Roles of polymerization dynamics, opposed motors and a tensile element in governing the length of *Xenopus* extract meiotic spindles.

T.J. Mitchison\(^1,2,6\), P. Maddox\(^1,3\), J. Gaetz\(^1,4\), A. Groen\(^1,2\), M. Shirasu\(^1,2\), A. Desai\(^1,5\), E.D. Salmon\(^1,3\) and T. M. Kapoor\(^1,4\).

\(^1\) Marine Biological Laboratory, Woods Hole, MA  
\(^2\) Dept. Systems Biology, Harvard Medical School, MA  
\(^3\) Dept. Biology, U. Nth Carolina, NC  
\(^4\) Rockefeller U., NY  
\(^5\) Dept Cell and Molec. Med., U. Ca. San Diego, CA  
\(^6\) To whom correspondence should be addressed: timothy_mitchison@hms.harvard.edu
Abstract

Metaphase spindles assemble to a steady state in length by mechanisms that involve microtubule dynamics and motor proteins, but are incompletely understood. We found that *Xenopus* extract spindles recapitulate the length of egg meiosis II spindles, using mechanisms intrinsic to the spindle. To probe these mechanisms, we perturbed microtubule polymerization dynamics and opposed motor proteins, and measured effects on spindle morphology and dynamics. Microtubules were stabilized by hexylene glycol and inhibition of the catastrophe factor MCAK (a kinesin 13, previously called XKCM) and destabilized by depolymerizing drugs. The opposed motors Eg5 and dynein were inhibited separately and together. Our results are consistent with important roles for polymerization dynamics in regulating spindle length, and for opposed motors in regulating the relative stability of bipolar vs. monopolar organization. The response to microtubule destabilization suggests that an unidentified tensile element acts in parallel with these conventional factors, generating spindle shortening force.
Introduction

A mitotic, or meiotic, spindle at metaphase can maintain a steady state in size and shape for prolonged periods, despite rapid turnover of subunits, movement of internal components and dissipation of free energy. In this paper we address the mechanisms that govern metaphase spindle length. Length is important for spindle function, since it influences the distance over which chromosomes are segregated. Furthermore, probing the factors that govern length will provide information on assembly and force producing mechanisms. Metaphase spindle length tends to be relatively constant within a given cell type, but varies considerably between species and between cell types in an organism. Spindle length typically increases with cell size and genome size, but this relationship can break down in specialized cells. In large eggs, female meiotic spindles are typically small compared to the egg cell. This is appropriate for meiosis biology; the egg meiotic spindle segregates one set of chromosomes a short distance into a polar body, retaining the other set near the cortex. In *Xenopus laevis*, the egg is ~1000µm in diameter, and unfertilized eggs arrest at metaphase of meiosis II containing a spindle ~25 µm in length attached at one pole to the cortex at the top of the egg (Cha et al 1998). Spindles assembled in cytoplasmic extracts made from unfertilized *Xenopus* eggs recapitulate meiosis II morphology (Sawin and Mitchison 1991a), but their length has not been systematically studied.

Spindle length per se has received relatively little attention, but many models have been proposed for how forces on chromosomes and poles are generated. These forces are thought to also govern spindle length, with steady state length arising from a balance of pushing and pulling forces. Force balance models can be divided into those that highlight the role of microtubule polymerization dynamics (Inoue and Sato 1967, Margolis and Wilson 1981, Mitchison et al 1986, Inoue and Salmon 1995), those that highlight action of ATPase motor proteins (McIntosh et al 1969, Hoyt et al 1993, Gaglio et al 1996, Sharp et al 2000, Nedelec 2002, Cytrynbaum et al 2003), and those that highlight the role of the “spindle matrix”, a hypothetical, non-microtubule, tensile element (Pickett-Heaps et al
Recently, a different type of model was proposed, in which spindle length is set not by a balance of forces, but by a concentration gradient of morphogens diffusing from a source at chromatin to a global sink in the cytoplasm (Karsenti and Vernos 2001). Most of these models have in common that they seek to explain spindle length with the microtubule system, including dynamics regulators, motors and cross-linkers, as the sole mechanochemical element. Exceptions are the original polymerization dynamics model, that preceded the discovery of tubulin (Inoue and Sato 1967), and spindle matrix models, that explicitly propose a non-microtubule, tensile element (Pickett-Heaps et al 1997).

The most widely discussed models have been those in which spindle length is governed by some combination of polymerization dynamics and opposed motor proteins, and the purpose of this study was to critically evaluate these models in *Xenopus* extract spindles.

A useful distinction in considering models for spindle length regulation is between mechanisms that act extrinsic to the spindle, vs. intrinsic mechanisms. Potential extrinsic mechanisms include limiting amounts of some subunit, and forces generated at the cell cortex. Intrinsic mechanisms include balanced forces within the spindle, and a possible morphogen gradient emanating from chromatin. In mammalian tissue culture mitosis, the spindle incorporates ~50% of the cell’s tubulin (Zhai and Borisy 1994), suggesting component limitation is a significant factor. Pulling forces from the cortex acting on astral microtubules are known to play a significant role in length regulation in several mitotic systems (Sharp et al 2000). Thus extrinsic and intrinsic factors probably act in concert to govern the length of typical mitotic spindles. In contrast, extrinsic mechanisms are probably much less important in egg meiosis. The meiotic spindle is small compared to the egg (~$10^{-5}$ of the egg volume in *Xenopus*), and presumably does not deplete a significant fraction of the egg’s tubulin. Pulling from the cortex operates mainly on one spindle pole in egg meiotic spindles (Lutz et al 1988), and is probably a minor factor in governing spindle length. In this study we formally demonstrate that the length of spindles assembled in *Xenopus* egg extracts is governed by intrinsic mechanisms, and we investigate these mechanisms by perturbation experiments.
Results

To measure spindle length in a convenient and non-perturbing way, mitotic extract (3-10 µl) containing spindles with replicated chromosomes (“cycled spindles”, Desai et al 1999a) was spread in a thick (~100µm) layer under mineral oil, and imaged by polarization microscopy (Figure1A). Spindles were remarkably stable in this preparation, and retained a constant length for at least 30 min by time lapse imaging (not shown). The spindles usually rotated and translated slowly, showing they were free of interactions with the substrate that might influence their length. To test if the amount of any extract component is limiting for spindle length, we assembled spindles in parallel at three different concentrations of added sperm, then measured the distribution of spindle lengths (Figure 1A,B). The morphology, birefringence, mean length, and length distribution, was similar in each case. A confocal fluorescence image of a meiosis II spindle at similar magnification in an unfertilized egg is shown for comparison (Figure 1C). The average length of extract spindles varied slightly from prep to prep, in part due to variability in the extent to which spindles fused together. Extract spindles are, on average, a little longer than egg meiosis II spindles (~25µm, Cha et al 1998), but overall the extract system does a good job of recapitulating meiosis II morphology. Since spindle length was independent of spindle concentration, and spindles appeared not to interact with the substrate, we conclude that length is regulated by mechanisms intrinsic to the spindle.

