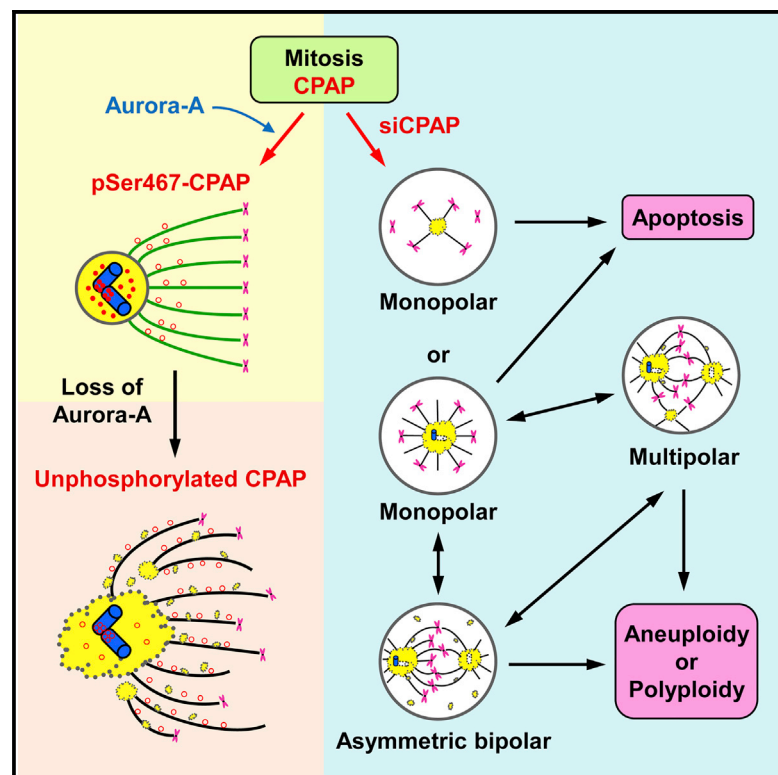Article

Phosphorylation of CPAP by Aurora-A Maintains Spindle Pole Integrity during Mitosis

Graphical Abstract

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In Brief

Chou et al. show that CPAP is essential for proper mitotic progression and maintenance of spindle pole integrity. CPAP is phosphorylated by Aurora-A during mitosis and phosphorylated CPAP coheres PCM proteins and maintains centrosome integrity.

Highlights

- Depletion of CPAP induces multiple mitotic abnormalities

- CPAP is phosphorylated by Aurora-A at Ser 467 during mitosis

- Phosphorylated CPAP coheres PCM proteins and maintains centrosome integrity

- Phosphorylated CPAP has a high affinity for PCM proteins but a low affinity for MTs

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Phosphorylation of CPAP by Aurora-A Maintains Spindle Pole Integrity during Mitosis

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SUMMARY

CPAP is required for centriole elongation during S/G2 phase, but the role of CPAP in mitosis is incompletely understood. Here, we show that CPAP maintains spindle pole integrity through its phosphorylation by Aurora-A during mitosis. Depletion of CPAP induced a prolonged delay in mitosis, pericentriolar material (PCM) dispersion, and multiple mitotic abnormalities. Further studies demonstrated that CPAP directly interacts with and is phosphorylated by Aurora-A at serine 467 during mitosis. Interestingly, the dispersal of the PCM was effectively rescued by ectopic expression of wild-type CPAP or a phospho-mimic CPAP-S467D mutant, but not a non-phosphorylated CPAP-S467A mutant. Finally, we found that CPAP-S467D has a low affinity for microtubule binding but a high affinity for PCM proteins. Together, our results support a model wherein CPAP is required for proper mitotic progression, and phosphorylation of CPAP by Aurora-A is essential for maintaining spindle pole integrity.

INTRODUCTION

The centrosome comprises a pair of centrioles surrounded by pericentriolar material (PCM). It acts as the microtubule-organizing center (MTOC), regulating cell motility, polarity, and intracellular material transport during interphase and spindle bipolarity during mitosis (Nigg and Raff, 2009; Woodruff et al., 2014). Centrosomes normally duplicate only once per cell cycle (Firat-Karalar and Stearns, 2014). Recent studies have identified several key centrosome-associated proteins (ZYG-1/PLK4, SAS-5/STIL, SAS-6, SAS-4/CPAP, and CEP135) that are essential for centriole duplication during interphase (Gönczy, 2012; Firat-Karalar and Stearns, 2014; Hirono, 2014; Jana et al., 2014). When cells enter mitosis, the duplicated centrosomes ensure the formation of a bipolar spindle, which is essential for accurate segregation of the chromosomes into the two daughter cells. At the onset of mitosis, the cytoplasmic microtubules (MTs) are disassembled and PCM proteins (e.g., pericentrin,

CDK5RAP2, and γ-tubulin) are recruited to the duplicated centrosomes. This process, which is called centrosome maturation, is necessary for astral and spindle MT nucleation during mitosis (Bettencourt-Dias and Glover, 2007; Woodruff et al., 2014). Depletion of pericentrin was found to reduce the recruitment of CDK5RAP2 and γ-tubulin to the centrosomes, disrupting MT nucleation from mitotic centrosomes and leading to mitotic spindle disorganization (Dictenberg et al., 1998). Depletion of proteins that are essential for centriole duplication (e.g., PLK4, CPAP, STIL, SAS6, and CEP135) has been shown to increase the population of cells with monopolar, abnormal bipolar, and multipolar spindles (Habedanck et al., 2005; Cho et al., 2006; Strnad et al., 2007; Kitagawa et al., 2011; Tang et al., 2011; Lin et al., 2013; Lee et al., 2014). However, the mechanism responsible for generating these variable mitotic phenotypes is not yet completely understood.

Aurora-A is a serine/threonine protein kinase that plays an important role in various steps of mitotic progression (i.e., centrosome separation, centrosome maturation, mitotic entry, spindle MT assembly, and genomic integrity) (Glover et al., 1995; Marumoto et al., 2005; Carmena et al., 2009). These events are thought to be regulated by the phosphorylation of specific substrates. For example, the Aurora-A-mediated phosphorylation of NDEL1 has been shown to promote centrosome separation, centrosome maturation, and mitotic entry (Mori et al., 2007), while Aurora-A-mediated phosphorylation of the kinesin-related protein Eg5 was shown to be essential for centrosome separation and spindle assembly (Giet et al., 1999; Dutertre et al., 2002).

