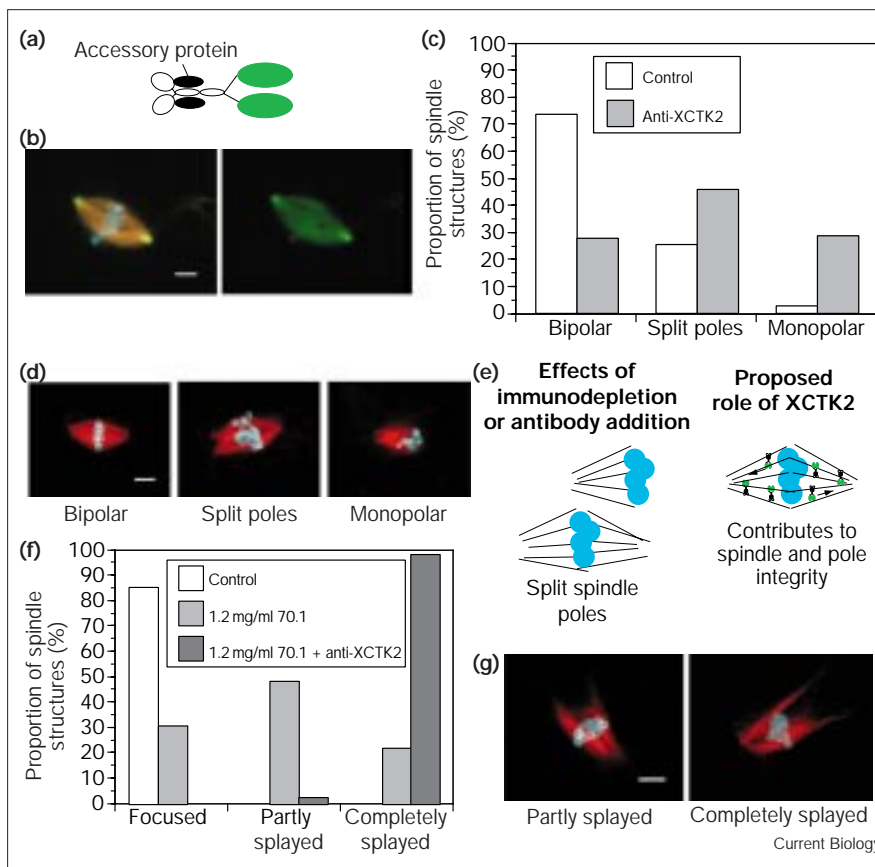
XCTK2 contributes to spindle integrity and pole formation. Colors and immunofluorescence staining are as for Figure 1. **(a)** The structure of XCTK2, a dimeric motor protein with the motor domain at the carboxyl terminus and which complexes with associated proteins. **(b)** Localization of XCTK2 by immunofluorescence. **(c)** Quantification of spindle structures formed in the presence of anti-XCTK2 antibodies shows a fourfold increase in monopolar spindles compared to control reactions, and an increase in the proportion of spindles with split poles ($n = 959$, five experiments for control antibody addition; $n = 973$, five experiments for anti-XCTK2 antibody addition). **(d)** Representative micrographs of a normal bipolar spindle, a monopolar spindle, and a spindle with split spindle poles. **(e)** Proposed model of XCTK2 function on the basis of its stabilizing effects on spindles and poles. XCTK2 might crosslink microtubules and move poleward, stabilizing microtubule bundles and contributing to the focusing of spindle poles. **(f)** Quantification of the spindle structures formed when both dynein and XCTK2 were inhibited ($n = 303$, two experiments). In a partially blocked dynein background, XCTK2 inhibition dramatically increased the proportion of completely splayed poles. **(g)** Representative micrographs of the effects of XCTK2 and dynein co-inhibition. The bars are 10 μm.

cerevisiae, *Schizosaccharomyces pombe* and *Aspergillus nidulans*, there exists an antagonistic force relationship between the KinC family members and the bipolar kinesins [40–42]. However, the precise function of KinC family members is unclear. In *Xenopus* egg extracts, inhibition of XCTK2 results in spindle instability whereas increasing the amount of XCTK2 promotes formation of bipolar spindles around sperm nuclei [20].