To test if protein polymerization dynamics play an important role in governing spindle length in Xenopus extracts, as they do in other spindles (Inoue and Sato 1967), we first measured the effect of an agent that non-specifically promotes protein assembly. We used 2-methypentane-2,4-diol, called “hexylene glycol” in the mitosis literature (suggested by Robert Palazzo (RPI)). This solvent promotes aggregation of many proteins, and is used for this purpose in crystallography. It also promotes microtubule polymerization, and stabilizes spindles and asters (Rebhun et al. 1975; Harris and Clason 1992). At 3% v/v and above, hexylene glycol promoted rapid nucleation of microtubule asters throughout the extract. At 2% it only slowly promoted nucleation, but had a remarkable effect on preformed spindles, causing them to increase in birefringent retardation (a measure of
spindle microtubule density), and to grow progressively in length and volume (Figure 2A, movie M1). Spindle length increased at a constant rate of $\sim 1.7 \mu$m/min, at least to the point where length doubled (figure 2B). To probe the mechanics of elongation in hexylene glycol, we imaged fluorescent tubulin at speckle levels by spinning disc confocal microscopy (Figure 2C, movie M2). Visual inspection and kymograph analysis (Figure 2D) showed that speckles throughout the spindle moved polewards at a rate similar to the rate of pole separation. We conclude that spindles elongate in hexylene glycol by anti-parallel sliding between the two half-spindles, with little or no microtubule depolymerization. This situation is reminiscent of poleward flux with depolymerization blocked. The antiparallel sliding component of poleward flux can be blocked by AMPPNP (Sawin et al 1991b) and Eg5 inhibitors including monastrol (Miyamoto et al 2004), so we tested the effect of these agents on spindle elongation. Monastrol caused spindle collapse when added alone, but this collapse could be suppressed by also adding p50 dynamitin (discussed below). Hexylene glycol induced elongation was blocked by both agents (Figure 2B). We conclude that spindle elongation in hexylene glycol occurs by anti-parallel sliding between the half spindles, most likely driven by Eg5. Consistent with this view, extract spindles also elongate by anti-parallel sliding when pole organization is disrupted (Gaetz and Kapoor 2004), dependent on Eg5 activity (Shirasu-Hiza et al 2004). The rate of spindle expansion in hexylene glycol ($\sim 1.7 \mu$m/min) is slower than that rate of anti-parallel sliding during flux in control spindles ($\sim 4 \mu$m/min). Eg5 motors appear to work against an unknown mechanical load to drive flux (Miyamoto et al 2004), and we suspect that hexylene glycol increases this load.

We next tested a more specific microtubule stabilizing agent, inhibitory antibody to *Xenopus* MCAK (a kinesin 13, formerly called XKCM1). This kinesin promotes microtubule catastrophes in an ATP-dependent reaction (Desai et al 1999b). It is the most potent known catastrophe factor in *Xenopus* egg extract (Tournebize et al 2000), and removing or inhibiting it induces massive microtubule polymerization in M phase extracts (Walczak et al 1996). Plus ends in extract spindles are thought to undergo bounded dynamic instability, meaning that they do not grow indefinitely, but rather catastrophe frequently enough to have a defined average length (Verde et al 1992).
Titrating a catastrophe inhibitor into extract is predicted to first increase this average length, and then cause a transition to the unbounded regime, where plus ends grow indefinitely. We titrated anti-MCAK into extract, adding the inhibitor well before spindle assembly, and fixing at two time points, to ensure we were measuring spindle length at steady state. Increasing concentrations of anti-MCAK up to 10 µg/ml caused a small, dose-dependent increase in spindle length, and a larger, dose-dependent increase in total microtubules per spindle (figure 3). At 15 µg/ml (figure 3) and above (figure 4), microtubules elongated dramatically, forming large asters. When MCAK was inhibited to this extent before spindle assembly, disorganized structures formed whose length could not be defined (figure 3). We infer that decreasing the catastrophe rate while staying in the bounded regime modestly increased spindle length. Decreasing catastrophe to the point of entering the unbounded regime resulted in disorganization, and not a dramatic increase in length.

To better understand the effects of unbounding microtubule length, we imaged preformed spindles live after adding saturating amounts of anti-MCAK. Microtubules rapidly extended away from the spindle, converting them into large asters (Figure 4A, movie M3). During this outgrowth, the spindle itself did not appear to elongate, and in some cases the poles even moved slightly closer together. Although the principle effect of inhibiting MCAK is inhibiting plus end catastrophes, this protein also has the biochemical capability to depolymerize minus ends at poles (Desai et al 1999b), and other kinesin 13 family members have been implicated in this activity (Rogers et al 2004, Gaetz and Kapoor 2004). To probe effects on poles, we imaged a fluorescent spindle pole marker as well as tubulin speckles after inhibiting MCAK (Figure 4B, movie M4). The pole marker was affinity purified antibody raised to a carboxy terminal peptide from *Xenopus* NuMA, labeled with Alexa488. NuMA accumulates at the poles of *Xenopus* extract spindles by a dynein-dynactin dependent mechanism, and has often been used as a pole marker (Merdes et al 1996). Our antibody was specific by immunoprecipitation (supplementary figure 1), gave the reported localization for NuMA in live and fixed spindles, and had no discernable effect on spindle assembly or dynamics when added to extracts, or on the response to inhibiting MCAK as judged by comparing effects in the
tubulin channel with and without the probe. Confocal imaging confirmed massive outgrowth of microtubules from the spindle starting a few minutes after adding anti-MCAK. Anti-parallel microtubules sliding in the center of the spindle that is characteristic of polewards flux continued (movie M4). By tubulin imaging alone, and more informatively by tubulin + NuMA imaging, we observed progressive disorganization of poles. In about half the spindles, this disorganization takes the form of the pole elongating and curling backwards towards the equator, appearing to track back along the outer surface of the spindle (Figure 4B, movie M4). Tubulin speckles follow this pole movement, moving out from the main body of the spindle, and curling around with the moving pole (blue arrows in Figure 4B). Due to the disorganization of pole structure, and lack of a direct assay for depolymerization, we were unable to quantify microtubule depolymerization at poles in this experiment.

