Aurora A Phosphorylates MCAK to Control Ran-dependent Spindle Bipolarity

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During mitosis, mitotic centromere-associated kinesin (MCAK) localizes to chromatin/kinetochores, a cytoplasmic pool, and spindle poles. Its localization and activity in the chromatin region are regulated by Aurora B kinase; however, how the cytoplasmic- and pole-localized MCAK are regulated is currently not clear. In this study, we used *Xenopus* egg extracts to form spindles in the absence of chromatin and centrosomes and found that MCAK localization and activity are tightly regulated by Aurora A. This regulation is important to focus microtubules at aster centers and to facilitate the transition from asters to bipolar spindles. In particular, we found that MCAK colocalized with NuMA and XMAP215 at the center of Ran asters where its activity is regulated by Aurora A-dependent phosphorylation of S196, which contributes to proper pole focusing. In addition, we found that MCAK localization at spindle poles was regulated through another Aurora A phosphorylation site (S719), which positively enhances bipolar spindle formation. This is the first study that clearly defines a role for MCAK at the spindle poles as well as identifies another key Aurora A substrate that contributes to spindle bipolarity.

INTRODUCTION

In most cells, both centrosomes and chromatin cooperate to nucleate microtubules (MTs) for bipolar spindle formation. However, neither of them is absolutely required for spindle assembly. In certain cell types, such as plant cells and some oocytes that do not have centrosomes, MTs nucleate from the chromosomes to form the mitotic or meiotic spindle (De Mey *et al.*, 1982; Karsenti *et al.*, 1984; Steffen *et al.*, 1986; Karsenti and Vernos, 2001; Raff, 2001). The *Xenopus* egg extract system is a cell-free model system that is commonly used to study mitosis because bipolar spindles form by the simple addition of demembranated sperm nuclei to cytostatic factor (CSF)-arrested extracts (Lohka and Maller, 1985; Murray and Kirschner, 1989; Sawin and Mitchison, 1991). During sperm-induced spindle assembly, chromatin directs nucleation, polymerization, and organization of MTs independently of kinetochores, whereas the sperm-associated centrosome aids in pole formation (Heald *et al.*, 1997). These processes are supported by MT motor proteins and by the regulation of MT dynamics (Karsenti and Vernos, 2001). Spindle assembly in other organisms, including mammalian cells, can also be directed solely by chromatin, indicating that spindle assembly mechanisms are conserved across species (Bonaccorsi *et al.*, 1998, 2000; de Saint Phalle and Sullivan, 1998; Khodjakov *et al.*, 2000; Hinchcliffe *et al.*, 2001).

The assembly of chromatin-nucleated MTs and spindle assembly depends on the chromatin-bound component RCC1, a guanine-nucleotide-exchange factor for the GTPase

protein ras-related nuclear protein (Ran) (Bischoff and Ponstingl, 1991a,b; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999). Chromosome-bound RCC1 locally generates a Ran-GTP gradient, which releases spindle assembly factors from their inhibitors, called importins (Carazo-Salas *et al.*, 1999; Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wiese *et al.*, 2001; Wilde *et al.*, 2001; Ems-McClung *et al.*, 2004). These spindle assembly factors then facilitate MT polymerization and bipolar spindle formation through changes in MT dynamics, MT polymerization and MT motor activities (Ciciarello *et al.*, 2007). Furthermore, in *Xenopus* egg extracts the nonhydrolyzable RanGTP mutants RanL43E or RanQ69L can promote bipolar spindle assembly in the absence of sperm nuclei and centrosomes (Kalab *et al.*, 1999; Wilde and Zheng, 1999; Gruss *et al.*, 2001). The downstream effectors of Ran that are involved in spindle bipolarity in the absence of centrosomes and chromatin include several microtubule-associated proteins (MAPs) (TPX2, NuMA, NuSAP, and HURP), a kinesin-5 (Eg5), and the kinase Aurora A (Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wilde *et al.*, 2001; Tsai *et al.*, 2003; Koffa *et al.*, 2006; Ribbeck *et al.*, 2006). TPX2 functions in conjunction with xRHAMM and γ -tubulin to promote MT nucleation and pole focusing (Schatz *et al.*, 2003; Groen *et al.*, 2004), and NuMA forms a meshwork that helps to anchor and focus MT minus ends (Gordon *et al.*, 2001; Du *et al.*, 2002; Khodjakov *et al.*, 2003). Conversely, NuSAP and HURP bundle and stabilize MTs at the plus ends to maintain the integrity of the spindle (Koffa *et al.*, 2006; Ribbeck *et al.*, 2006; Sillje *et al.*, 2006; Ribbeck *et al.*, 2007). Ran-induced bipolarity is also dependent on the activities of Aurora A and Eg5 (Giet and Prigent, 2000; Tsai *et al.*, 2003; Koffa *et al.*, 2006). Inhibition of either of these proteins results in the formation of monopolar spindles (Giet and Prigent, 2000; Wilde *et al.*, 2001). However, whether other downstream targets of Aurora A are required for spindle bipolarity is not known.

The pivotal role of Aurora A in Ran-induced spindle bipolarity is exemplified by the fact that Aurora A-coated beads or excess Aurora A can induce bipolar spindle assem-

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Abbreviations used: GFP, green fluorescent protein; MCAK, mitotic centromere-associated kinesin; MT, microtubule; Ran, ras-related nuclear protein.

bly in the absence of chromatin and centrosomes (Tsai and Zheng, 2005; our unpublished results). The ability of Aurora A to induce bipolar spindles is due to its Ran-regulated activation by TPX2 and subsequent phosphorylation of downstream targets. Recently, Aurora A, Eg5, XMAP215, TPX2 and HURP were found to be in a complex important for the transition from asters to bipolar spindles in egg extracts (Koffa *et al.*, 2006). Among the proteins in this complex, Aurora A is the most important because its kinase activity is required for the formation and function of the entire complex (Koffa *et al.*, 2006). Although Aurora A phosphorylates Eg5 (Giet *et al.*, 1999), TPX2 (Tsai *et al.*, 2003), and HURP (Yu *et al.*, 2005), Aurora A does not seem to phosphorylate XMAP215 directly. Rather, Aurora A phosphorylates the regulator of XMAP215, the TACC/Maskin family of proteins (Lee *et al.*, 2001; Giet *et al.*, 2002; Barros *et al.*, 2005; O'Brien *et al.*, 2005; Peset *et al.*, 2005). XMAP215 promotes plus end MT polymerization through a processive tubulin addition mechanism (Howard and Hyman, 2007; Brouhard *et al.*, 2008), but it may also stabilize the minus ends of MTs that have been released from their nucleation sites (Lee *et al.*, 2001). Although it is clear that the HURP complex is critical for spindle bipolarity, it is unclear whether there are additional targets of Aurora A that are important for this process.