To further explore the role of XCTK2, we assessed its function in spindle assembly around chromatin beads. XCTK2 was found on chromatin bead spindle microtubules and enriched at spindle poles (Figure 4b), as it is on spindles formed around sperm DNA. Addition of antibodies to XCTK2 increased the proportion of monopolar spindles from 2% to 27%. Spindle pole structure was also affected, with almost twice the number of 'split' poles, which fail to form a single minus-end focus (Figure 4c,d). Similar results were obtained if XCTK2 was immunodepleted from extracts (data not shown). These results indicate that XCTK2 contributes to spindle integrity, and they are consistent with the previously proposed model that XCTK2 bundles microtubules, thereby promoting antiparallel microtubule interactions and bipolarity (Figure 4e) [20].

Our results also indicate a role for XCTK2 in pole formation in the absence of centrosomes.

In order to test whether XCTK2 functions in conjunction with dynein to form spindle poles, we tested the effects of XCTK2 inhibition in the presence of 1.2 mg/ml of the anti-dynein antibody 70.1, which by itself causes only partial splaying of spindle poles (Figures 3c,4g). Under these conditions, co-inhibition of XCTK2 caused a dramatic increase in the proportion of spindles with completely splayed poles, from 21.5% to 98%. Furthermore, the bipolar spindle axis was often distorted, as microtubules failed to form a single bundle (Figure 4f,g). Therefore, XCTK2 appears to have a pole-forming function partially redundant with that of dynein, and XCTK2 also appears to contribute to the integrity of the bipolar spindle.

Xklp2 plays a minor role in spindle assembly in the absence of centrosomes
Xklp2 is a dimeric plus-end-directed motor with an amino-terminal motor domain (Figure 5a) [21]. A mouse homologue has been identified using a PCR screen, but its function has not been addressed [43]. Studies using *Xenopus* sperm nuclei indicate that Xklp2 is required for

Figure 5

Xklp2 plays a subtle role in spindle assembly. Colors and immunofluorescence staining are as for Figure 1. **(a)** Xklp2 is a plus-end-directed, dimeric motor protein with the motor domain at its amino terminus. **(b)** Xklp2 antibodies added to extracts are revealed by secondary antibodies to localize throughout spindle microtubules. **(c)** Quantification of structures formed in the presence of anti-Xklp2 antibodies shows only minor effects on spindle integrity (*n* = 959, five experiments for control antibody addition). Co-inhibition of Xklp2 and XCTK2 caused an increase in monopolar spindles (*n* = 594, four experiments). **(d)** Co-inhibition of Xklp2 and dynein also increased the proportion of completely splayed spindle poles (*n* = 222, two experiments). **(e,f)** Representative spindle structures from the experiments quantified in (c) and (d), respectively. The bars are 10 μm.
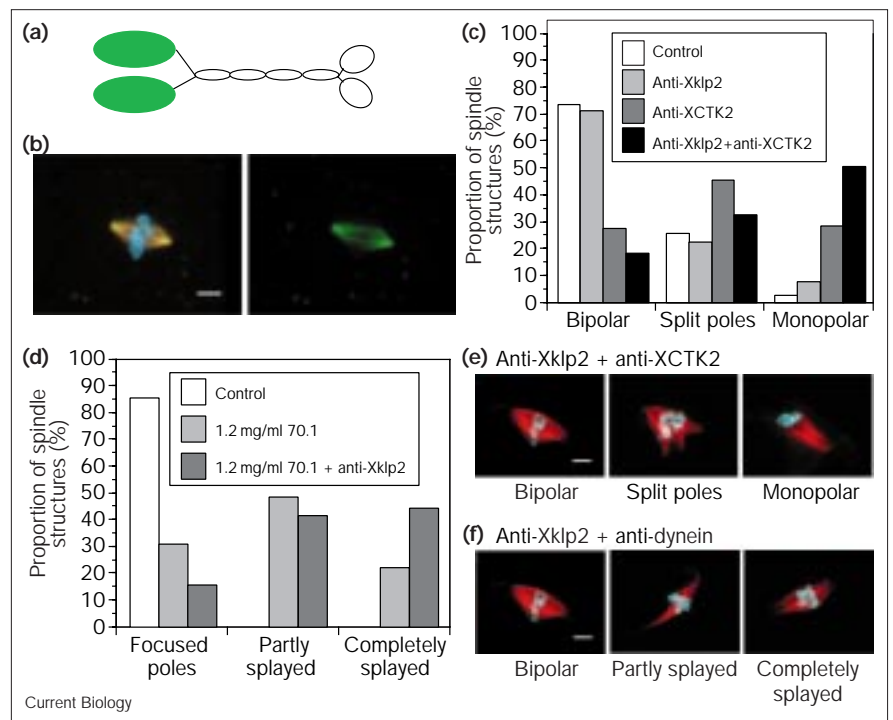
centrosome separation [21]. To determine whether Xklp2 plays a role in spindle assembly in the absence of centrosomes, we examined its function during spindle assembly around chromatin beads. Using two different polyclonal antibodies, we failed to detect Xklp2 on chromatin bead spindles by immunofluorescence. However, antibodies added to extracts could be visualized by a fluorescent secondary antibody and were localized on spindle microtubules (Figure 5b). As on sperm DNA spindles, an Xklp2 carboxy-terminal fusion protein localized to spindle poles (T. Wittmann, H. Boleti, C. Antony, E.K. and I.V., unpublished observations), indicating the presence of a spindle pole targeting domain in the tail of the protein. Addition of anti-Xklp2 antibodies or depletion of the protein from the extract, however, did not have a significant effect on spindle assembly around chromatin beads (Figure 5c and data not shown). Addition of the carboxy-terminal fusion protein altered pole morphology, but did not affect the percentage of bipolar spindles formed (data not shown and T. Wittmann, H. Boleti, C. Antony, E.K. and I.V., unpublished observations).