We and others previously showed that CPAP is required for centriole duplication and responsible for regulating centriole elongation (Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). CPAP and its *Drosophila* ortholog, dSAS-4, have been shown to localize at the centriole and PCM (Hung et al., 2000; Gopalakrishnan et al., 2011; Sonnen et al., 2012) and are reportedly associated with proteins of the PCM complex, such as γ-tubulin, pericentrin, and CDK5RAP2 (Hung et al., 2000; Gopalakrishnan et al., 2011; Zheng et al., 2014). Furthermore, ectopic expression of dSAS-4 in *Drosophila* embryos induces the formation of multiple MTOCs (Dzhindzhev et al., 2010), and dSAS-4 seems to be able to recruit PCM proteins either directly or indirectly (Dzhindzhev et al., 2010; Gopalakrishnan et al., 2011; Conduit et al., 2015). All of these findings suggest that

CPAP may play a role in regulating PCM function during mitosis. Here we provide evidence showing that Aurora-A-mediated phosphorylation of CPAP at serine 467 (S467) during G2/M phase is required for proper mitotic progression and maintenance of spindle pole integrity during mitosis. Furthermore, phosphorylated CPAP appears to have a high affinity for PCM proteins (e.g., pericentrin and CDK5RAP2), but a low affinity for MTs. Finally, we propose a possible model that could explain how phosphorylated CPAP maintains spindle pole integrity during mitosis.

RESULTS

CPAP Is Essential for Proper Mitotic Progression and Maintenance of Centrosome Integrity

Previous reports showed that depletion of CPAP caused severe defects in centriole duplication and increased the population of monopolar, asymmetric bipolar, and multipolar spindles in fixed mitotic cells (Cho et al., 2006; Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009; Kitagawa et al., 2011). As no previous study has examined the transitions and dynamics of these mitotic abnormalities in a single living CPAP-depleted cell during mitosis, we transfected HeLa-T-REx cells stably expressing mCherry-α-tubulin with a CPAP-targeting (siCPAP) small interfering RNA (siRNA), and we traced mitotic cell progression by time-lapse microscopy. Our live-cell imaging results showed that CPAP-depleted cells took longer to complete mitosis, with this process taking 204 ± 17 min in siCPAP-treated cells (n = 63) versus 75 ± 6 min in control cells (n = 39) (Figure 1A, a). Interestingly, most of the CPAP-depleted cells started with one monopolar spindle, but then further assembled asymmetric bipolar spindles (Figure 1A, c; Movie S2) or multipolar spindles (Figure 1A, d; Movie S3) before finally dividing into two separate cells. Fragmented centrosomal masses frequently were observed in CPAP-depleted cells during mitotic progression (arrows in Figure 1A, c). A small portion of the CPAP-depleted cells that maintained monopolar spindles throughout mitosis progressed to apoptosis at the end of the process (Figure 1A, e; Movie S4). Some cells divided into three separate cells (5/63, 8%) in a one-to-three pattern (Figure S1A; Movie S5), while others formed large polyploid cells (4/63, 6%) in a one-to-one pattern due to failure of cytokinesis (Figures 1A, f and S1B; Movie S6). The proportions of the various mitotic phenotypes in CPAP-depleted cells are shown in Figure 1A, f. The results from our live-cell imaging demonstrate that multiple mitotic spindle defects (e.g., monopolar, asymmetric bipolar, and multipolar spindles and fragmented centrosomal masses) may dynamically coexist in a single CPAP-depleted cell and such cells can endure different cell fates (i.e., becoming two or three divided cells, becoming a single polyploid cell, or entering apoptosis).

Notably, when bipolar spindles were seen, one tended to be moved closer to the cell cortex in CPAP-depleted cells (Figure 1A, c, 176–204 min; Figures 1A, f, and 1B), whereas those of the control group usually oscillated in the cytoplasm during mitosis (Figure 1A, b; Movie S1). Immunofluorescence staining revealed that the spindle pole closer to the cell cortex always contained the centriole(s) in CPAP-depleted cells (Figure 1B). We propose that the astral MTs emanating from the centriole-containing spindle pole could be efficiently captured by the cell cortex and thereafter erect a force pulling the centrosome toward the cortex.

We also observed that the mitotic centrosomes in some CPAP-depleted cells were torn apart to form multipolar spindles (Figure 1A, d) or fragmented centrosomal masses (Figure 1A, c, arrows), suggesting that CPAP is essential for spindle pole integrity. To investigate whether the observed mitotic abnormalities were produced via defects in spindle pole integrity, we examined the centriole numbers in fixed CPAP-depleted cells. Most control cells had the expected four centrioles during mitosis, whereas more than 90% of CPAP-depleted cells had fewer than four centrioles (Figure S2B, right panel). Interestingly, some CPAP-depleted cells with three or fewer centrioles still possessed multipolar spindles (Figure S2B, left panel), usually accompanied by fragmented PCMs (as assessed by pericentrin-positive signals; Figure S2C). Thus, it appears that the multipolar spindles in CPAP-depleted cells were generated via PCM fragmentation rather than centriole amplification. Furthermore, most CPAP-depleted cells showed dispersed PCM signals (as assessed by monitoring of pericentrin, CDK5RAP2, and γ-tubulin; Figure 2A, row 3), while siControl cells exhibited compact and focused signals at their mitotic poles (Figure 2A, row 1). The quantitation of each dispersed PCM protein and its corresponding area are illustrated in Figures 2B and 2C, respectively.

Interestingly, the dispersed pericentrin in siCPAP-treated cells seemed to be spread along the MTs (Figure 2D). When we cold-treated CPAP-depleted cells to depolymerize MTs, the dispersion of PCM was significantly reduced (Figures 2A and 2B). Together, these results suggest that CPAP is required to maintain centrosome integrity in an MT-dependent manner. Finally, most mitotic abnormalities, including monopolar (Figure S3C, i), asymmetric bipolar (Figure S3C, ii), fragmented PCM (Figure S3C, iii, arrows), multipolar with fewer centrioles (less than four as assessed by centrin-positive signals, Figure S3C, iv), and one pole moving closer to the cortex (Figure S3D), could be effectively rescued by exogenous expression of siRNA-resistant CPAP-WT-myc in siCPAP-depleted cells (Figures S3C, v and vi, S3D, and S3E). However, expression of CPAP-WT-myc did not appear to rescue abnormal multipopular spindles carrying multiple centrioles (more than four centrin- or myc-positive signals; Figures S3C, vii and viii). The underlying mechanism is not clear. Together, our results suggest that CPAP is essential for proper mitotic progression and maintenance of spindle pole integrity.