Mitotic centromere-associated kinesin (MCAK) is a kinesin-13 that destabilizes MTs and plays important roles in MT dynamics, mitotic spindle assembly, and chromosome segregation (Moore and Wordeman, 2004). Globally, the MT-destabilizing activity of MCAK is counteracted by MT-stabilizing MAPs, such as XMAP215/TOGp, Tau, MAP4, and EB1 (Tournibize *et al.*, 2000; Kinoshita *et al.*, 2001; Holmfeldt *et al.*, 2002; Moore *et al.*, 2005; Noetzel *et al.*, 2005). In interphase MCAK activity is low because of the increased association of MAPs with MTs, whereas during the transition to mitosis MAPs become hyperphosphorylated, causing them to dissociate from MTs allowing MCAK to be more active (Niethammer *et al.*, 2007). During mitosis MCAK localizes to chromatin/kinetochores, to spindle poles, and it is found in a cytoplasmic pool (Wordeman and Mitchison, 1995; Walczak *et al.*, 1996). MCAK activity on chromatin/kinetochores is regulated by Aurora B kinase, which is mostly restricted to the chromatin (Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004) and functions to ensure proper kinetochore-MT attachments (Kline-Smith *et al.*, 2004). Specifically, MCAK MT depolymerization activity is regulated by Aurora B phosphorylation of residue S196, and localization to chromatin is regulated by phosphorylation of T95 and S110 (Zhang *et al.*, 2007). How the cytoplasmic and pole localized MCAK activity and location are regulated is currently not clear. Recently, MCAK was also found to play a role in spindle bipolarity in the absence of centrosomes and chromatin (Ems-McClung *et al.*, 2007). However, the mechanism of its function in spindle bipolarity is unknown.

In this study, we found that MCAK localization and activity at the poles are regulated by Aurora A in Ran asters through two distinct sites to promote spindle bipolarity. In particular, MCAK colocalizes with NuMA and XMAP215 at the center of Ran asters where its activity is regulated by Aurora A-dependent phosphorylation of S196, which contributes to proper pole focusing. In addition, we find that MCAK localization is regulated through another Aurora A phosphorylation site (S719), which is critical for bipolar spindle formation. Overall, our studies suggest that MCAK has an important role in regulating MT dynamics at the poles to promote aster formation and spindle bipolarity and that its activity and localization is regulated through temporal phosphorylation by Aurora A.

MATERIALS AND METHODS

Protein Expression and Purification

The glutathione transferase (GST)-tagged RanQ69L expression construct was a kind gift from Yixian Zheng (Carnegie Institute). GST-RanQ69L was purified from cell lysates with glutathione agarose and loaded with GTP as described previously (Wilde and Zheng, 1999), and then it was dialyzed extensively into XB buffer (10 mM HEPES, pH 7.7, 1 mM MgCl₂, 0.1 mM CaCl₂, and 100 mM KCl). The dialyzed protein was quantified by Bradford assay, its concentration was adjusted to 0.5 mM (20 mg/ml), sucrose added to 50 mM, aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C.

For the in vitro kinase assays of MCAK and its mutants, Aurora B kinase was purified as reported previously (Resnick *et al.*, 2006), and His-xAurora A was purified with Ni-NTA agarose (QIAGEN, Valencia, CA), and dialyzed into XB buffer. Green fluorescent protein (GFP)-tagged MCAK (GFP-MCAK) proteins containing the S719A and S719E mutations were created using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA) in the pFBGFP-MCAK clone (Ems-McClung *et al.*, 2007). The resulting constructs were verified by sequencing, and then they were used to create baculovirus for protein expression in Sf9 cells by using the Bac-to-Bac expression system (Invitrogen, Carlsbad, CA). GFP-MCAK and the S196A/E and S719A/E GFP-MCAK mutant proteins were expressed and purified to near homogeneity as described previously (Desai *et al.*, 1999; Ems-McClung *et al.*, 2007). Proteins were quantified by densitometry with ImageJ software (National Institutes of Health, Bethesda, MD) from colloidal Coomassie-stained SDS-polyacrylamide gels by using bovine serum albumin (BSA) as a standard. Protein concentrations are expressed in terms of monomer concentration.

Spindle Assembly and Immunodepletion in *Xenopus* Egg Extracts

CSF-arrested *Xenopus* egg extracts were prepared as described previously (Murray, 1991). We included X-rhodamine-labeled tubulin in all extracts at 50 µg/ml to visualize MTs. MCAK and Aurora A immunodepletions and add-back of recombinant proteins were performed as described previously (Zhang *et al.*, 2007) by using 10 µg of antibodies/25 µl of protein G Dynabeads (Invitrogen)/100 µl of extract. Endogenous MCAK was depleted using sheep α-MCAK-NT, and control reactions were mock depleted with sheep immunoglobulin (IgG). Recombinant GFP-MCAK or its phosphorylation mutants were added back to a final concentration equivalent to the endogenous concentration of 100 nM MCAK or to 300 nM. In some experiments, monastrol was added to extracts at a final concentration of 100 µM by initial dilution in extracts to 1 mM. To assemble Ran asters, His-RanL43E (Wilde and Zheng, 1999) or GST-RanQ69L was added to a final concentration of 25 µM on ice to CSF extracts. Total protein addition did not equal >10% of the reaction volume. Reactions were incubated at room temperature (RT) for 45 min, unless noted otherwise, fixed, sedimented onto coverslips, and processed for immunofluorescence as described previously (Zhang *et al.*, 2007). For quantification of aster formation/spindle assembly, ~300 structures were counted per experiment, and a minimum of three independent extracts were scored. All values are presented as mean ± SD.