These results raised the possibility that Xklp2 function is critical only in systems that contain centrosomes. Alternatively, Xklp2 might play a minor or redundant role in spindle assembly in the chromatin bead system. To examine the role of Xklp2 in the context of other motor proteins involved in pole organization, we co-inhibited Xklp2 and XCTK2, or Xklp2 and dynein (Figure 5c–f).

Xklp2 inhibition increased the proportion of monopolar spindles from 28% to 50% in an XCTK2-inhibited background (Figure 5c,e). As for XCTK2, Xklp2 co-inhibition increased the severity of pole defects caused by partial dynein inhibition, leading to an increase in the proportion of completely frayed spindle poles from 21.5% to 44% (Figure 5d,f). This increase was not nearly as dramatic as that seen when both dynein and XCTK2 were inhibited (see Figure 4f). Therefore, Xklp2 appears to play a minor role in bipolar spindle formation, acting in combination with XCTK2 and dynein to stabilize bipolar spindles and poles. Its primary function is probably in centrosome-dependent spindle assembly reactions.

Discussion

Multiple motor proteins act in concert to build a spindle

We have examined the roles of five different motor proteins in determining the bipolar arrangement of microtubules using a system in which spindles form around DNA-coated beads. Because spindles form by a microtubule self-organization mechanism in the absence of centrosomes, this system has allowed us to evaluate motor protein function independent of focal microtubule nucleation. Our finding that multiple motor proteins are necessary to build a mitotic spindle is not unexpected. Our approach is unique, however, in that we have been able to define the functions of individual motor proteins in a simplified system that is completely dependent on motor proteins as organizing forces to generate a bipolar array. Spindle assembly around