To further probe the role of microtubule dynamics in governing spindle length, we rapidly depolymerized microtubules. We added 20 µM nocodazole to an aliquot of extract containing spindles on a microscope slide, mixed, put on a coverslip, and initiated imaging using a dry 40x lens to facilitate rapid location of spindles. Spindles shortened and depolymerized completely in 2-3 minutes with this treatment, and it was necessary to locate them within ~10 seconds of drug addition to obtain useful information on early events. Spindle shortening in nocodazole was previously argued to occur by pulling at kinetochores (Cassimeris et al 1990), so we used a non-perturbing kinetochore probe (Alexa488-antiCenpA IgG; Maddox et al 2003) to observe possible action of such forces. As expected from previous work (Inoue and Sato 1967, Salmon et al 1984, Cassimeris et al 1990) the microtubule density dropped rapidly, and the pole-pole distance decreased (figure 5A, movie M5). Note that the images in figure 5 and movie M5 are normalized to peak intensity, optimizing visualization of remaining structures, but giving a misleading impression of microtubule density, which is quantified as total tubulin fluorescence in figure 5B. We expected to see stretched kinetochores pulling the poles inwards in this experiment, and were surprised to observe that the distance between sister kinetochores invariably decreased shortly after drug addition, indicating loss of tension (red dots in figure 5A, triangles in figure 5B, see movie M5). All kinetochores visualized
experienced this relaxation (>50 kinetochore pairs in 13 spindles in 10 sequences). In some cases, kinetochores appeared to experiencing compression during spindle collapse. This was evident from lateral movement away from the spindle axis, twisting of the kinetochore pair (figure 5A 119 sec), and apparent curving or buckling of kinetochore microtubules (figure 5A 119 sec, note microtubule bundle connected to the upper sister of the pair marked with blue lines, see also movie M5).

Further investigation of the forces in collapsing spindles required high resolution imaging, which was difficult using nocodazole addition because it took too long to find and focus on a spindle. Therefore, we synthesized a photochemically “caged” microtubule depolymerizing drug that allowed us to find and image a spindle before and after triggering depolymerization (Figure 6A). We started with N-(2-napthyl)-3-trifluoromethylbenzene sulfonamide (“105D”), a depolymerizing drug that was found by phenotypic screening of a combinatorial library (Mitchison 2003). It arrests cells in mitosis in tissue culture cells with a phenotype similar to nocodazole, and an IC$_{50}$ of $\sim$3 µM ($\sim$20-fold less potent than nocodazole). It inhibited polymerization of pure tubulin (supplementary Figure 2) and depolymerized extract spindles with an IC$_{50}$ of $\sim$20µM, again $\sim$20-fold less potent than nocodazole. 105D has several advantages for making a caged derivative. It is simple to synthesize and modify (supplementary Figure 3). The caged drug had no detectable effect on microtubules or extract spindles at its solubility limit ($\sim$400 µM). 105D is weakly fluorescent in the DAPI channel, while its caged derivative is not. Thus photorelease and subsequent movement of the drug can be imaged and quantified by fluorescence microscopy (supplementary Figure 4).

Time lapse imaging of spindles before and after photorelease of 105D showed effects broadly similar to nocodazole addition. The intensity of the tubulin signal rapidly decreased after photorelease (figure 6B, D), the spindle shortened (figure 6B,C,D, movies M6, M7) and sister kinetochores moved together and twisted (figure 6D,movie M7). 105D did not depolymerize spindle microtubules as efficiently as nocodazole, and a subset of microtubule bundles were stable for several minutes after photorelease. Most of these stable microtubules terminated at kinetochores (figure 6D, movie M7), implying
they are kinetochore microtubules, which are known to be selectively resistant to
depolymerizing drugs in other systems (Cassimeris et al 1990). In nocodazole,
kinetochore fibers eventually disappeared (figure 5A), but after uncaging 105D they did
not, perhaps because 105D partially stabilizes kinetochore microtubules. Other tubulin
drugs are known to have stabilizing as well as destabilizing effects (Wilson et al 1999).
Both wide-field (figure 6C) and confocal (figure 6D) sequences suggested that the
spindle poles are pulled (or pushed) together after photorelease, resulting in compression
of attached microtubules. These forces caused buckling of selectively stable kinetochores
fibers, movement of sister kinetochores towards each other, and twisting or sideways
movement of the sister pair. If the poles were pulled together by microtubules, we would
expect to see straight bundles of microtubules connecting them. By through-focus
imaging of >10 collapsing and fully collapsed spindles using wide-field and confocal
microscopy we were unable to find any straight bundles of microtubules connecting
poles, or any sister kinetochore pairs still under tension. Figure 6D 941”, 948” show two
images from a through focus confocal image series where all remaining microtubule
bundles were either buckled, or pushed out sideways from the spindle axis, and all
kinetochore pairs are close together. We tentatively conclude that when overlap
microtubules are rapidly removed with a drug, the poles are pulled (or pushed) together
by something other than microtubules. Spindle collapse is unlikely to depend on F-actin,
since our extracts routinely included cytochlasin D (∼3μM), and addition of latrunculin B
to 30 μM (in addition to cytochalasin D) had no effect on the speed, extent or
morphology of collapse induced by photorelease of 105D (not shown). The photorelease
experiment made it possible to collect before and after data on spindle length and
microtubule density. For 7 representative spindles, the peak rate of shortening (measured
pole-to-pole) averaged 7 μm/min (range 2-13 μm/min). Length plateaued after 350 sec on
average (n=6, range 300-450 sec), when the spindle was 47% on average of its initial
length (range 31-57%). Total tubulin fluorescence plateaued ∼ 300 sec after initiating
depolymerization, at an average of 8% of the initial fluorescence (range = 5-11%). The
half-time between initiating depolymerization and reaching the plateau in total
fluorescence was 55 sec (n=7, range = 53-60 sec).
We next probed the role of opposed motor proteins in governing spindle length, focusing on Eg5 and dynein. Eg5 is essential for bipolarity in extract spindles (Sawin et al 1992, Kapoor et al 2000), where it drives anti-parallel sliding associated with poleward flux and spindle elongation (Figure 2, Miyamoto et al 2004, Shira-Hizu et al 2004). Dynein works together with dynactin and NuMA to organize and focus the poles (Merdes et al 1996), and appears to be the dominant minus end directed motor in *Xenopus* extract spindles on the basis of inhibition experiments (Heald et al 1998). Using polarization microscopy, we confirmed the effects of inhibiting Eg5 with monastrol, and dynactin with excess p50 dynamitin, (Figure 7B,C). Unexpectedly, when both these inhibitors were added together, they counteracted each other (Figure 7D). Almost all spindles were now bipolar, and their poles were more organized than with p50 alone. We quantified length and morphology for spindles in the presence of inhibitors, adding them before and after spindle assembly (table 1). p50 almost completely rescued the effect of monastrol on bipolarity, whether it was added before or after assembly. Monastrol partially rescued the effect of p50 on poles morphology. When both inhibitors were added before spindle assembly, length was almost completely rescued (32 µm vs 39 µm in controls; table 1). This was less true when both inhibitors were added after assembly (23 µm vs 37 µm in controls; table 1), probably reflecting reduced effectiveness of p50 when added after assembly. Although the double inhibited spindles had relatively normal morphology and length by polarization imaging, they were much more fragile than control spindles. Unlike control spindles, they were easily damaged by squashing between a slide and coverslip, or by touching with microneedles, and their average length in replicate experiments was more variable than controls. We note that the concentrations of p50 we used was probably insufficient to completely block pole organization. Complete inhibition of pole organization tends to increase spindle length (Gaetz and Kapoor 2004, Shirazu-Hiza et al 2004), perhaps due to displacement of a kinesin 13 depolymerization factor from the poles (Gaetz and Kapoor 2004).