Antibody Production and Immunostaining

Rabbit polyclonal Aurora A antibodies were raised against *Xenopus laevis* His-tagged Aurora A (His-xAurora A) by using the services of Covance Research Products (Denver, PA). The His-xAurora A antigen construct was a kind gift from Yixian Zheng. Antibodies were affinity-purified, dialyzed into 10 mM HEPES, pH 7.2, 100 mM KCl, aliquoted, and flash frozen in liquid nitrogen.

For immunofluorescence, the coverslips were blocked in AbDil-Tx (20 mM Tris, pH 7.5, 150 mM NaCl [TBS], 0.1% Triton X-100, 2% BSA, and 0.1% azide) for 1 h at RT or at 4°C overnight, incubated in primary antibody for 30 min, washed with TBS and 0.1% Triton X-100 (TBS-TX), and then incubated in 1/50 dilution of donkey anti-rabbit Alexa 488 or anti-rabbit Cy5 secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. The coverslips were then washed with TBS-TX, mounted onto slides with mounting media (90% glycerol; 20 mM Tris-HCl, pH 8.8, and 0.5% *p*-phenylenediamine), and sealed with nail polish. All primary and secondary antibodies were diluted in AbDil-Tx. The primary antibodies and the concentrations used for immunofluorescence were as follows: α-NT2-xMCAK (0.5 µg/ml), α-pS196 (2 µg/ml) (Lan *et al.*, 2004), α-γ-tubulin (1/1000) (Sigma-Aldrich, St. Louis, MO), α-Eg5-tail (0.6 µg/ml) (Sawin *et al.*, 1992), α-XMAP215 (1/500) (a kind gift of Mimi Shirasu-Hiza, Stanford University, Stanford, CA), α-NuMA (1/500) (a kind gift of Andreas Merdes, Institut de Sciences et Technologies du Médicament de Toulouse, Toulouse, France), α-Aurora A (1/500), and α-Aurora B (1 µg/ml) (Lan *et al.*, 2004). For staining with rabbit α-NT2-xMCAK and another rabbit antibody, Alexa Fluor 488 labeled α-NT2-xMCAK was used as described previously (Zhang *et al.*, 2007).

Imaging and Quantitative Immunofluorescence Analysis

Images were acquired with a 40× 1.0 Plan Apo, 60× 1.4 Plan Apo VC or a 100× 1.4 Plan Apo VC objective mounted on a Nikon 90i microscope with a Photometrics CoolSnap HQ cooled charge-coupled device camera. The mi-

croscope, camera, and filter wheels were controlled by MetaMorph (Molecular Devices, Sunnyvale, CA). For control and experimental reactions, images were taken with the same exposure time and scaled identically. All images were processed in Adobe Photoshop (Adobe Systems, Mountain View, CA) and assembled in Adobe Illustrator (Adobe Systems). Line scans of structures were performed on the color-combined images using MetaMorph. The fluorescence intensities and distances in pixels were imported to Excel (Microsoft, Redmond, WA), the distance was converted to micrometers, and plotted against the fluorescence for each color channel.

Quantification of immunofluorescence staining was determined using MetaMorph. To quantify the pS196 to total MCAK ratio or the MCAK intensity at the center of asters in control or Aurora A-depleted extracts, five to six images were taken for each channel and treatment in three independent experiments. For each image, three asters were quantified so that ~15 asters were quantified per treatment per independent experiment (~50 total asters). The total integrated fluorescence intensity was measured in a 40×40 pixel box drawn around the center of the aster or spindle pole, and then the background was subtracted using a 40×40 pixel box drawn outside of the aster or spindle. The average ratio of pS196 to total MCAK intensities from the ~15 asters in a single experiment was determined, and then the mean ratio and SD were calculated from the three independent experiments. A Student's *t* test performed in Excel was used to compare the means, and a statistical difference was considered when $p < 0.05$. Quantification was similar for measuring the fluorescent intensity of α -tubulin and γ -tubulin in asters versus spindles.

Western Blot Analysis

For all Western blots, samples were separated on 10% or 15% SDS-polyacrylamide gels and transferred to Protran nitrocellulose membrane (Whatman Schleicher and Schuell, Dassel, Germany). The blots were blocked in blocking buffer (TBS, 0.1% Tween 20 [TBST], and 5% nonfat dry milk) for 1 h at RT. Primary antibodies, α -NT2-MCAK (0.5 μ g/ml), DM1 α -tubulin (1/5000), or α -x-Aurora A (0.3 μ g/ml), were diluted in AbDil-T (TBST, 2% BSA, and 0.1% sodium azide). α -pH3-Ser10 (Cell Signaling Technology, Danvers, MA) was diluted 1/1000 in blocking buffer. Blots were incubated with primary antibodies for 1 h, washed in TBST, incubated for 1 h with 1/5000–1/10,000 dilution of goat- α -mouse or donkey- α -rabbit horseradish peroxidase secondary antibodies (GE Healthcare, Piscataway, NJ) diluted in blocking buffer, washed in TBST, washed in TBS, and then developed with SuperSignal West Pico Chemiluminescence Substrate (Pierce Chemical, Rockford, IL)).

In Vitro Kinase Assays

The in vitro kinase assays were performed essentially as described previously (Lan *et al.*, 2004), and Aurora B kinase was purified as reported previously (Ribbeck *et al.*, 2006). Specifically, for every 10- μ l reaction, 100 ng of kinase (His-x-Aurora A or Aurora B) was incubated with 5 μ M substrate and ATP mix (0.15 mM ATP and 0.4 μ M of [γ - 32 P]ATP) in CSF-XB (10 mM HEPES, pH 7.7, 2 mM MgCl₂, 0.1 mM CaCl₂, 100 mM KCl, 5 mM EGTA, and 50 mM sucrose) at RT for 30–60 min. Reactions were stopped by the addition of 2 $\times$ sample buffer (0.125 M Tris, pH 6.8, 3% SDS, 20% glycerol, and 2% β -mercaptoethanol). Equal volumes of each reaction were then separated on 10 or 15% gels. Gels were stained with Coomassie G250, and then they were scanned using a Typhoon PhosphorImager (GE Healthcare).