CPAP Is Associated with and Phosphorylated by Aurora-A at S467 during G2/M Phase

We next investigated the potential role of CPAP in maintaining centrosome integrity and the mechanism underlying the dispersal of PCM proteins in CPAP-depleted cells during mitosis. Since Aurora-A kinase plays a critical role in regulating mitotic progression and centrosome integrity, we first examined whether CPAP could be associated with Aurora-A. Our co-immunoprecipitation (coIP) experiments showed that exogenously expressed GFP-CPAP could co-precipitate with Flag-Aurora-A in transfected cells synchronized at G2/M phase by nocodazole treatment (Figure 3B). This suggests that CPAP is associated with Aurora-A during mitosis.
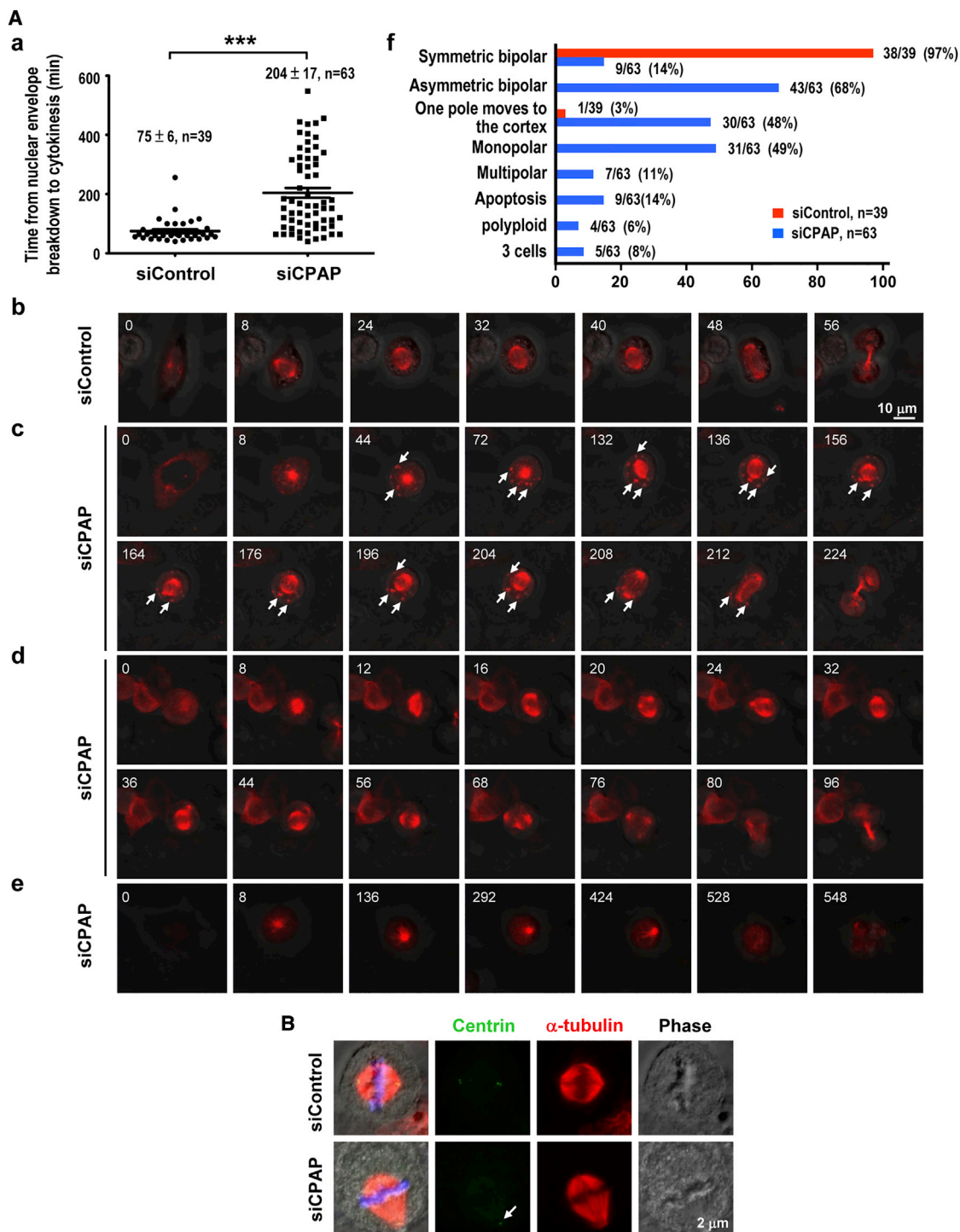
Figure 1. CPAP Depletion Induces Various Mitotic Abnormalities, as Observed in Live Cells

(A) HeLa-T-REx-derived mCherry-α-tubulin-expressing cells were transfected with siControl (b) or siCPAP (c–e). Then, 60 hr post-transfection, live-cell images were taken with an LSM780 confocal system at the indicated time (minutes). Depletion of CPAP prolonged mitosis (a), resulting in mitotic abnormalities, such as asymmetric bipolar spindles with fragmented PCM (c, white arrows), multipolar spindles (d), and apoptosis (e). Error bars (a) indicate the means ± SEM from three independent experiments; ***p < 0.001 (an unpaired t test). (f) Histogram illustrates the percentages of the indicated phenotypes observed in time-lapse live-cell images. See also Figure S1 and Movies S1, S2, S3, S4, S5, and S6.