Eg5 is still present in spindles after monastrol treatment (Kapoor et al 2001), and might influence the response to dynein inhibition even when inhibited. We therefore tested if p50 could also rescue the effect of Eg5 depletion. Depletion of Eg5 to <5% of normal
resulted in assembly of almost entirely monopolar spindles as previously reported, and addition of p50 before assembly rescued this effect (figure 8). Eg5 depleted p50 spindles were mostly bipolar, their poles were more focused than in p50 alone, and they were approximately the right length. We conclude that while inhibition or removal of Eg5 caused spindles to collapse to monopoles, co-inhibition of dynein reversed that effect, and allowed assembly of spindles that are physically fragile, but nevertheless able to achieve an approximately normal steady state length. Rescue of bipolarity in Eg5 inhibited spindles by p50, discovered here, was a key technical advance for probing the role of Eg5 in flux (Miyamoto et al 2004).
Discussion

In this paper we begin a systematic experimental attack on the mechanisms that govern spindle length in the *Xenopus* extract system. Extract spindles, and by implication egg meiosis II spindles, achieve a steady state in length and mass by purely intrinsic mechanisms (figure 1). We performed perturbation experiments to test standard models for length regulation based on polymerization dynamics and opposed motor proteins, finding they can account for some, but not all, of the results. To account for the response to rapid microtubule depolymerization, we propose adding a non-microtubule tensile element. Since egg meiotic spindles are small relative to the cell than contains them, and their assembly is largely chromatin-driven rather than MTOC-driven (Karsenti and Vernos 2000), they may employ length-governing mechanisms that are different from somatic mitotic spindles.

Polymerization dynamics models predict that increasing microtubule length by increasing polymerization or decreasing depolymerization should cause spindles to elongate. They can account for the response of extract spindles to hexylene glycol (figure 2), and also for the slight increase in spindle length observed when the catastrophe factor MCAK is partially inhibited, but dynamic instability is still bounded (figure 3). They fail to account for the response of spindles to stronger MCAK inhibition, when microtubules go into unbounded growth and plus ends leave the spindle, but the spindle poles do not separate further, and rather curl back towards the equator (figure 4). This curling phenomenon might be due to inhibition of minus end depolymerization at poles by anti-MCAK, or simply to disorganization of poles by misdirected motor activity (discussed below). We currently lack an assay for measuring depolymerization at poles that is required to distinguish these possibilities. Kymographs of tubulin speckles do not provide a reliable assay for depolymerization at poles, since converting sliding rates into depolymerization rates requires knowing whether minus ends are static or moving, which has not been measured in extract spindles. Previous interpretation of kymographs assumed static minus ends depolymerizing at poles during metaphase (eg Sawin and Mitchison 1991, Rogers et al 2004), and Kinesin 13 family members (previously called KinI kinesins)
were implicated in depolymerization (Rogers et al 2004, Gaetz and Kapoor 2004). However, an alternative model can be proposed, in which stable minus ends are distributed throughout the spindle. These ends move polewards at the flux rate without depolymerizing, and loose stability when they reach the poles, leading to loss of the microtubule from the plus end. To distinguish these models, we need to localize minus ends in extract spindles, and measure their dynamic behavior. Perhaps the largest discrepancy from standard polymerization dynamics models was the response to microtubule depolymerizing drugs. In previous work, drug- or pressure-induced spindle shortening was interpreted as a consequence of pulling forces at kinetochores generated by microtubule depolymerization (Cassimeris et al 1990, Inoue and Salmon 1995). Instead, we found that depolymerizing drugs induced a switch from tension at kinetochores in unperturbed spindles (Maddox et al 2003) to compression, arguing that kinetochores were not pulling the poles together. In fact our imaging suggested that the poles are pulled together by something other than microtubules, discussed below.

Opposed motor models predict that when the zone of microtubule overlap in the spindle is increased by microtubule polymerization, more plus end directed motors are recruited, increasing the sliding forces between anti-parallel microtubules, and elongating the spindle (Sharp et al 2000, Cytrynbaum et al 2003). Such models predict spindle elongation in hexylene glycol, its sensitivity to AMPPNP and Eg5 inhibitors, and elongation with partial inhibition of MCAK. A motor model might also account for the effect of complete MCAK inhibition on pole morphology (figure 4B). Minus end directed motor complexes at the pole faced with large numbers of invading microtubules of the wrong polarity may track towards the minus ends of the invaders, causing curling of the poles. We tried to test this by inhibiting dynein and MCAK at the same time, but the results were ambiguous due to disorganization of poles. Opposed motor models predict opposite effects of inhibiting Eg5 and dynein/dynactin, and compensating effects when both are inhibited (Hoyt et al 1993, Gaglio et al 1996, Sharp et al 2000). In motor inhibition experiments, we tended to see either relatively normal length bipoles, or monopoles, rather than intermediate length bipoles (Table 1, inhibitors added before assembly). This suggests that opposed motor activity controls the relative stability of
monopolar vs bipolar organization as a switch-like transition, rather than controlling
spindle length as a continuous variable. More work is required to address this point
experimentally. In a systematic investigation of different classes of opposed-motor
models, Nedelec (2002) found several stable bipolar solutions. In all cases the relative
stability of bipolar vs. monopolar organization depended on motor activities, but in most
cases motor activities did not control pole-pole distance, which was instead governed by
microtubule polymerization dynamics. One of the functions of Eg5 in bipolar spindles is
to drive the sliding components of poleward flux. At the monastrol concentration used in
figure 7 and table 1 (200 µM), rate of anti-parallel sliding at metaphase is reduced to
~10% of control values (near the detection limit; Miyamoto et al 2004), yet average
spindle length in spindles treated with p50 plus monastrol is reduced by less than 2 fold.
Insensitivity of steady state length to the rate of antiparallel sliding is surprising, since
sliding should tend to increase length, and spindles indeed elongate when microtubule
destabilization at poles is inhibited (Gaetz and Kapoor 2004, Rogers et al 2004, Shirasu-
Hiza et al 2004). To account for these observations, either minus end depolymerization
rate must decrease in concert with sliding rate after Eg5 inhibition/removal, or spindle
length must be governed by a mechanism that is insensitive to sliding rate. New methods
for probing minus end dynamics are required to distinguish these hypotheses.