MT EC₅₀ Assay

MTs were polymerized using guanylyl-(a,b)-methylene-diphosphonate (Jena Scientific, Jena, Germany) to create stable tubulin polymer and resuspended in BRB49 (49 mM PIPES pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT) as previously described (Desai and Walczak, 2001; Ems-McClung *et al.*, 2007). MT EC₅₀ assays were then performed and analyzed as described (Ems-McClung *et al.*, 2007). Specifically, 1 μ M of stabilized MTs were incubated with 0–100 nM GFP-MCAK, GFP-MCAK(S719A) or GFP-MCAK(S719E) for 30 min at RT centrifuged at 45,000 rpm in a Beckman TLA100 for 5 min, the supernatant and pellets separated, and the samples electrophoresed on 10% SDS-polyacrylamide gel electrophoresis (PAGE). Gels were stained with Colloidal Coomassie Blue, and the tubulin bands analyzed for the percentage of tubulin released into the supernatant. The percentage of soluble tubulin was plotted against the log of the concentration of enzyme, and the best fit EC₅₀ dose response curve determined using GraphPad Prism (San Diego, CA) (Ems-McClung *et al.*, 2007).

RESULTS

MCAK Localizes to the Center of Ran Asters and It Is Important for Pole Focusing

To address the role of MT dynamics in aster and spindle assembly in the absence of chromatin and centrosomes, we assembled Ran asters in *Xenopus* egg extracts (Ohba *et al.*, 1999; Wilde and Zheng, 1999; Zhang *et al.*, 1999; Carazo-Salas *et al.*, 2001) and studied the localization of known MT dynamics regulators (MCAK and XMAP215), MT-associated pro-

teins (NuMA and Eg5), and the microtubule nucleating protein γ -tubulin. Immunofluorescence analysis revealed that endogenous MCAK localized to the center of RanQ69L-induced asters in *Xenopus* egg extracts along with γ -tubulin, Eg5, XMAP215, and NuMA (Figure 1, A–D). Previous studies showed that γ -tubulin, Eg5, XMAP215, and NuMA are important for Ran-induced aster formation and/or contribute to aster formation either by nucleating, cross-linking, or stabilizing MTs at poles (Gaglio *et al.*, 1995; Ohba *et al.*, 1999; Wilde and Zheng, 1999; Wiese *et al.*, 2001). However, MCAK function in Ran-dependent aster formation is unclear.

By examining more closely the localization of MCAK and these other proteins at the center of asters at high resolution, we found that there were distinct classes of localization patterns. In about half of the Ran asters, γ -tubulin was found as a “donut” at the center of asters that occurred as two peaks of fluorescence in a line scan (Figure 1, A and E). Eg5 staining also showed the same donut distribution as γ -tubulin (Figure 1, B and F). However, MCAK was found inside of the donut where it colocalized with XMAP215 and NuMA (Figure 1, C and D and G and H). In the other half of the Ran asters, γ -tubulin staining did not form a donut at the center of asters, rather γ -tubulin was concentrated in discrete foci at the center of asters (Supplemental Figure 1, A and E). In these asters, γ -tubulin, Eg5, MCAK, XMAP215, and NuMA staining all seemed to be colocalized at the center of asters (Supplemental Figure 1, A–H). When Ran asters were examined in a side-on view, it was apparent that γ -tubulin and Eg5 colocalized with the peak intensity of MTs, presumably marking the MT minus ends (Figure 1, I and J). In contrast, MCAK, XMAP215 and NuMA colocalized internally to the peak fluorescence of γ -tubulin and the MTs (Figure 1, I and J). Because XMAP215 and NuMA are important for MT nucleation and/or pole focusing (Merdies *et al.*, 1996; Du *et al.*, 2001; Popov *et al.*, 2002; Cassimeris and Morabito, 2004; Tsai and Zheng, 2005), it is perhaps not surprising that they colocalized near the MT minus ends, but it is not necessarily clear why their localization is even more internal than that of the bulk of the tubulin and MT minus ends (Figure 1K).

During our immunofluorescence analysis of MCAK in Ran-induced aster assembly, we noticed that MCAK localized differentially to the center of asters and bipolar spindle poles (Figure 2). In particular, MCAK localized more robustly to the center of asters than to bipolar spindle poles (Figure 2A). It is interesting that γ -tubulin showed the opposite pattern: it localized more at poles of the bipolar spindle than at the center of asters (Figure 2A). We therefore asked how the other MT-associated proteins important for Ran aster assembly behaved. Similar to MCAK, XMAP215 and NuMA localized more robustly to the center of asters than to bipolar spindle poles (Figure 2, B and C). Eg5 localization did not differ much between asters centers and bipolar spindle poles (Figure 2D). Thus, not only do MCAK, XMAP215, and NuMA localize to similar positions within the aster but they also are more concentrated on asters than on bipolar spindles. This suggests that MCAK, XMAP215, and NuMA may be critical for the establishment of asters. In addition, the finding that MCAK localization is similar to NuMA and XMAP215 raises the question of whether MCAK may be needed for proper minus-end MT dynamics at the aster pole, which could contribute to pole organization.

In the absence of centrosomes, the formation of Ran asters requires γ -tubulin (Wilde and Zheng, 1999). However, we found that the levels of γ -tubulin visualized in Ran asters were very low in comparison with its levels in bipolar spindles (Figure 2A). One possible explanation is that the minus ends of MTs in asters are not as focused as they are in

Figure 1. Localization of MCAK at the center of Ran asters. (A–D) Top down view of Ran asters. Ran asters were assembled in egg extracts in the presence of rhodamine-labeled tubulin (red), and then they were costained with antibodies to MCAK (green) and γ -tubulin (A), Eg5 (B), XMAP215 (C), or NuMA (blue) (D). The dotted line across each merged image represents the area viewed in the line scans of

bipolar spindles so that the γ -tubulin staining seems diluted and faint. We therefore measured the integrated fluorescence intensity of γ -tubulin, MTs, and MCAK staining in both asters and bipolar spindles. We found that the amount of detectable γ -tubulin in bipolar spindles was about two-fold of that in asters (Supplemental Figure 2, A and B), whereas α -tubulin or MCAK were not increased in bipolar spindles ($p < 0.05$). Together, these data suggest that the distribution of MT nucleation and MT-destabilizing proteins are differentially localized in asters and bipolar spindles.