Figure 6
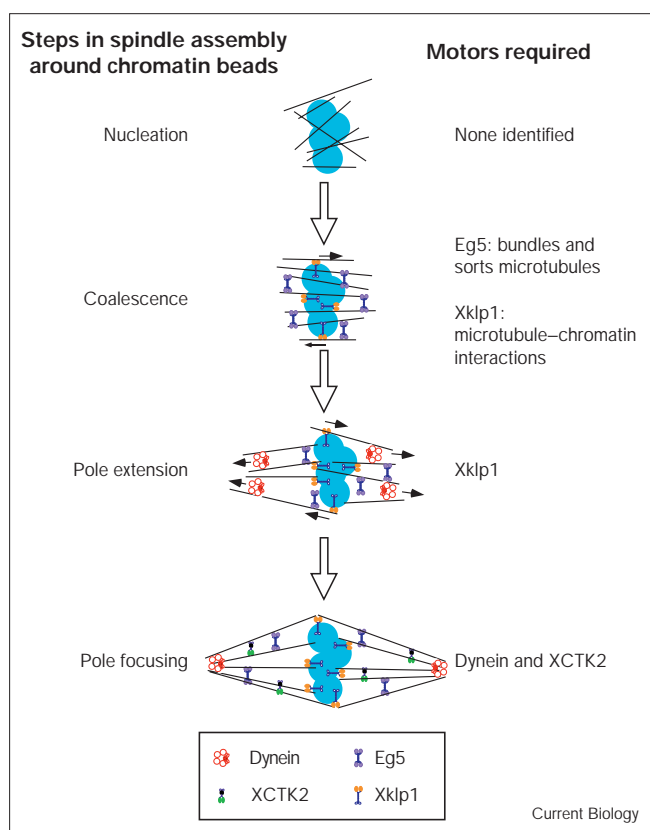
Summary of the roles of motor proteins during the process of bipolar spindle assembly around chromatin beads. Microtubules are nucleated in the region around the chromatin beads. Eg5 bundles and sorts microtubules during the coalescence phase. Xklp1 is required for spindle pole extension, and both dynein and XCTK2 focus spindle poles.

chromatin beads can be separated into several processes (Figure 6): first, nucleation and random growth of microtubules around chromatin beads; second, coalescence of those microtubules into bundles and sorting of bundles into an antiparallel array; third, extension of spindle poles; and fourth, focusing of spindle poles. We have been able to define the motor protein activities required in three of these processes.

Nucleation of microtubules around chromatin beads
None of the motor proteins we examined here had any effect on the nucleation of microtubules around chromatin beads. XKCM1, however, a KLP with a central motor domain, has been shown to influence global microtubule dynamics in *Xenopus* extracts [19]. A future project will be to study whether its activity is regulated locally to promote microtubule stabilization around chromatin beads. It is likely that non-motor proteins — such as Stathmin/Op18, microtubule-associated proteins, and factors involved directly in microtubule nucleation such as gamma tubulin

— are important in generating a population of stable microtubules in this first step of spindle assembly [44–48].

Coalescence of microtubules into bundles and antiparallel microtubule sorting
A key step in spindle assembly is the bundling and sorting of microtubules that is necessary to set up the bipolar axis of the spindle. We have shown here that Eg5 function is required for this process. Inhibition of the protein resulted in monopolar-like microtubule structures emanating from chromatin beads, with their plus ends extending outward. In contrast, in *Xenopus* sperm spindle reactions, or in mammalian cells containing centrosomes, Eg5 inhibition resulted in astral or rosette structures with microtubules emanating from unseparated centrosomes and extending outward toward chromosomes [17,29,31]. We propose that the difference is due to the sites of microtubule nucleation, which in the absence of centrosomes are on or near the chromatin. Minus-end-directed motors are presumably still active when Eg5 is inhibited and can focus the microtubules into astral arrays, but because antiparallel pushing forces are compromised, the microtubule focus remains on the beads. The outwardly splayed structures formed upon Eg5 inhibition, both in the presence and absence of centrosomes, also support a role for Eg5 in establishing the bipolar axis of the spindle by microtubule bundling. These results indicate a role for Eg5 beyond its role in centrosome separation, to form and stabilize parallel and antiparallel microtubule interactions. Support for this model comes from experiments with a *Drosophila* Eg5 homologue, KLP61F [49]. Strong mutant *Klp61f* alleles completely block formation of bipolar spindles, while weaker *Klp61f* alleles result in monoastral bipolar spindles, in which centrosome separation has failed, but a bipolar spindle still forms. Presumably, partial function of the protein allows some antiparallel arrays to form by a self-organization mechanism, although centrosome separation still fails.

XCTK2 also contributes to spindle integrity, as inhibition of its function caused an increase in the proportion of monopolar spindles. As with sperm DNA spindle reactions, the addition of excess XCTK2 protein enhanced spindle formation around DNA beads [20] (unpublished observations). These findings are consistent with the model that XCTK2 bundling activity is important for bipolar spindle formation, independent of the spindle assembly pathway [20].