The most surprising observation in our study was the response of spindles to microtubule
depolymerizing drugs, which indicated that poles can be pulled (or pushed) together by
something other than microtubules. It is possible that our through-focus imaging missed a
small number of straight microtubules running pole-pole that pulled poles together in
response to 105D, but we consider this unlikely since kinetochore bundles remained
buckled for several minutes after photorelease, implying the pulling factor is does not
depolymerize. We also consider it unlikely that 105D causes microtubules to curve on
their own, since it did not have this action on pure microtubules, and the response to
nocodazole also showed evidence for kinetochore compression and microtubule buckling.
We hypothesize that an unidentified tensile element pulls the poles together, and that this
element also opposes elongation in unperturbed spindles. We consider two possible
candidates for this element, external membranes and an internal matrix. Extract spindles
are surrounded by a sheath of membranous organelles, including mitochondria and endoplasmic reticulum, that appear physically connected in thin section EM images (Coughlin and Mitchison, unpublished). Membranes may be important for spindle assembly, since it failed in high speed supernatants of *Xenopus* extracts, unless they were supplemented with purified membranes (Shirazu-Hiza and Mitchison, unpublished). Membranes also surround meiotic spindles in Drosophila oocytes, where their importance for spindle assembly was revealed by genetics (Kramer and Hawley 2003). Perhaps membranes serve as a tensile element encapsulating the spindle, tending to oppose spindle expansion, and driving collapse when microtubules are depolymerized. Alternatively, spindles may contain some internal polymer or gel that exerts tension between the poles, as proposed in the spindle matrix hypothesis (reviewed in Pickett-Heaps et al 1997). Detergent treated, isolated sea urchin embryo spindles contracted when microtubules were removed using calcium (Salmon and Segall 1980), an observation more consistent an internal matrix than tension from membranes. A molecular candidate for an internal matrix is poly(ADP-ribose), a non-protein macromolecule that is required for bipolar organization of *Xenopus* extract spindles, and appears to turn over much less rapidly than microtubules (Chang et al 2004).

In figure 9 we combine polymerization dynamics, motors, and a hypothetical tensile element to try and account for all our data. The model is more explicit in molecular terms than the data justify, but we hope it will provoke discussion and attempts at experimental falsification. We envisage the matrix as a cross-linked gel that attaches to poles and plus end directed motors (Kapoor and Mitchison 2001), and thus become stretched, storing elastic energy and pulling the poles inward at steady state. Hexylene glycol, a non-specific protein aggregating reagent, promotes recruitment of more matrix in addition to stabilizing microtubules, resulting in balanced growth, and increased spindle length while retaining approximately normal morphology. MCAK inhibition leads to increased tubulin polymerization without a parallel increase in matrix assembly, and unbounded growth of plus ends through the poles and out of the spindle. Although plus end directed motors try to push the elongated half spindles apart, this is opposed by matrix stretched between the poles, and curling of poles back towards the equator driven by dynein-containing
complexes moving on microtubules of the wrong polarity that invaded the poles (orange arrows). Drug induced depolymerization causes microtubules to disassemble faster than the matrix. Tensile forces focus onto remaining kinetochore microtubules, causing spindle collapse with buckling of kinetochore fibers. Dynactin inhibition by p50 leads to splaying out of poles, and detachment of matrix. Partial destruction of poles by p50 (this paper) did not increase spindle length, perhaps because some matrix remains attached. Complete destruction of poles cause spindles elongation (Gaetz and Kapoor 2004, Shirazu-Hisa et al 2004). Eg5 inhibition promotes movement of the poles together by a combination of matrix contraction and dynein pulling. Inhibition of both dynactin and Eg5 results in bipolar spindle that are physically fragile and lack poleward flux (Miyamoto et al 22004) but are relatively normal in length (table 1). We propose these lack matrix as well as the opposed motor systems, and that they regulate length by dynamic instability alone. Figure 9 does not address other potentially important processes in spindle length regulation, including signals diffusing from chromatin and poleward flux, and new experiments are required to integrate these processes into a complete model. The spindle matrix hypothesis has long been controversial, but the experiments we report should help in the design of future experiments to test molecular candidates.
Acknowledgements

This work was funded by NIH grants GM39565 (TJM), GM24364, GM606780 (EDS), and by MBL fellowships from UIC Inc. and Nikon Inc. We thank other members of the MBL Cell Division Group and our winter labs for comments, and David Gard for information on the egg meiosis II spindle.
Methods

*Xenopus* egg extract sand spindles with replicated DNA were made by standard methods (Desai et al 1999a), and used within 90’ of assembly. Spindle assembly and imaging were performed at 19-20°C. For polarization imaging we used a Nikon (Melville, NY) TE-300 inverted microscope equipped for DIC using DeSernamont compensation, a 20x long working distance objective, a heat reflection filter and a cooled CCD camera. The Wollaston prisms were removed, and the polarizer set slightly away from extinction. A chamber was made by drilling a 22mm round hole in a 1mm thick sheet of stainless steel the shape of a microscope slide. A 25mm round coverslip was cemented under the hole with valap (33% paraffin wax, 33% beeswax, 33% lanolin). 3-10 µl of extract containing spindles was deposited on the coverslip and smeared out into a disc 5-10mm wide with the pipette tip. The spread extract was immediately covered with 250µl of mineral oil.

Length measurements were made by capturing images of many random fields and measuring all spindles, ignoring multipolar structures. Perturbing reagents were added and mixed immediately before spreading the extract in the chamber. Microscopes for widefield and confocal fluorescence imaging, and fluorescent probes for tubulin and kinetochores have been described elsewhere (Desai et al 1999a, Maddox et al 2003). Optimal concentrations of X-rhodamine tubulin for confocal speckle imaging were determined empirically for each extract, with ~50nM typical. Affinity purified, inhibitory antibody to *Xenopus* MCAK was made and characterized as described (Walczak et al 1996). Affinity purified anti-NuMA IgG was made by immunization with the C-terminal peptide (C)TAKSPRASNLFEERKQRNK coupled to KLH, and affinity purified using the same peptide coupled to agarose, using the methods described in (Field et al 1998). Specificity was tested by immunoprecipitation as described in the legend to supplemental figure 1. Anti-NuMA pulled down primarily a band of the expected mol wt, whose identity was confirmed by western blotting of the immunoprecipitate (supplemental figure 1). Western blotting of whole extract was negative for NuMA, presumably because the high protein concentration interfered with transfer to nitrocellulose, a problem we have noticed with other antibodies. For imaging, the IgG was labeled with Alexa488-NHS ester (Molecular Probes, Eugene OR) according the manufacturers
recommendations. p50 dynamitin was made as described (Heald et al 1997). Immunodepletion with magnetic beads was performed as described (Funabiki and Murray 2000).