The alteration of MT dynamics by addition of paclitaxel to extracts depleted of Eg5 results in asters with unfocused poles that often seem to have holes at the center (Sawin and Mitchison, 1994; Gaglio *et al.*, 1996). To address the function of MCAK in the organization of the poles in Ran asters, we immunodepleted endogenous MCAK and analyzed the phenotype of Ran asters in the presence and absence of the Eg5 inhibitor monastrol (Figure 3, A and B). Compared with control IgG-depleted extract (Δ IgG), depletion of MCAK (Δ M) not only resulted in large asters with long MTs but also resulted in enlarged poles as judged by the enlarged densities of tubulin fluorescence (Figure 3A). In the presence of monastrol, we found that MCAK depletion resulted in a high number of very large asters that had significantly reduced MT fluorescence in the middle of the structure (~62%), which were not found in IgG-depleted extracts, and they were rarely found (~8%) in MCAK depleted extracts that were not treated with monastrol (Figure 3C). Both the long MTs and the “holey” aster phenotypes were rescued by the addition of purified MCAK back to the depleted extracts that were treated with monastrol (Figure 3, A and B). This finding supports the idea that MCAK contributes to the focusing of poles in Ran asters, presumably by regulating MT dynamics at the poles.

MCAK Localization at the Center of Ran Asters Is Regulated by Aurora A

The localization of MCAK at the center of Ran asters, the occurrence of holey asters in the absence of MCAK and Eg5 activity, and the differential localization between spindle poles and aster centers suggests that MCAK plays a role in regulating MT dynamics at spindle poles. However, it seems counterintuitive to have an MT-destabilizing enzyme placed at the aster center, which is where most MT nucleation is thought to occur. We therefore wondered whether the MCAK that was enriched at the aster centers was active or inactive. We showed previously that phosphorylation of MCAK at S196 inhibits its MT depolymerization activity (Lan *et al.*, 2004). We therefore analyzed the distribution of total MCAK and phospho-S196-MCAK at the center of asters. We found that at an early time point of Ran aster formation (5 min), the ratio of phospho-S196-MCAK to total MCAK on asters was 80% higher than at later time points (30 min) (Figure 4A; our unpublished results) ($p < 0.05$). However, when comparing this ratio on bipolar spindles relative with asters, we found that the ratio was 67% higher ($p < 0.05$) at bipolar spindle poles relative to aster centers (Figure 4A; our unpublished results). This indicates that MCAK depolymeriza-

tion activity is likely inhibited by S196 phosphorylation at early time points to favor MT nucleation and at later points after the bipolar spindle formed to stabilize the spindle.

Our previous work showed that Aurora B was the kinase that phosphorylated MCAK at S196 (Lan *et al.*, 2004); however, we found that Aurora B did not localize to Ran-induced aster centers or spindle poles, whereas Aurora A localized robustly to the aster centers and spindle poles (Figure 4B) as shown previously (Tsai and Zheng, 2005). The lack of staining of the aster centers by the Aurora B antibodies is not due to a failure in the antibody staining because these Aurora B antibodies stained chromatin in chromatin-induced asters and spindles in CSF extracts (Figure 4C). Considering the similarity between Aurora A and Aurora B consensus sequences for phosphorylation, it is likely that Aurora A is the major kinase that phosphorylates MCAK S196 at the center of Ran asters. To test this hypothesis, we analyzed the ability of Aurora A to phosphorylate MCAK in vitro (Figure 4D). For this assay, we used a version of GFP-MCAK (amino acids 187–731) that does not contain the N-terminal domain (Ems-McClung *et al.*, 2007) because the N-terminal domain of MCAK is phosphorylated at multiple sites by Aurora B (Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004), which would make the kinase assay difficult to interpret. We found a significant decrease in phosphorylation of the GFP-MCAK-187-731(S196A) mutant compared with wild-type GFP-MCAK-187-731, by using either Aurora A or Aurora B as the kinase (Figure 4D). However, it should be noted that the overall level of phosphorylation of MCAK at S196 by Aurora A was weaker than it was by Aurora B. These data are consistent with a model in which S196 serves as a major site for regulating MCAK activity at the spindle poles by Aurora A and at the centromeres and chromatin by Aurora B.

Because Aurora B phosphorylation of MCAK controls the localization of MCAK to centromeres, we speculated that Aurora A may also influence the localization of MCAK to aster centers. To address this possibility, we first compared the localization of endogenous MCAK to Aurora A. Immunofluorescence staining of endogenous MCAK and Aurora A revealed that when Aurora A localization was high at the aster center, MCAK localization was low. Conversely, in asters that had high MCAK staining, Aurora A staining was low (Figure 5A). This inverse localization of Aurora A and MCAK suggests that Aurora A may regulate MCAK localization. To test this hypothesis, we analyzed the distribution of MCAK in Ran asters assembled in IgG-depleted and Aurora A-depleted extracts. MCAK localization at the center of Ran asters was increased approximately twofold in Aurora A-depleted extracts (Figure 5B; our unpublished results), indicating that Aurora A phosphorylation of MCAK may inhibit MCAK binding to the center of asters.

To determine which sites on MCAK may be phosphorylated by Aurora A, we took advantage of a large number of MCAK deletion constructs and phosphomutant derivatives that we had available in our laboratory. We found that all Aurora A sites resided in the N-terminal and C-terminal domains of MCAK (our unpublished results). In vitro kinase assays showed that S196 was the major Aurora A site in the N-terminal domain but it was not the only site. Because Aurora A phosphorylation of GFP-MCAK-187-731(S196A) did not completely inhibit the phosphorylation of GFP-MCAK-187-731 and our finding that mutation of S196 had no effect on the localization of MCAK to Ran asters (our unpublished results), it is likely that Aurora A phosphorylates the MCAK C-terminal domain to affect MCAK localization at aster centers. To this end, we identified S719 as a

Figure 1 (cont). each fluorescent image shown in E–H. (I) Side view of Ran asters (red) costained with antibodies to MCAK (green) and γ -tubulin, Eg5, XMAP215, or NuMA (blue). (J) Corresponding line scans of the images shown in I. (K) Schematic illustrations showing the localization of MCAK, Eg5, γ -tubulin, XMAP215, and NuMA relative to the MTs of Ran asters. Bar, 10 μ m.