(B) In CPAP-depleted cells, the centriole-containing spindle pole is located close to the cell cortex. U2OS cells were transfected with siControl or siCPAP for 72 hr and analyzed by immunofluorescence staining with the indicated antibodies. The arrow indicates the centriole(s) (marked by centrin) near the cell cortex.
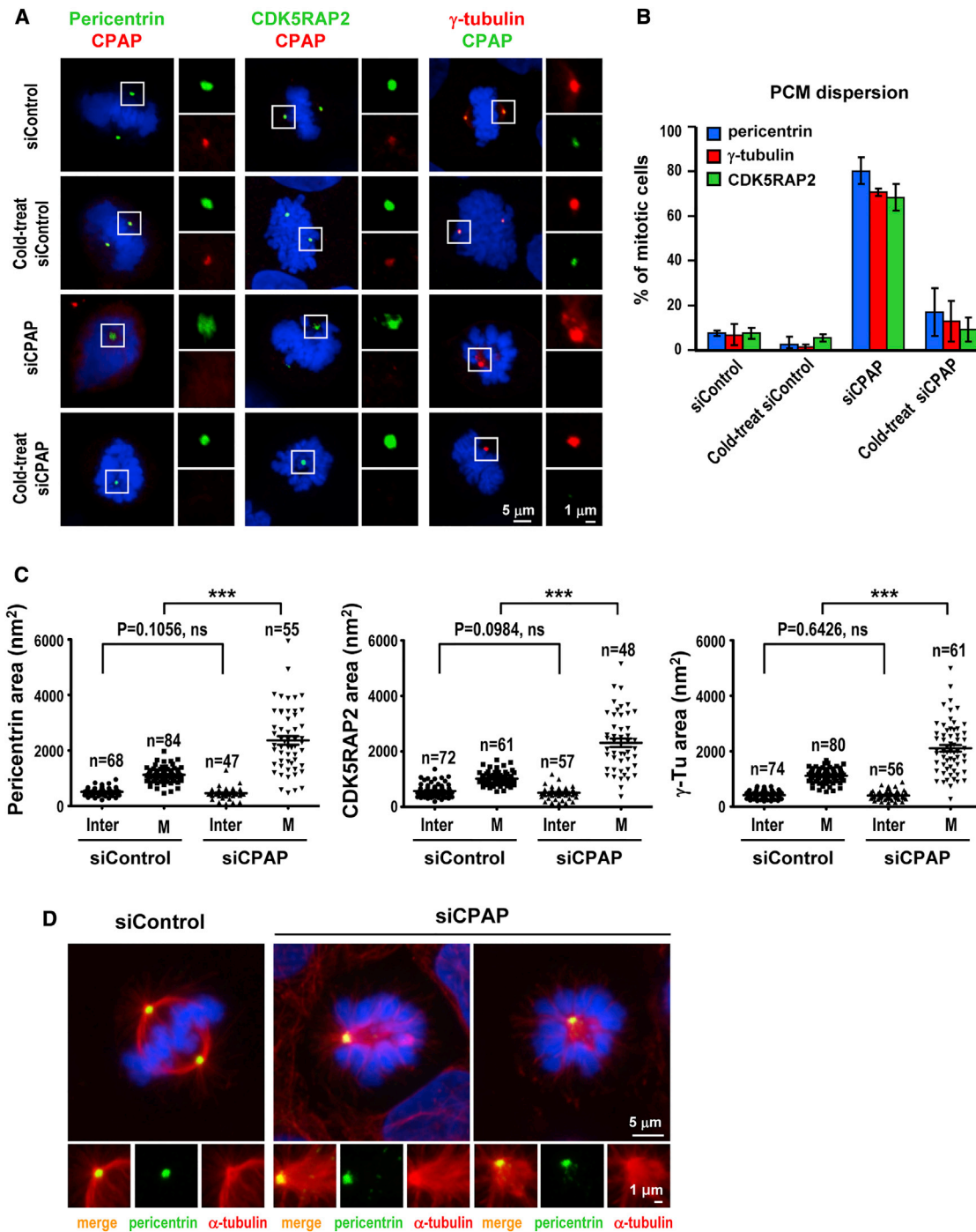
Figure 2. CPAP Is Required to Maintain Centrosome Integrity in an MT-Dependent Manner

(A and B) U2OS cells were transfected with siControl or siCPAP for 72 hr and analyzed by immunofluorescence staining with or without cold treatment. In cold-treated experiments, the transfected cells were put on ice for 2 hr, immediately fixed in methanol, and then analyzed by immunofluorescence staining (A) with the indicated antibodies. (B) Histogram illustrates the percentages of dispersed PCM signals in siControl or CPAP-depleted mitotic cells with or without cold treatment. Error bars represent means ± SD of 100 cells from three independent experiments.

(C) Quantification of the areas labeled for pericentrin, CDK5RAP2, or γ-tubulin (nm^2) at the poles of siControl and siCPAP-treated cells during interphase (Inter) or mitosis (M). The PCM dispersion was found in the CPAP-depleted mitotic cells, but not the interphase cells. The areas were measured by encircling the edges of the stained regions. Error bars represent means ± SEM; ***p < 0.001 (an unpaired t test); ns, not significant.

(D) The scattered pericentrin signal (green) co-localized with that of MTs (labeled with α-tubulin, red).

See also Figure S3.

To identify the Aurora-A-associated domain in CPAP, we performed glutathione S-transferase (GST) pull-down assay. We transfected HEK293T cells with a series of constructs encoding GFP-tagged truncated versions of CPAP, used nocodazole to synchronize the cells at G2/M phase, and incubated cell lysates with affinity-purified GST-Aurora-A recombinant proteins. As shown in Figure 3C, the N-terminal CPAP fragment (residues 1–758 and 132–607), but not the C-terminal fragment (residues 607–1,338), was specifically pulled down by recombinant GST-Aurora-A (Figure 3C, a). Similarly, endogenous Aurora-A could be pulled down by GST-CPAP-truncated proteins (residues 1–758 and 311–607) (Figure 3C, b). These findings indicate that CPAP associates with Aurora-A through its N-terminal domain that maps to residues 311–607 (Figure 3A).

The consensus sequence for Aurora-A phosphorylation was predicted to be R-X-pS/T-L/V (Ohashi et al., 2006). Interestingly, this putative consensus site is found in the N-terminal region of CPAP ([465]RKSL[468]), within the region herein found to interact with Aurora-A (Figures 3B and 3C). We thus examined whether CPAP could act as a substrate for Aurora-A by subjecting various affinity-purified GST recombinant proteins, including GST-Aurora-A wild-type (WT), GST-A5N (CPAP residues 423–607), and GST-A5C (CPAP residues 1,070–1,338), to in vitro kinase assays. Our results revealed that Aurora-A phosphorylates CPAP primarily in the A5N fragment, with some very weak phosphorylation in the A5C polypeptide (Figure 3D, a). Heat inactivation of Aurora-A at 65°C blocked its activity to phosphorylate the A5N fragment of CPAP (Figure 3D, b). We further analyzed the site at which CPAP is phosphorylated by Aurora-A using mass spectrometry, and we found that S467 of CPAP is phosphorylated by Aurora-A in HeLa cells (Figure S4A). To confirm the specificity of this phosphorylation, GST-tagged A5N cDNA constructs containing point mutations that substituted Ser467 and/or Ser469 to alanine (S467A and S469A) were generated by site-directed mutagenesis, and the resulting recombinant proteins were subjected to in vitro kinase assays. As shown in Figure 3D (c), A5N-WT and A5N-S469A, but not A5N-S467A or A5N-S467A/S469A, could be phosphorylated by Aurora-A. This confirms that Ser467 of CPAP is the site of Aurora-A-mediated phosphorylation.