The microtubule depolymerizing drug 105D was tested for effects on pure tubulin polymerization as described in the legend to supplemental figure 2. Its caged derivative had no effect in this assay. Synthesis of 105D and its and its caged derivative are described in the legend for supplemental figure 3. Both were >95% pure by TLC and LC/MS, and gave the expected molecular ions. On photolysis in methanol using a hand-lamp, caged 105D released 105D with ~100% yield by TLC and LC/MS. For experiments in extracts, caged 105D was dissolved in DMSO as a 200mM stock by warming to 60°, and added to extracts to a final concentration of 400µM. This concentration is approximately the solubility limit, and it was chosen so that only a fraction of the compound had to be photocleaved to cause microtubule depolymerization, thus limiting the UV dose delivered to spindles. The caged drug had no discernable effect on spindle assembly in the absence of UV light. To measure the rate of uncaging using a microscope in extracts, we collected sequential images with a DAPI filter set. 105D is weakly fluorescent in the UV while its caged derivative is not, so the field gets brighter as uncaging proceeds (see supplemental figure 4). We estimated a half-time for photorelease in extract of ~5 seconds with 360nm illumination from a 100W Hg bulb through a DAPI filter cube to a 60x/1.4NA PlanApo objective in a Nikon (Melville NY) 800e upright microscope. For spindle depolymerization experiments, caged 105D was added to preformed spindles in extracts that also contained X-rhodamine tubulin (~200nM) and Alexa488-anti-CenpA (~1µg/ml; Maddox et al 2003). Spindles were located using dim rhodamine illumination and time lapse imaging initiated with a double label filter cube (widefield) or no filter cube (confocal). After a few pre-uncaging frames had been collected, a DAPI filter cube was brought into the epi path, and the field illuminated with 360nm light for 1-2 sec. Then the cube was changed back and the time-lapse sequence continued. 1-2 sec of UV illumination in extracts containing 400µM caged 105D generated sufficient free105D to depolymerize spindles in the field, and had no effect on spindles when the caged drug was not present. The effect of uncaging was
remarkably local. A spindle in the microscope field (~200µm circle) subject to 360nm illumination with caged-105D present rapidly disassembled, while spindles outside the field were unaffected. Thus we were able to trigger and follow depolymerization of several spindles in each slide-coverslip preparation. To determine why the effects of photoreleasing 105D are local and persistent, we imaged the drug diffusing away from a UV illuminated area, using its intrinsic fluorescence, and limiting the observation light to minimize further photorelease. We observed that 105D partitions into membranes, and moves only very slowly through the extract after photorelease (supplementary Figure 4), explaining its local effects on minute time scales.
References.


Table 1.

Affect of motor protein perturbation on spindle morphology and length scored by polarization microscopy.

Spindle assembly, drug concentrations and polarization imaging as per Figure 7. Motor perturbing agents were added either before spindle assembly, at the time of CSF add-back, with images taken 90-120 min later, or 60 min after CSF add-back, when steady state spindles were already assembled, with images taken 30-60 min later. Spindles were scored as monopolar if they appeared as a single aster at 20x, otherwise bipolar. Multipolar and aggregated structures represented <20% of the total in all cases and were not counted. Any bipolar spindles with poles separated by less than ~3µm would be

<table>
<thead>
<tr>
<th>Agents added before spindle assembly</th>
<th>% Bipolar spindles</th>
<th>Average pole focus index (bipolar spindles)</th>
<th>Average pole to pole length (µm)</th>
<th>S.D. of pole to pole length (µm)</th>
<th>Number scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>1.8</td>
<td>39</td>
<td>4</td>
<td>115</td>
</tr>
<tr>
<td>p50</td>
<td>98</td>
<td>0.1</td>
<td>37</td>
<td>9</td>
<td>59</td>
</tr>
<tr>
<td>Monastrol</td>
<td>2</td>
<td>*</td>
<td>1</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>p50 + monastrol</td>
<td>100</td>
<td>0.8</td>
<td>32</td>
<td>5</td>
<td>104</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Agents added after spindle assembly</th>
<th>% Bipolar spindles</th>
<th>Average pole focus index (bipolar spindles)</th>
<th>Average pole to pole length (µm)</th>
<th>S.D. of pole to pole length (µm)</th>
<th>Number scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>1.9</td>
<td>37</td>
<td>7</td>
<td>107</td>
</tr>
<tr>
<td>p50</td>
<td>100</td>
<td>1.0</td>
<td>43</td>
<td>4</td>
<td>65</td>
</tr>
<tr>
<td>Monastrol</td>
<td>6</td>
<td>*</td>
<td>1</td>
<td>3</td>
<td>81</td>
</tr>
<tr>
<td>p50 + monastrol</td>
<td>79</td>
<td>1.3</td>
<td>23</td>
<td>2</td>
<td>87</td>
</tr>
</tbody>
</table>
scored as monopolar. To quantify pole focusing each spindle was assigned a score of 2 if both poles were well focused, 1 if both poles were partially focused or one pole was focused and the other unfocussed, and 0 if neither pole was focused. Pole focusing was scored only for bipoles. * means too few bipoles present to reliably calculate the index. Pole to pole length was averaged for all structures, and counted as 0 for monpoles.
Figure Legends

Figure 1. Length of in vitro spindles is independent of spindle density.

Cycled spindles were assembled at a final density of 50, 16.7 and 5.5 sperm nuclei/µl and imaged in oil overlay chambers by polarization microscopy 90-120 minutes after addition of CSF extract.
   A. Representative images at each dilution. Bar = 5µm.
   B. Histograms of spindle lengths at each dilution measured from polarization images. The mean and standard deviation (in µm) is written on each histogram. Note they are similar at each dilution.
   C. Immunofluorescence image of the meiosis II spindle in an unfertilized egg, at a comparable magnification, for comparison. The animal cortex of the egg is to the left. Anti-tubulin staining and laser confocal imaging. Image kindly provided by David Gard, U. Utah. See (Cha et al 1998).

Figure 2. Hexylene glycol promotes spindle expansion.

2%v/v Hexylene glycol was added to cycled spindles from a 20% stock in water immediately before imaging.

   A. Polarization images from a time lapse sequence. Elapsed time shown in minutes and seconds. Hexylene glycol was added ~1 minute before the first time point. Note the growth in spindle length and width. Time elapsed shown as min:sec. Length of time box = 10µm. See movie M1
   B. Spindle length as a function of time after addition of hexylene glycol alone (2%; black circles), hexylene glycol plus AMPPNP (1.5mM; open squares) or hexylene glycol plus monastrol (400µM) and p50 dynamitin (0.9mg/ml; grey diamonds).
C. Spinning disc confocal fluorescence images of a spindle containing speckle level tubulin to which 2% hexylene glycol has been added. The line in the second panel was used to make the kymograph in D. See movie M2. Bar = 5\(\mu\)m

D. Kymograph through the upper pole of the spindle shown in C. Time is vertical, distance along the line horizontal. Note the movement of the pole away from the spindle equator, which is to the right in the panel. Note also that many thin lines, which are speckle trajectories, parallel the movement of the pole (black lines highlight examples). Near the equator, speckles are also moving in the other direction (white line), in parallel with the opposite pole.

Figure 3. Titration of anti-MCAK antibody effects.