Figure 2. MCAK localization is reduced at the poles of bipolar spindles relative to the center of asters. Localization of MCAK (green) relative to γ -tubulin (A), XMAP215 (B), NuMA (C), or Eg5 (blue) (D) at the center of asters and bipolar spindle poles (red). For each image, the top left panel shows the front view of an aster, whereas the top right panel shows a side view of an aster. The bottom two panels show bipolar

predominant Aurora A site in the C-terminal domain of MCAK (Figure 5C). Mutation of S719 to A or to E decreased the phosphorylation of GFP-MCAK-187-731 by Aurora A, but not by Aurora B, suggesting that S719 is an Aurora A-specific phosphorylation site on MCAK (Figure 5C).

Because our Aurora A depletion experiment showed that Aurora A regulates MCAK localization at the center of asters, we wanted to test whether S719 is the site through which Aurora A controls MCAK localization. To analyze the localization of MCAK with the S719A (dephospho) and S719E (phosphomimic) mutations we used a GFP-tagged version of MCAK in depletion and add-back assays to be able to specifically detect the exogenously added protein. Because MCAK localization at the center of Ran asters and bipolar spindle poles was very different, it would be complicated to compare the localization of the MCAK phosphomutants at the center of asters unless we had a uniform population of asters. To obtain this population, we depleted endogenous MCAK and added back varying amounts of GFP-MCAK, GFP-MCAK(S719A), or GFP-MCAK(S719E) to Ran extracts in the presence of monastrol to inhibit bipolar spindle formation. We found that GFP-MCAK(S719E) had greatly reduced binding to the center of asters compared with wild type (Wt) or GFP-MCAK(S719A), which is consistent with Aurora A depletion increasing MCAK localization to the center of asters (Figure 5, B and D). At an add-back concentration of 100 nM, which is equivalent to the endogenous level of MCAK, both GFP-MCAK and GFP-MCAK(S719A) formed bright focused dots at the center of $\sim 50\%$ of the asters, but GFP-MCAK(S719E) was not bright at the aster centers. At 300 nM, GFP-MCAK formed bright focused dots at the center of $\sim 80\%$ of the asters, and GFP-MCAK(S719A) formed bright focused dots at the center of $>95\%$ of the asters, whereas GFP-MCAK(S719E) was still not bright at the center of asters at this concentration. The lower binding of S719E to the center of asters suggests that Aurora A phosphorylation at this site decreases MCAK binding to the center of asters. This is consistent with our finding that Aurora A depletion causes increased MCAK localization at the center of asters.

Aurora A Phosphorylation of MCAK Facilitates Bipolar Spindle Formation

To ask whether Aurora A phosphorylation of MCAK at S719 had a functional consequence, we depleted endogenous MCAK and added back GFP-MCAK or the S719 mutants at endogenous concentrations to examine their activity in Ran aster/spindle formation. We found that GFP-MCAK(S719E) induced bipolar spindles at earlier time points and to a greater extent than Wt GFP-MCAK or GFP-MCAK(S719A) (Figure 6, A and C). Specifically, when GFP-MCAK(S719E) was added back to MCAK-depleted extracts, bipolar spindles formed in as early as 10 min (Figure 6A), which was 10 min earlier than when wild-type GFP-MCAK or GFP-MCAK(S719A) was the added extract (our unpublished results). In addition, there was an approximately fourfold increase in the percentage of bipolar spindles that formed when GFP-MCAK(S719E) was added

Figure 2 (cont). spindles. The line scans to the far right are of the region indicated in the merged image to its left. Note that the γ -tubulin staining at the center of asters in A is dimmer than in Figure 1 because these figures were scaled differently; in A, the images were scaled to minimize the saturation of γ -tubulin at the bipolar spindle poles. To make the MCAK staining in aster centers visible, some MCAK staining at bipolar spindle poles is saturated when they are scaled accordingly. Bar, 10 μ m.

Figure 3. MCAK is important for Ran aster pole focusing. Ran asters were assembled in IgG-depleted (Δ IgG) or MCAK-depleted (Δ M) extracts in the presence (+MA) or absence of monastrol. MCAK was added back to the MCAK-depleted extract in the presence (Δ M+M+MA) or absence of monastrol (Δ M+M). Bar, 10 μ m. (B) Western blot showing the depletion of endogenous MCAK and add-back of GFP-MCAK to endogenous levels (100 nM). (C) Quantification of the percentage of asters with large holes in the center in extracts depleted of MCAK in the absence (Δ M) or presence of monastrol (Δ M+MA). At least 100 structures were scored from three independent experiments.

back to the extracts compared with add-back of wild-type GFP-MCAK or GFP-MCAK(S719A) (Figure 6, B and C). The effects on spindle bipolarity after Ran addition are not due to differences in MT depolymerization activity because the activities of GFP-MCAK and its S719A and S719E mutants were similar *in vitro* (Figure 7). Instead, GFP-MCAK(S719E) localized more robustly to bipolar spindle poles (Figure 6D), which may be the reason that it increases bipolar spindle formation.

DISCUSSION

In this study, we used *Xenopus* egg extracts to form spindles in the absence of chromatin and centrosomes, and we found that MCAK localization and activity are tightly regulated by Aurora A, which is important to focus MTs at aster centers and to facilitate the transition from asters to bipolar spindles. This is the first study that clearly defines a role for MCAK at the spindle poles and elucidates another key Aurora A substrate that contributes to spindle bipolarity.

MCAK Functions at Poles to Facilitate Pole Organization

Our data establish that MCAK is found at the center of Ran asters. Initially, it seems counterintuitive to place an MT-

Figure 4. MCAK is an Aurora A substrate. (A) Immunofluorescence staining of MCAK (green) and pS196-MCAK (blue) on Ran asters (red) at two different time points. (B and C) Aurora A and B are shown in green, MTs are shown in magenta or red, and DNA is shown in blue. (B) Immunofluorescence staining of Aurora A (top) or Aurora B (bottom) in Ran-induced asters. (C) Immunofluorescence staining of Aurora B in spindles of CSF extracts (top) and chromatin-induced asters (bottom). (D) In vitro phosphorylation of Wt (GFP-MCAK-187-731) or S196A (GFP-MCAK-187-731(S196A)) by Aurora A and Aurora B.