Extension of spindle poles
It has been proposed that KLPs on chromosome arms contribute to the molecular mechanism responsible for holding mono-oriented chromosomes away from the pole in prometaphase and for ejection of severed chromosome arms from the spindle [34]. This so-called 'polar ejection force' could be produced by plus-end-directed chromosomal motors, driving chromosomes toward the metaphase

plate [50,51]. Alternatively, it could be produced by microtubule polymerization pushing attached chromosomes toward the spindle equator. We show here that inhibition of Xklp1 during spindle assembly around chromatin beads resulted in a failure to extend spindle poles. We also found that inhibition of Xklp1 with Ab03 blocks the migration of chromatin from the center of the aster to the ends of the microtubules in a sperm half-spindle reaction (unpublished observations). Taken together, these results are consistent with a role for Xklp1 in the polar ejection force.

Focusing of spindle poles

We have shown previously that cytoplasmic dynein is required to focus microtubule minus ends into spindle poles [13]. Despite the different pathways of centrosome-dependent and centrosome-independent spindle assembly, dynein function in pole assembly appears to be conserved in both systems [12,35,52]. In *Drosophila*, the KinC family member Ncd is important for focusing poles. *Ncd* mutants form abnormal meiotic and mitotic spindles that contain multiple or splayed poles [36–38]. We show here that the KinC family member XCTK2 contributes to spindle pole formation in *Xenopus* extracts, but only in the absence of centrosomes. In sperm DNA spindle reactions containing centrosomes, inhibition of XCTK2 had no effect on pole morphology [20]. We propose that the role of XCTK2 in pole formation in *Xenopus* extracts is minor relative to dynein and is only revealed in the absence of focal nucleation by centrosomes.

Our studies on Xklp2 function in DNA bead spindles suggest that this motor is not important for spindle formation in our assay, but that its activity might be redundant with that of other motors for pole organization. Inhibition of Xklp2 in combination with dynein or XCTK2 aggravated the effects of inhibition of either XCTK2 or dynein alone, though not dramatically. We favor the idea that Xklp2 activity is more important in the cycled spindle reactions of sperm DNA which contain duplicated centrosomes. Classical centrosome separation has been difficult to document in this system; however, the observation that Xklp2 inhibition causes the collapse of spindles that contain duplicated centrosomes, but has only minor effects on spindles formed by fusion of half-spindles or around DNA beads, suggests that Xklp2 is required to push or hold centrosomes apart [21].

Common mechanisms of spindle assembly

The formation of DNA bead spindles is a model for meiotic spindle assembly, which occurs in the absence of centrosomes. It is important to note that, although spindles in meiotic and somatic cells form by different pathways, many of the same motor proteins are involved in both cases. In mitotic cells, centrosomes dominate as the point of focused microtubule nucleation and provide a kinetic advantage to spindle assembly [12]. Microtubule-based motor proteins are still essential under these conditions, but their precise mechanism of action might be partially masked by the organizational properties of centrosomes. It is likely that motor-dependent microtubule self-organization still occurs in the presence of centrosomes and serves as a redundant mechanism to ensure the accurate formation of a bipolar array. Thus, an analysis of motor protein function in different spindle assembly pathways is essential to our understanding of spindle morphogenesis.

Conclusions

We have examined the roles of different motor proteins during spindle assembly around beads coated with plasmid DNA to generate a model of motor-dependent microtubule organization during spindle formation. We found that spindle bipolarity depends on the activity of Eg5 to bundle and sort microtubules, and on Xklp1 to tether microtubules to chromatin and extend spindle poles. XCTK2 plays a supporting role in maintaining spindle integrity and spindle pole formation, whereas dynein is the dominant motor that focuses microtubules into spindle poles in our system. Thus, we have now identified roles for several motor proteins in the global organization of microtubules into bipolar spindles. It is possible, however, that we have not yet identified all of the motor proteins involved. Furthermore, we will not fully understand how the activities of the motor proteins are integrated to form the dynamic structure of the spindle until we can examine their temporal activation and regulation during the spindle assembly process. In the long run, these analyses will contribute significantly toward reconstituting spindle assembly using purified components.