Affinity purified, inhibitory antibody to MCAK (Walczak et al 1996) was added to spindle assembly reactions containing X-rhodamine tubulin 20’ after bring the extract back into M phase after replication, well before spindle assembly. Samples were squash-fixed at 80’ and 120’ minutes after bringing the extract into M phase.

A. shows representative spindles fixed at 80’ as a function of final antibody concentration. Note the massive microtubule growth out of the spindle at 15 \(\mu\)g/ml (and higher, not shown), indicative of transition to unbounded dynamic instability. Bar = 10\(\mu\)m.

B. Quantification of spindle length in this experiment. Open bars are 80’ and black 120’ samples. The error bars indicate one standard deviation above and below the mean, and the number in each bar the sample size.

C. Quantification of total microtubules per spindle measured as integrated fluorescence intensity minus local background signal. Labeling as B.

Figure 4. Inhibition of MCAK promotes microtubule outgrow and pole curling, but not spindle elongation.

Affinity purified, inhibitory antibody to MCAK was added at 150 \(\mu\)g/ml final to cycled spindles immediately before imaging.
A. Polarization images from a time lapse sequence. Note the dramatic outgrowth of microtubules from the spindle (arrows in second panel). There is some alteration of spindle shape and pole structure over the sequence, but pole-pole distance remains approximately constant. Elapsed times shown in minutes:seconds. Antibody was added ~1 minute before the first time point. Time bar = 18µm. See movie M3.

B. Spinning disc confocal fluorescence images of a spindle containing speckle level X-rhodamine tubulin (shown green) and alexa-488 labeled antibodies to NUMA at 5 µg/ml as a pole marker (shown red). Note that the upper pole, visualized by anti-NUMA localization (white arrow), is curled over and apparently attached to the body of the spindle. By the last time point the pole has moved towards the spindle equator, and become partially disorganized. In the tubulin channel, the movements of the microtubules that are associated with this pole movement can be visualized (blue arrows). In the center of the spindle, microtubules slide apart in both directions. In the upper part, most of the flow is upwards. At the top of the spindle, the flow curls over, correlating with movement of the pole back towards the equator. Bar = 5µm. See movie M4.

Figure 5.
Rapid microtubule depolymerization using nocodazole.

Cycled spindles containing labeled tubulin (green) and a kinetochore marker (anti-CenpA, red) were mixed with nocodazole (20µM final) and widefield time lapse imaging was initiated ~10 sec after mixing using a 40x dry objective.

A. Imaging of spindle collapse. Elapsed time is shown in sec. relative to the first image, tubulin fluorescence is normalized to peak brightness in each image, to highlight the organization of remaining microtubules. Insets show 3x mag. of marked kinetochores. Note the sister kinetochores are initially well separated. The spindle rapidly looses fluorescence, shortens, and the sister kinetochore move together. Note some evidence of buckling of kinetochore microtubules at 59, 119 sec. Bar = 5µm main panels, 1.7µm in insets. See movie M5.
B. Quantification of the sequence in A, showing distance between the marked kinetochores in A (k-k), pole to pole distance (p-p) and integrated tubulin fluorescence after subtracting local background signal. Note that k-k distance drops faster (in % terms) than p-p distance.

Figure 6.
Microtubule depolymerization using a caged drug. Cycled spindles containing labeled tubulin, kinetochore and pole markers in some cases, and caged 105D, were imaged by time lapse widefield (B,C) or confocal (D) fluorescence. At t = 0 105D was photoreleased by 1-2 sec illumination with a UV filter set. All times are in sec. relative to photorelease. Bar = 7.5 µm in B, 6 µm C, 5 µm in D and 1.25 µm in D insets.

A. Structure of caged 105D, and the photochemical reaction that releases active drug.
B. Example of a spindle before and after photorelease of 105D (widefield). Tubulin fluorescence is presented with brightness and contrast held constant throughout the sequence to highlight the rapid decrease in microtubule density. This sequence also contained a fluorescent kinetochore probe, see movie M6.
C. Second widefield example. Tubulin fluorescence is normalized to peak brightness in each image, to highlight the organization of remaining microtubules. Note the decrease in spindle length, and buckling of remaining stable microtubules. See movie M7.
D. Example of photorelease of 105D (confocal). Probes are for tubulin (green), kinetochore (anti-CenpA, red) and poles (anti-NUMA; red). Tubulin fluorescence is normalized to peak brightness in each image. Insets show marked kinetochore pairs at 4x magnification. The focal plane was changed several times during this sequence, and different kinetochore pairs are shown in each panel. At late time points most of the remaining stable microtubules connect to kinetochores. Note that the poles (large red dots) move progressively together. Sister kinetochores first move together, then twist and move away from the spindle axis, while their attached microtubules either buckle or moving outwards, suggesting kinetochore fibers experience compression from the collapsing poles. The spindle was
optically sectioned twice during this sequence, and the images at 941,978 sec. are two focal planes from one through-focal series. Note the absence of straight microtubule bundles directly connecting the poles at any time point or focal plane.

Figure 7. Eg5 and dynein/dynactin play antagonistic roles in spindle assembly. Cycled spindles were assembled in the presence of no drug, the dynactin inhibitor p50 dynamitin (0.9mg/ml), the Eg5 inhibitor monastrol (200µM), or both. Drugs were added at the time of CSF addition, and spindles were imaged live in oil overlay chambers by polarization microscopy 90-120 minutes later. A panel of representative images are shown for each condition. Note the expected appearance of p50 spindles with unfocussed poles, and monopolar monastrol spindles. When both drugs were added, bipolarity was completely rescued, and pole focusing was partially rescued. Bar = 10µm. See table 1 for quantitation.

Figure 8. Antagonizing dynein/dynactin rescues the effects of Eg5 depletion.

Extracts were depleted of Eg5 using affinity purified antibody and magnetic beads. 3 rounds of depletion were used to remove all Eg5 that could be detected on western blots (>95%).

A. Western blot analysis of Eg5 and mock depleted extracts. Eg5 is removed to below the detection limit in the depleted extract.

B. Quanatitation of spindle morphology. Eg5 and mock depleted extracts were used to assemble cycled spindles in the presence or absence of p50 dynamitin (0.9mg/ml). In Eg5 depletion alone, the vast majority of spindles were monopolar asters. Addition of p50 promoted assembly of mostly bipolar spindles.

C. Typical spindle assembled in mock depleted extract.

D. Typical spindle assembled in mock depleted extract + p50. Note bipolar organization with splayed poles.

E. Typical spindle assembled in Eg5 depleted extract. Note monopolar organization.

F. Typical spindle assembled in Eg5 depleted extract + p50. Note rescue of both bipolarity and pole morphology. Bar = 5µm
Figure 9. Interpretation of results. See text for details.
Supplementary Figure legends

Figure S1. Testing anti-NuMA IgG

Approximately 20µg of affinity purified antibody to Xenopus NuMA C terminal peptide, or random rabbit IgG) was adsorbed to to 50µl of a suspension of magnetic beads coated with protein A (2.8 micron diameter; DYNAL Biotech). The beads were incubated with 200µl of meiotic Xenopus egg extracts for 1 hr at 4°C. The beads were retrieved and washed 6 times with TBS (0.15M NaCl, 20mM TrisCl pH 7.4) + 0.1% Triton-X100. Bound proteins were removed in 20µl sample buffer at 100º, and analyzed by SDS-PAGE. 5µl of each sample was analyzed by Coomassie staining and by western blotting with the same anti-NuMA antibody and luminescent detection. Note the band of expected mol wt in the immunoprecipitate, that was positive for NuMA on the western blot. Western blotting of whole extract was negative for NuMA, probably because the high protein concentration and low NuMA concentration make transfer and detection difficult.