The left panels are autoradiograms, and the right panels show the corresponding Coomassie-stained gels. Bar, 10 μ m.

depolymerizing enzyme at aster centers, but we believe that MCAK acts with other MT nucleation and polymerization molecules to regulate MT dynamics that favors proper aster assembly. Consistent with this idea, addition of paclitaxel, an MT-stabilizing drug, to Eg5-depleted extracts results in

asters that have unfocused poles (Sawin and Mitchison, 1994; Gaglio *et al.*, 1996), indicating that MTs at aster centers must be dynamic to achieve a focused pole. We show here that changing the MT dynamics by depleting MCAK in the presence of monastrol also makes the center of the Ran

Figure 5. Aurora A controls MCAK localization at Ran spindle poles through a site on the C-terminal domain of MCAK. (A) Immunofluorescence staining of endogenous MCAK (green) and Aurora A (blue) at the center of Ran-induced asters (red). (B) Immunofluorescence staining of MCAK (green) at the center of asters (magenta) in IgG-depleted (Δ IgG) or Aurora A-depleted (Δ Aurora A) extracts. Right, Western blot showing Aurora A depletion. (C) In vitro phosphorylation of Wt (GFP-MCAK-187-731), 719A [GFP-MCAK-187-731(S719A)], or 719E [GFP-MCAK-187-731(S719E)] by Aurora A (left) or Aurora B (right). (D) Immunofluorescence staining of microtubules in Ran asters (magenta) after MCAK depletion and add-back of 100 or 300 nM Wt (GFP-MCAK), S719A [GFP-MCAK(S719A)], or S719E [GFP-MCAK(S719E)] (green). Bar, 10 μ m (A and B) and 20 μ m (D).

asters unfocused, indicating that MCAK is important for the regulation of MT dynamics in these aster centers.

Because MCAK regulates MT dynamics at the plus ends of kinetochore MTs and at the cell periphery (Kline-Smith and Walczak, 2002; Moore *et al.*, 2005), it is therefore possible that MCAK functions in asters to regulate short MT plus end dynamics near the aster center. This is consistent with studies showing that XMAP215, which localizes to poles, regulates plus-end dynamics to contribute to aster/pole formation (Wilde and Zheng, 1999; Tsai and Zheng, 2005; Brouhard *et al.*, 2008). Alternatively, it is possible that MCAK regulates MT minus-end dynamics at the aster centers. This is consistent with the observation that MCAK can depolymerize MTs from both ends (Desai *et al.*, 1999; Hunter *et al.*, 2003; Helenius *et al.*, 2006). How MT minus-ends are regulated by MCAK or by any other protein is virtually unknown due to the density of MT ends present at poles, which makes this an important area for future work. It is interesting to note that a second Kinesin-13, Kif2A, is found at spindle poles, and it is implicated in regulating MT flux (Gaetz and Kapoor, 2004; Ganem *et al.*, 2005). Because data suggest that MCAK is not needed for MT flux (Ganem *et al.*, 2005; Ohi *et al.*, 2007), we favor the idea that MCAK activity at the poles is more critical in the overall process of pole organization.

MCAK Activity and Localization at Poles Is Regulated by Aurora A

To facilitate aster formation, MCAK activity should be tightly regulated. Our previous work and that of others showed that MCAK MT depolymerization activity is inhibited through phosphorylation of S196 by the chromosome passenger kinase Aurora B (Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004). Our current study shows that spindle pole-associated Aurora A can also phosphorylate MCAK at the same site, which controls its MT depolymerization activity. We noted that there is a higher percentage of inactive phospho-S196 MCAK present at early time points of Ran aster formation, which likely favors MT nucleation (Figure 8A). At later time points, there is proportionally more active MCAK where it may cooperate with other molecules, such as XMAP215, to regulate MT dynamics and aid in aster and spindle assembly. Furthermore, Aurora A seems to exert an additional level of control on MCAK by controlling its association to spindle poles through phosphorylation of S719 on the MCAK C-terminal domain to regulate spindle assembly (Figure 8, B and C). Our present data are most consistent with the idea that this regulation is mediated directly through Aurora A on MCAK, but we cannot rule out the possibility that Aurora A mediates some of its effects on MCAK through phosphorylation of an intermediate sub-

Figure 6. Phosphorylation of MCAK at S719 increases spindle bipolarity. (A) Images of Ran aster formation (magenta) at 10 min in extracts depleted of endogenous MCAK and reconstituted with endogenous levels (100 nM) of Wt (GFP-MCAK) or S719E [GFP-MCAK(S719E)]. Arrow shows the bipolar spindle that formed at 10 min in the GFP-MCAK(S719E) add-back reaction. (B) Images of Ran aster/spindle formation at 45 min in extracts depleted of endogenous MCAK and reconstituted with endogenous levels (100 nM) of Wt (GFP-MCAK), S719A [GFP-MCAK(S719A)], or S719E [GFP-MCAK(S719E)]. Only the MT channel is shown. (C) Quantification of asters versus spindles in reactions at 45 min that were depleted of endogenous MCAK and reconstituted with endogenous levels (100 nM) of GFP-MCAK, GFP-MCAK(S719A), or GFP-MCAK(S719E). The average percentage of asters and spindles are graphed with the SD. For each reaction, 300 total MT structures were counted from three independent experiments. Bipolar and multipolar spindles were grouped together in the spindle category. (D) The localization of Wt (GFP-MCAK) or S719E [GFP-MCAK(S719E)] (green) is shown on asters (top) and bipolar spindles (bottom) (magenta). Bar, 10 μ m.

strate. Overall, these phosphoregulatory events allow Aurora A to have precise spatial and temporal control over MCAK pole function.