Materials and methods

Antibodies

Anti-Xklp1 antibodies were raised to bacterially expressed fusion proteins containing the tail domain (Ab65) or part of the stalk (Ab03) as described [18]. Anti-Eg5 antibodies to the stalk and tail region were prepared as described [17]. Anti-XCTK2 antibodies raised to the stalk and tail domain were produced as described [20]. Xklp2 antibodies were generated to the tail region using a fusion of this region and glutathione-S-transferase (GST) [21]. The rabbits were immunized with this fusion protein, and the sera were depleted of anti-GST antibodies before specific anti-Xklp2-tail antibodies were affinity purified according to published procedures [53]. The monoclonal immunoglobulin M (IgM) anti-dynein-intermediate-chain antibody (70.1) and control IgG antibodies were obtained from Sigma Chemical Co. For antibody addition experiments, antibodies were dialyzed against 50 mM potassium glutamate, 0.5 mM $MgCl_2$, or 10 mM Hepes, pH 7.2, 100 mM KCl, concentrated, flash frozen, and stored in aliquots at –80°C. Thawed antibodies were stored at 4°C for up to several months.

Extract preparation and spindle assembly assays

Cytoplasmic extracts of unfertilized *Xenopus* eggs arrested in metaphase of meiosis II by colony stimulating factor (CSF) activity were prepared fresh as described [54,55]. Rhodamine-labeled tubulin prepared from calf brain tubulin was added to 0.2 mg/ml [56]. DNA beads and chromatin bead spindles were prepared as described [13,57]. For antibody addition experiments, all antibodies were added to the reaction before spindle assembly at a dilution of 1:10 or 1:15 of the final reaction volume. In some double-inhibition experiments, it was necessary to

dilute each antibody to 1:15, which resulted in a final dilution of antibodies of 1:7.5 in the extract. In each case, the control IgG antibody was diluted accordingly so that each series of experiments was consistently performed. All inhibition results were confirmed in sperm DNA spindle assembly reactions to show that the reagents were functioning in the same manner as described previously for each motor protein.

Immunodepletion experiments were carried out as described previously [19,55]. The amount of antibody necessary for depletion varied between proteins in a given 200 µl depletion reaction. We used 4 µg antibody for XCTK2 and Xklp2 and 10 µg antibody for Eg5 and Xklp1. For all depletions except that of Xklp1, chromatin was assembled onto the beads in a non-depleted extract. The beads containing assembled chromatin were isolated and then washed with the depleted extract before assembling spindles in the depleted extract. This procedure helped increase the life span of the extract which is greatly shortened after depletion. Because Xklp1 associates with chromatin in the absence of spindle assembly, it was necessary to assemble chromatin and spindles using an Xklp1-depleted extract to ensure that no Xklp1 was present on the chromatin. The efficiency of depletion was assayed by immunoblot of mock and depleted samples, as well as by immunofluorescence on the spindles assembled after depletion.

It should be noted that the DNA–bead spindles are much more sensitive to the effects of depletion than sperm DNA spindles. In general, the efficiency of spindle formation of DNA–bead spindles is routinely lower than that of sperm DNA in the same extract. Immunodepletion also lowers the efficiency of spindle assembly of any given extract, and this is even more apparent in the DNA–bead spindles. After immunodepletion, at least 50% of the extracts were no longer competent to form DNA–bead spindles even though they still formed spindles around sperm DNA. In addition, for Xklp1 it was very difficult to get complete depletion of all detectable protein, and this proved important in interpreting the results. Any residual Xklp1 in the extract after depletion was sufficient to assemble onto chromatin beads and function in spindle assembly. For all other motors, an incomplete depletion still severely compromised the ability to form spindles in the extracts.

Immunofluorescence
Spindle assembly reactions were diluted and spun onto coverslips as described [55,58]. The samples were post-fixed with methanol and then processed for immunofluorescence as described [19,20,58]. Antibodies to Eg5 were used at a final concentration of 1 µg/ml; all other antibodies were used at a final concentration of 5 µg/ml.

Data acquisition
To evaluate structures formed in spindle reactions, samples were examined from at least three independent experiments. In most experiments, the same results were obtained qualitatively in at least five independent experiments. Coverslips were examined under a 40× lens field-by-field, and bead–microtubule arrays were classified accordingly. Data presented are summations of two to five experiments; between 220 and 1000 spindle structures were evaluated for each motor or motor combination. Photomicrographs were taken on either a Nikon Optiphot-2 or a Nikon E-600 with a 40× objective (Planfluor 0.75NA) and a cooled charged-coupled device camera (Princeton Instruments). Images were transferred to Adobe Photoshop and processed.

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