The Subcellular Localization of pS467-CPAP during the Cell Cycle

To investigate the spatiotemporal localization of phosphor-S467-CPAP (pS467-CPAP) during the cell cycle, we generated a phosphor-specific antibody from a rabbit immunized with a phosphor-synthetic peptide (see the Supplemental Experimental Procedures). The antibody was affinity purified and its specificity was confirmed by western blot analysis (Figures S4B–S4D). The pS467-CPAP antibody could specifically recognize pS467-CPAP in a reaction mixture containing CP3-6-His (CPAP residues 311–607) and Aurora-A-WT recombinant proteins, but not in a reaction containing the Aurora-A-K162M kinase-dead mutant (Figure S4B). Co-transfection experiments further demonstrated that the S467 of GFP-CPAP-WT was phosphorylated in cells that exogenously expressed Aurora-A-T288D (an activated form of Aurora-A; Walter et al., 2000; Figure S4C) and that this S467 phosphorylation could be specifically blocked

by treating the G2/M cell lysates with lambda phosphatase (NZλ; Figure S4D).

The subcellular localizations of CPAP, pS467-CPAP, Aurora-A, and pT288-Aurora-A were examined by confocal immunofluorescence microscopy. As shown in Figure 4A (a), we did not detect pS467-CPAP in interphase cells, while during mitosis pS467-CPAP co-localized with endogenous CPAP at the centrosomes. Interestingly, a significant portion of pS467-CPAP signal was detected at the cell midbody (Figure 4A, a), suggesting that phosphor-CPAP may play a yet uncharacterized role during cytokinesis. The spatiotemporal localization of pS467-CPAP was quite similar to that of pT288-Aurora-A (Marumoto et al., 2005; Mori et al., 2007), and both showed compact and focused signals at the spindle poles during early mitosis (pS467-CPAP, Figure 4A, b; pT288-Aurora-A, Figure S4E). In contrast, the signal corresponding to endogenous Aurora-A was detected as a prominent umbrella pattern during prometaphase and metaphase (Figure 4A, b), which is consistent with the previous report (Kimura et al., 1997). Interestingly, when cells were treated with MLN8237, an Aurora-A kinase inhibitor (Manfredi et al., 2011), the signals for both pT288-Aurora-A (Figure 4B, a) and pS467-CPAP (Figure 4B, b) were significantly reduced. Taken together, these results indicate that CPAP is phosphorylated by Aurora-A during mitosis, and the phosphorylated forms of CPAP and Aurora-A are both detected at the mitotic spindle poles, but not interphase centrosomes.

Aurora-A Phosphorylation of CPAP Does Not Affect CPAP-Induced Centriole Elongation

Since CPAP is required for centriole duplication, particularly for centriole elongation (Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009), we next examined the possible involvement of pS467-CPAP in this process. We previously generated U2OS-based inducible transfectants that expressed full-length CPAP-WT-myc under the control of doxycycline, and we showed that CPAP overexpression can induce the formation of extra-long centrioles (Tang et al., 2009). Using a similar approach, we produced CPAP-S467A- and CPAP-S467D-inducible cell lines, and we compared the numbers of extra-long centrioles among these inducible cell lines. We found that the numbers of extra-long centrioles did not differ in doxycycline-induced CPAP-WT, CPAP-S467A, or CPAP-S467D cells (Figure S5A), suggesting that pS467-CPAP is not involved in centriole elongation. Furthermore, inhibition of Aurora-A kinase activity with various doses of MLN8237 (20, 50, or 100 nM) did not affect the centriole length in CPAP-WT-inducible cells (Figure S5B). Together, these results indicate that Aurora-A-mediated phosphorylation of CPAP does not affect CPAP-induced centriole elongation.

CPAP Coheres PCM Proteins and Maintains Centrosome Integrity during Mitosis through Aurora-A-Mediated Phosphorylation at S467

Our live-cell imaging results showed that CPAP depletion induced a prolonged M phase during which dynamic transitions among various mitotic phenotypes (e.g., monopolar, asymmetric bipolar, multipolar, and fragmented centrosomal masses; Figure 1) could be seen. This suggests that the
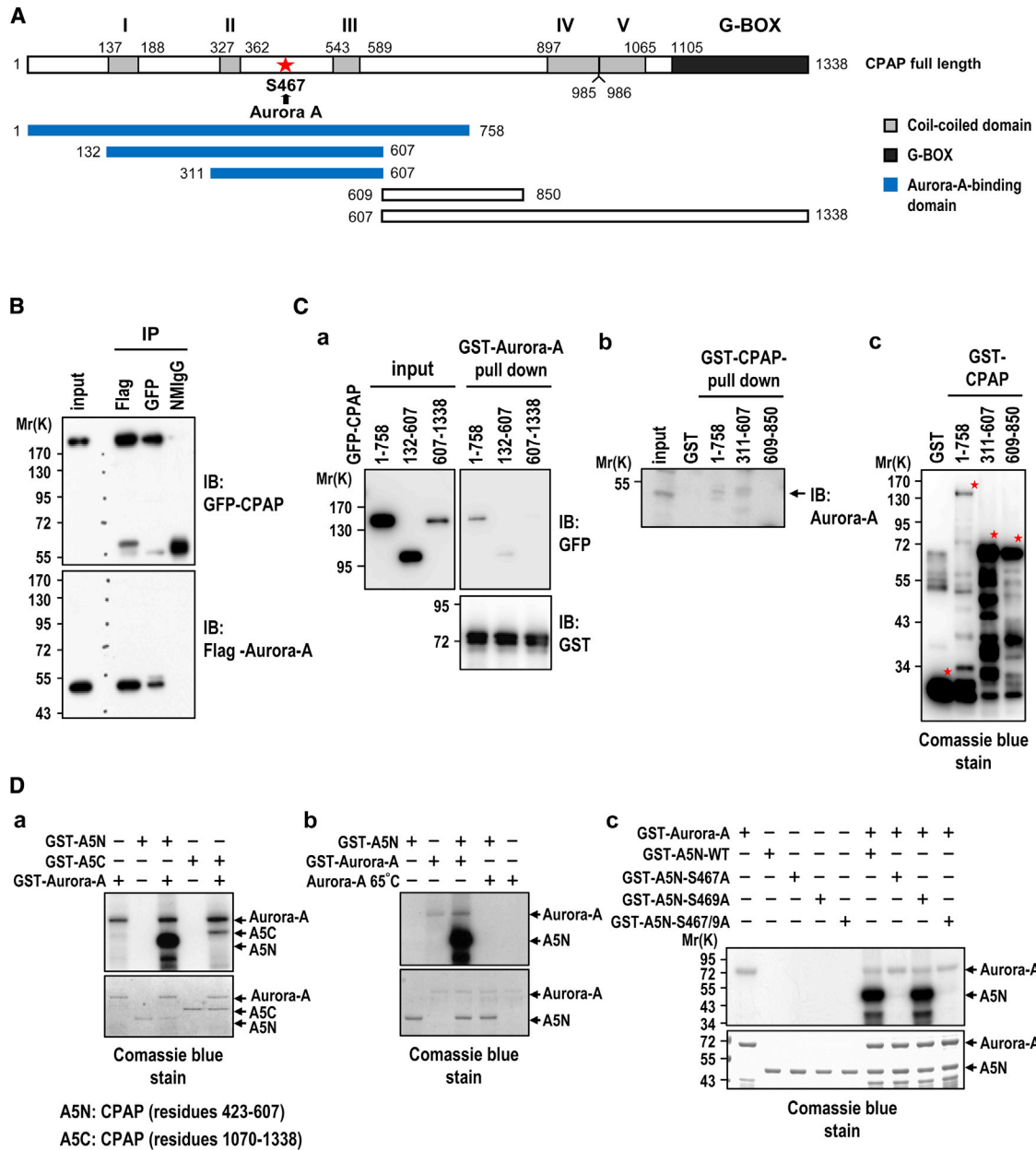
Figure 3. CPAP Is Associated with and Phosphorylated by Aurora-A at Ser467 during G2/M Phase