Figure S2. 105D inhibits polymerization of pure tubulin.

Each reaction contained 9 µl of tubulin at 40µM tubulin with ~5% of the dimers labeled with tetramethyl rhodamine in BRB80 (80mM KPIPES 6.8, 1mM MgCl2, 1mM EGTA) + 1mM GTP + 0.1% 2-mercaptoethanol. This was mixed on ice with 1 µl of DMSO containing 10x drug, to give the final concentration shown in the Figure, and a final DMSO concentration of 10% which promotes microtubule polymerization. The reactions were warmed to 37º for 20 min, then diluted 1000 fold into BRB80 + 0.1% glutaraldehyde + 60% glycerol to fix the microtubules. 5 µl aliquots were spread under a 22mm² coverslip, left for ~60 min for the microtubules to adhere to the glass surfaces, and imaged. Note that 105D inhibits polymerization with an IC50 in the mid µM range.

Figure S3. Synthesis of caged 105D.

All reagents were from Aldrich (Milwaukee WI). Solvents were anhydrous grade, and reactions were carried out under dry nitrogen. All products were >95% pure by TLC and LC/MS, and gave the expected molecular ions.
A: 3-trifluormethyl-benzenesulfonylchloride (4.8 mmol) was added in portions to 2-aminonaphthylene (4.8 mmol) + N-methymorpholine (6mmol) dissolved in 50ml 1,4 dioxane at 25°. The reaction was stirred overnight, taken up in ethyl acetate, washed with 1M HCl x3, 5% NaHCO₃ x3 and saturated NaCl x1, dried over Na₂SO₄, and evaporated dry. 105D was used for synthesis without further purification. Yield = ~100%. For biological work it was recrystalized from hexane/CH₂Cl₂.

B: 5-hydroxy-2-nitrobenzyl alcohol (20mmol), tButyl-bromoacetate (18mmol) and CsCO₃ (20mmol) were stirred in 10ml DMF at at 25° overnight. The product (compound 1) was worked up as for 105D and recrystalized from hexane/CH₂Cl₂. Yield = ~60%.

C: 105D (1mmol), compound 1 (1mmol) and triphenyl phosphine (1.5mmol) were mixed in 5ml CH₂Cl₂ and cooled to 0°. Diethyl azodicarboxylate (1.5mmol) was added slowly, and the reaction allowed to warm to at 25° overnight. The product was purified by flash chromatography on silica gel in 75% hexane, 25% ethylacetate, and taken on to the deprotection step. The product was dissolved in a minimal volume of CH₃Cl at 25°, and an equal volume of trifluoaetic acid was added, plus water to 1% final. After 60min volatiles were removed by adding benzene and evaporating twice, followed by high vacuum overnight. No purification necessary. Yield for both steps = ~90%.

Figure S4. Membrane partitioning and slow diffusion of 105D after photorelease in *Xenopus* extract.

105D is weakly fluorescent in the DAPI channel, while is caged derivative is not. Thus we could follow 105D activation and movement by imaging.

A) To observe diffusion of 105D, a field of extract containing 400 µM caged 105D was illuminated for 15 seconds at 360nm with a 20x objective to promote full photorelease in the illuminated field. Then the slide was re-positioned so the previously illuminated field was on the left, and the boundary of this field could be observed. This boundary region was followed for 10 minutes, taking images every 2 minutes. The exposure used to follow diffusion (100msec) was chosen to minimimize additional photorelease during imaging. The numbers in each panel are time in seconds after finishing the photorelease. There is little fluorescence in the pre-activation image. After photorelease and re-positioning, a prominent
boundary is visible for several minutes. 105D gradually moves into the un-
illuminated field, but even after 10 minutes the concentration of 105D is higher in
the original illuminated zone than the far side of the field. Thus most of the 105D
diffuses only slowly out of a microscope field. A fraction may diffuse faster,
indicated by the dim fluorescence in the non-activated zone at 50sec.

B) Shows an image at the same magnification as A of a fully activated zone taken
with longer exposures to visualize the distribution of 105D in more detail. Note
that the distribution is highly non-uniform.

C) Higher magnification image of a field like B taken with a 60x objective. Note that
most of the 105D accumulates in punctate structures. Given the morphology of
these structures, and the hydrophobic nature of 105D, we believe these are the
membranous organelles that are abundant in the extract.

Bar = 30µm in A, B and 10µm in C.
Movies

M1. Polarization imaging of spindle treated with 2% v/v hexylene glycol. See figure 2 for details.

M2. Confocal fluorescence imaging of spindle treated with 2% v/v hexylene glycol. Probe is Xrhodamine-tubulin at speckle level. See figure 2 for details.

M3 Polarization imaging of spindle treated with anti-MCAK at 150 µg/ml final. See figure 4 for details.

M4 Confocal fluorescence imaging of spindle treated with anti-MCAK at 150 µg/ml final. Probes are Xrhodamine tubulin at speckle level (shown green) and Alexa488-antiNUMA IgG (shown red). See figure 4 for details.

M5 Widefield fluorescence imaging of a spindle treated with nocodazole at 10µM final. Elapsed time is shown relative to the first image. The sequence starts ~10 sec after nocodazole addition and mixing, the time it took to mount the squash, find a spindle, and start imaging. Probe is Xrhodamine-tubulin (green) and Alexa488-anti-CenpA (red). See figure 5 for details.

M6. Spindle subject to uncaging of 105D between the 5th and 6th frame. This sequence is presented with images normalized to peak fluorescence in each image. Widefield imaging, probes are Xrhodamine tubulin (green) and Alexa488-anti-CenpA (red). Note that the sister kinetochores rapidly collapse towards each other as the spindle shortens. Note also that many of the selectively stable microtubule bundles appear to terminate at kinetochores. See figure 6 for details.

M7. Spindle subject to uncaging of 105D after the first frame. This sequence is presented with images normalized to peak fluorescence in each image, to allow visualization of residual microtubules. Widefield imaging, probe is Xrhodamine-tubulin. Note buckling
of the remaining microtubules as the spindles collapse. Many of the stable bundles appear to be kinetochore fibers, as evidenced by the \(~1.5 \, \mu\text{m}\) gap in fluorescence at the equator in the first few frames. See figure 6 for details.