Both Aurora A and MCAK Are Required for Spindle Bipolarity

Aurora A was initially discovered as a mediator of spindle bipolarity (Glover *et al.*, 1995; Roghi *et al.*, 1998). Its activity is pivotal in the transition from asters to bipolar spindles in

egg extracts (Roghi *et al.*, 1998; Giet and Prigent, 2000; Tsai and Zheng, 2005; Liu and Ruderman, 2006). Thus far, only a few downstream effectors of Aurora A-induced spindle assembly are known, and the detailed regulatory mechanisms and its substrates remain to be illustrated (Giet and Prigent, 2000; Tsai *et al.*, 2003; Peset *et al.*, 2005). The HURP complex, which contains Aurora A, Eg5, XMAP215, and TPX2, was identified as a major factor needed for bipolarity of Ran-mediated spindle assembly (Koffa *et al.*, 2006). Our studies

Figure 7. Mutation of residue S719 to a dephospho- or phosphomimic site does not affect the in vitro MT depolymerization activity of MCAK. (A) GMPCPP-stabilized MTs (1 μ M) were incubated with 0–100 nM GFP-MCAK, GFP-MCAK(S719A), or GFP-MCAK(S719E) protein for 30 min in saturating MgATP at RT. The soluble tubulin was separated from MTs by centrifugation, and the supernatant and pellet fractions run on SDS-PAGE. The percentage of soluble tubulin was determined by densitometry, and the mean percentages $\pm$ SEM were plotted against the log of each protein concentration. (B) The data from three individual experiments were fit to the four-parameter logistic equation using GraphPad Prism to determine the EC₅₀ concentration for each protein. The 95% confidence intervals for each EC₅₀ are also given.

suggest that MCAK needs to be added to this list of important mediators of spindle bipolarity in Ran extracts. It is possible that MCAK is a previously unidentified member of the HURP complex, but we think this is unlikely. It is more

Figure 8. Model for regulatory actions of Aurora A on MCAK. (A) At early time points, MCAK is phosphorylated at S196 in aster centers (MCAK-P-S196), which correlates with inactive MCAK. As asters become organized over time, proportionally less MCAK is phosphorylated, resulting in a net increase in MT depolymerase activity. (B) Phosphorylation at S719 controls association with asters and bipolar spindles. MCAK that is dephosphorylated at S719 (MCAK-deP-S719) associates predominantly with aster centers, whereas MCAK phosphorylated at this site (MCAK-P-S719) is only weakly associated with aster centers but is associated with spindle poles of bipolar spindles. (C) Aurora A stimulates bipolar spindle formation at least in part by phosphorylation of MCAK at S719.

likely that the HURP complex, which contains Aurora A, may mediate some of its actions through MCAK.

Our study shows that MCAK is critical for bipolar spindle formation in Ran-mediated spindle assembly (Figure 8C). We believe that control extracts may be limiting for Aurora A activity because addition of exogenous Aurora A or the Aurora A-activating protein TPX2 to extracts results in a dramatic increase in spindle bipolarity (Koffa *et al.*, 2006; our unpublished results). One possibility is that this limited amount of Aurora A results in insufficient MCAK phosphorylation that causes it to be lost from the spindle poles. Consistent with this idea, we find that a phosphomimic of MCAK at S719 has a reduced association with aster centers but an increased association with bipolar spindle poles (Figure 8B). This suggests that Aurora A is not needed for MCAK localization to aster centers but is needed to recruit MCAK to or maintain it at spindle poles. It will be critical to determine how MCAK association/dissociation with poles is mediated, and more critically how this contributes to bipolarity.

One potential model for MCAK-mediated spindle bipolarity is through regulatory mechanisms on the MCAK C-terminal domain. We showed previously that a version of MCAK missing the C-terminal domain, GFP-MCAK-2-592, also increases the percentage of bipolar spindles in Ran extracts (Ems-McClung *et al.*, 2007). In our previous work, we proposed the idea that there is a cooperative relationship between the C-terminal domain and the N-terminal domain such that deletion of the C-terminal domain positively influences the function of MCAK during the transition between asters and bipolar spindles. It is possible that the MCAK C-terminal domain is normally folded in a manner in which it interacts with the N-terminal domain, perhaps through autoinhibition as seen with conventional kinesin and CENP-E (Hackney *et al.*, 1992; Coy *et al.*, 1999; Espeut *et al.*, 2008). On deletion of the C-terminal domain, or perhaps by phosphorylation of S719, this inhibition is relieved, and MCAK becomes active in promoting bipolarity. We are currently testing this model.

MCAK Activity and Localization Are Controlled by Two Aurora Kinases

Our data highlight that precise regulation of MCAK activity is needed both spatially and temporally in the spindle. Our previous work established that there is a two-site regulatory network to control MCAK association with chromosomes and centromeres via its N-terminal domain (Zhang *et al.*, 2007). MCAK activity at these locations is then precisely modulated by phosphorylation at S196. In this study, we now show that MCAK activity is controlled by a second two-site phosphoregulatory network in which Aurora A controls MCAK targeting to spindle poles via phosphorylation at S719, and it also regulates its activity through phosphorylation at S196. It is intriguing that MCAK-regulated activity occurs in a spatial manner with an emphasis on one critical site that can be modulated by two separate kinases. An elucidation of the mechanism of how phosphorylation at this site affects the catalytic cycle of MCAK should be a high priority for future studies. We know that Aurora B phosphorylation of MCAK is critical in controlling its dynamic association with the centromere (Andrews *et al.*, 2004). Our data showing that MCAK phosphorylation at S719 changes its association with aster centers and spindle poles strongly supports the hypothesis that Aurora A controls the dynamic association of MCAK with spindle poles.

Remaining Questions of RanGTP-induced Aster and Spindle Assembly

Although the results shown here demonstrate that MCAK likely plays an important role in the transition from asters to bipolar spindles, they raise more questions and leave many others unanswered. For example, how γ -tubulin directs minus-end focusing in Ran asters is not clear. It is believed that in the absence of centrosomes, MT minus ends are focused through a combination of multiple MT-binding proteins and motor proteins, such as NuMA, the dynein/dynactin complex, Eg5, and XMAP215 (Theurkauf and Hawley, 1992; McKim and Hawley, 1995; Walczak *et al.*, 1997, 1998; Gaetz and Kapoor, 2004). The finding that MCAK, XMAP215, and NuMA have distinct localization patterns separate from Eg5 and γ -tubulin within aster centers and spindle poles is surprising. Does γ -tubulin simply cap the minus ends but MCAK, XMAP215, and NuMA provide an anchorage for MT minus ends in the absence of centrosomes? Perhaps this is one of the reasons why Ran asters fail to form in the absence of XMAP215 or NuMA (Gaglio *et al.*, 1995; Wilde and Zheng, 1999). In addition, whether there are other proteins that colocalize with MCAK, XMAP215, and NuMA is still unknown. Moreover, we show that MCAK differentially binds to asters and spindle poles through phosphorylation, but the regulatory mechanisms for XMAP215 and NuMA localization remain to be fully elucidated. In the future, it will be necessary to elucidate how MCAK interacts with these other players and how all of these activities are coordinated to organize functional spindle poles.

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