(A) Schematic structure of CPAP. Five α-helical coiled-coil domains (I–V) and the G-box (glycine repeat box)/Tcp10 are shown (Hung et al., 2000). The Aurora-A-binding domain of CPAP is shown (residues 311–607) as is the Aurora-A-phosphorylated site (Ser467, red star). The association of CPAP with Aurora-A was demonstrated by coIP (B) and GST pull-down (C) assays.

(B) HEK293T cells were co-transfected with constructs encoding the GFP-CPAP and FLAG-Aurora-A. After transfection, cells were treated with nocodazole (NZ) to induce arrest at the G2/M phase. Cell lysates were immunoprecipitated (IP) with the indicated antibodies and immunoblotted (IB) with anti-FLAG or anti-GFP antibodies.

(C) GST pull-down assays. GST, GST-Aurora-A, and GST-CPAP truncated proteins were affinity purified and stained with Coomassie blue (c, asterisks mark the predicted sizes of the purified proteins). (a) HEK293T cells were transfected with the indicated GFP-CPAP fragments, and cell lysates were incubated with equal amounts of GST-Aurora-A and analyzed by western blotting using the indicated antibodies. (b) HEK293T cells were arrested at G2/M with NZ, and lysates were incubated with various GST-CPAP truncated recombinant proteins. Endogenous Aurora-A was detected in the pulled-down proteins using an anti-Aurora-A antibody.

(D) CPAP is phosphorylated in vitro at Ser467 by Aurora-A. The indicated GST recombinant proteins were affinity purified and stained with Coomassie blue (a–c, bottom panels). Equal amounts of the indicated purified recombinant proteins were used to perform in vitro kinase assays. GST-A5N (CPAP residues 423–607) is
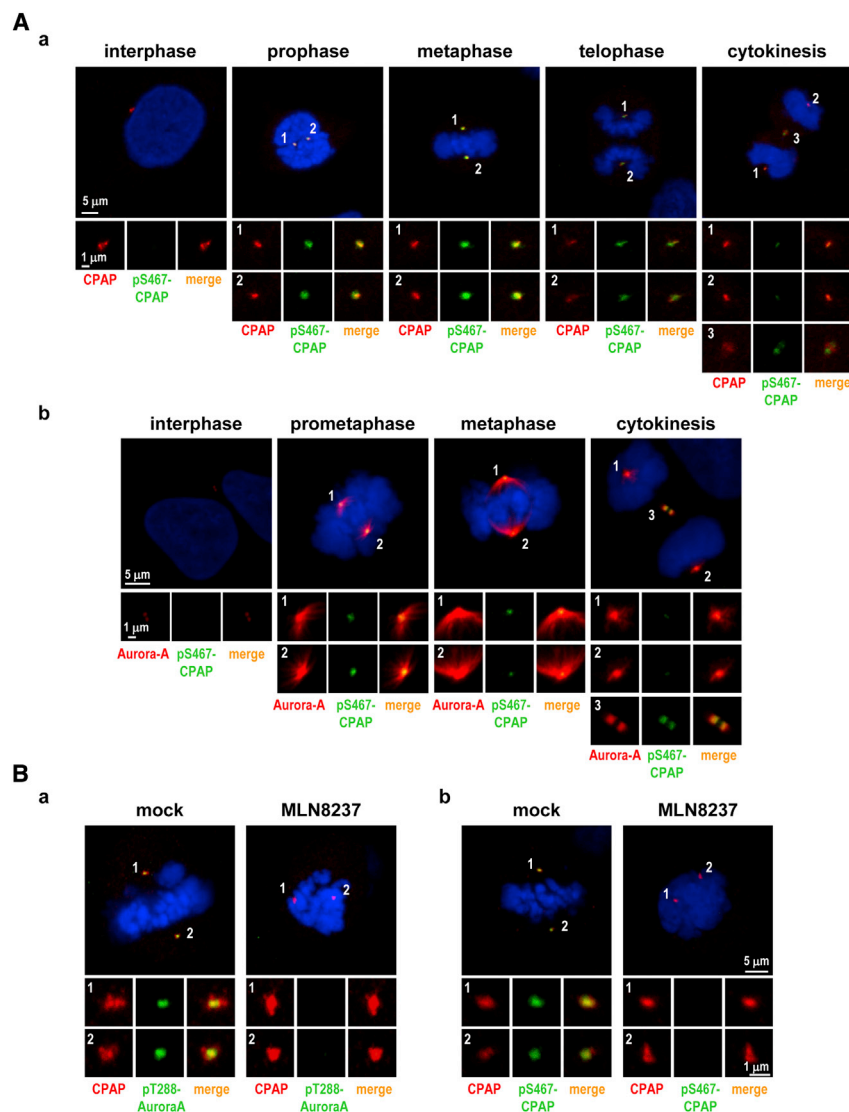
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Figure 4. Endogenous CPAP Phosphorylated at Ser467 Is Detected at the Mitotic Spindle Poles, but Not Interphase Centrosomes

(A) U2OS cells were fixed with methanol and analyzed by confocal fluorescence microscopy using the indicated antibodies. (a) The subcellular localization of pS467-CPAP during the cell cycle is shown. (b) The pS467-CPAP signal co-localizes with a portion of the Aurora-A signal at the centers of mitotic spindle poles and the midbody.

(B) The pS467-CPAP signal is greatly reduced in cells treated with MLN8237, an Aurora-A kinase inhibitor. U2OS cells were mock treated or treated with 50 nM MLN8237 for 24 hr, and then they were analyzed with antibodies against pT288-Aurora-A/CPAP (a) or pS467-CPAP/CPAP (b), followed by confocal fluorescence microscopy.

WT, CPAP-N (residues 1–758), or CPAP-C (residues 607–1,338), and we arrested the cells in S (thymidine treatment) or G2/M phase (nocodazole treatment). CoIP experiments were performed using the indicated antibodies. As shown in Figure 5A, a, the PCM proteins (pericentrin and CDK5RAP2) showed increased affinities for CPAP during mitosis (as revealed by nocodazole treatment) than in interphase (as revealed by thymidine treatment) (Figure 5A, a). Moreover, CPAP-N, but not CPAP-C, showed strong associations with pericentrin and CDK5RAP2 (Figure 5A, b). This finding is consistent with the previous report showing that the N-terminal domain of dSAS-4 (the *Drosophila* ortholog of CPAP) is required for it to interact with a number of PCM proteins, including D-PLP (a pericentrin-like protein) and CNN (a CDK5RAP2-related protein) (Gopalakrishnan et al., 2011).

cohesive force that maintains centrosome (spindle pole) integrity in normal mitotic cells is sensitive to CPAP. Furthermore, PCM protein dispersion and PCM fragmentation were commonly observed in CPAP-depleted cells (Figures 1A, c, 2A, and S2C) and in cells treated with siAurora-A or the Aurora-A kinase inhibitor MLN8237 (Figure S6). These findings prompted us to hypothesize that CPAP may cohere PCM proteins and maintain centrosome integrity by Aurora-A-mediated phosphorylation.

To test this hypothesis, we examined whether phosphorylated CPAP associates with PCM proteins. We transiently transfected HEK293T cells with constructs encoding GFP-tagged CPAP-

To investigate whether Aurora-A-phosphorylated CPAP was involved in PCM protein association and/or centrosome integrity (as judged by PCM protein dispersion), we transfected HEK293T cells with plasmids encoding myc-tagged CPAP-WT, -S467A, or -S467D and performed coIP experiments. Our results revealed that the CPAP-S467A mutant had lower affinities for pericentrin (Figure 5B, a and b) and CDK5RAP2 (Figure 5B, c and d) than did CPAP-WT or -S467D. Furthermore, CPAP depletion induced the dispersions of pericentrin (Figure 5C, b) and CDK5RAP2 (Figure 5D, b) in mitotic cells, whereas such effects could be effectively rescued by ectopic expression of siRNA-resistant CPAP-WT-myc (Figures 5C, c and 5D, c) or CPAP-S467D-myc (which

strongly phosphorylated by Aurora-A (a), while its phosphorylation is significantly inhibited by incubation with heat-inactivated Aurora-A (b, 65°C for 20 min). The specific Aurora-A phosphorylation site in CPAP was mapped to Ser467 by our in vitro kinase assays. Little or no phosphorylation was observed on the GST-A5N-S467A or GST-A5N-S467A/S469A mutant proteins (c). See also Figure S4.
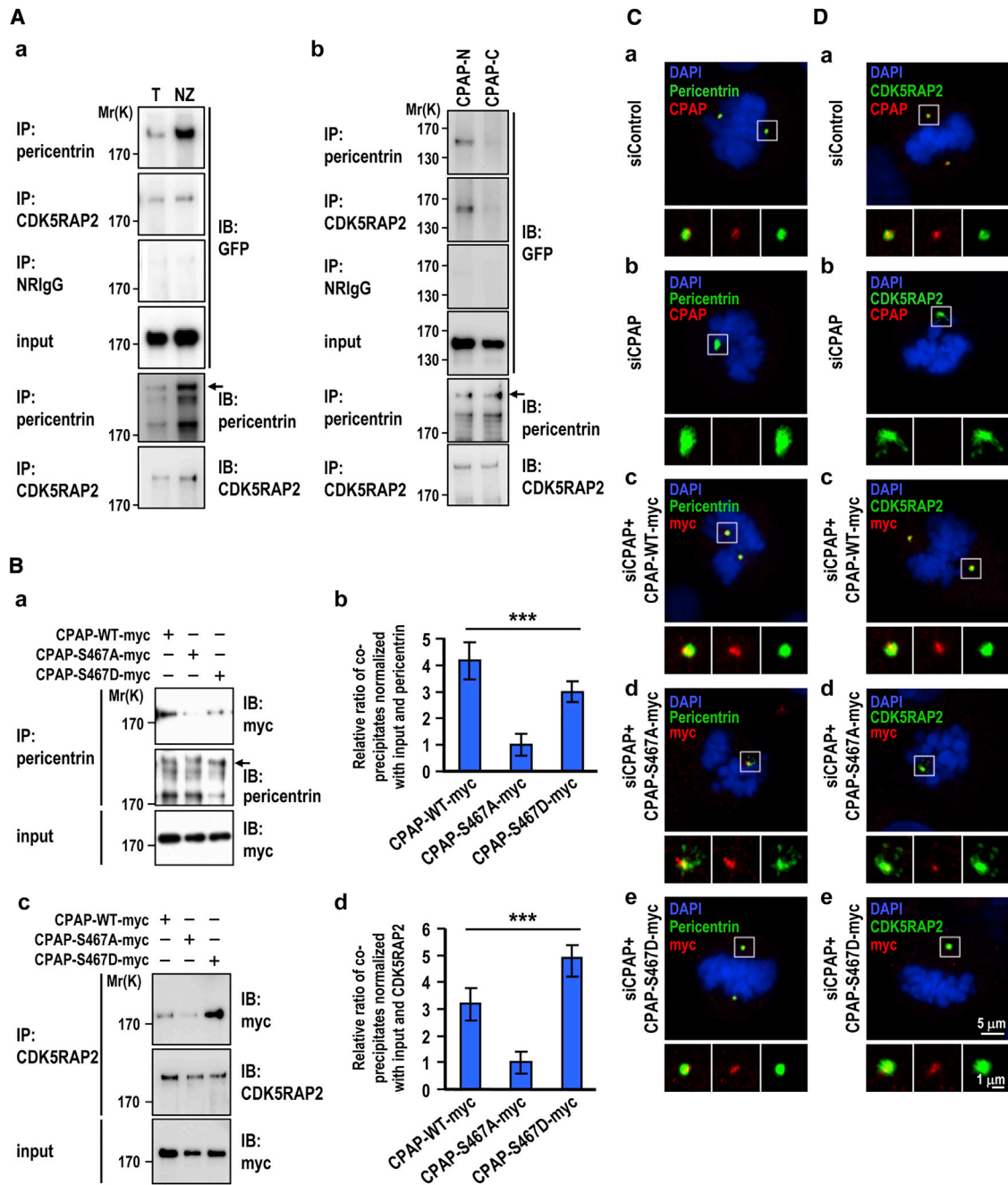
Figure 5. CPAP Associates with PCM Proteins and Maintains Spindle Pole Integrity during Mitosis

(A) CPAP associates with pericentrin and CDK5RAP2 via its N-terminal domain during G2/M phase. HEK293T cells were transfected with constructs encoding GFP-tagged CPAP-WT (a), CPAP-N (residues 1–758), or CPAP-C (residues 607–1,338) (b) for 8 hr, and then they were treated with thymidine (T) (a) or NZ (a and b) for an additional 16 hr. Cell lysates were IP with antibodies against pericentrin, CDK5RAP2, or normal rabbit IgG (NRIgG) and then analyzed by western blotting using the indicated antibodies.

(B) CPAP-S467D-myc has higher affinities for pericentrin and CDK5RAP2 compared to the affinities of CPAP-WT-myc and CPAP-S467A-myc. HEK293T cells were transfected with constructs encoding CPAP-WT-myc, CPAP-S467A-myc, or CPAP-S467D-myc for 8 hr, and then they were treated with NZ for an additional 16 hr. Cell lysates were IP with pericentrin (a) or CDK5RAP2 (c) and analyzed by western blotting using the indicated antibodies. Histograms illustrate the relative ratio of co-precipitates normalized with input and pericentrin (b) or CDK5RAP2 (d). Error bars represent means ± SEM from seven independent experiments. Quantification was analyzed using the ImageJ software (***p < 0.001, one-way ANOVA).
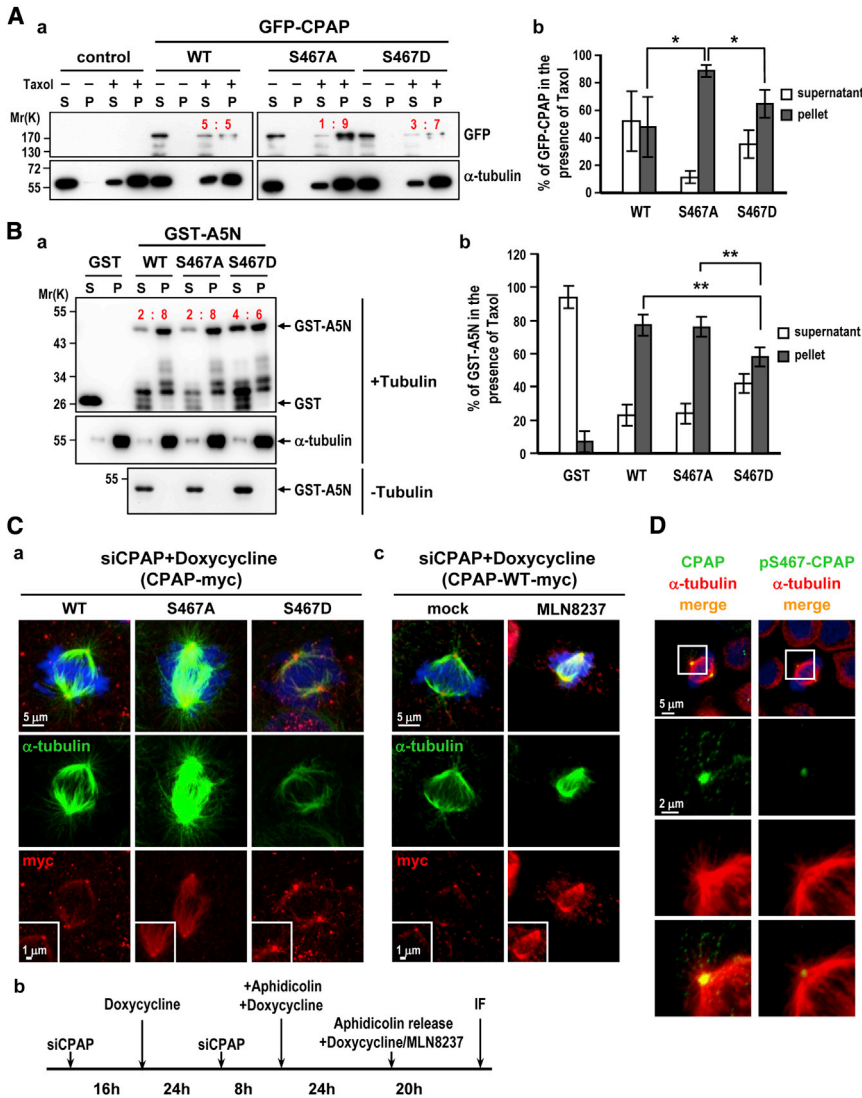
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Figure 6. CPAP-S467A Has an Increased Affinity for MT Binding

(A) GFP-S467A shows increased MT binding in transfected cells. HEK293T cells were transfected with constructs encoding GFP-CPAP-WT, GFP-CPAP-S467A, or GFP-CPAP-S467D for 8 hr, and then they were treated with NZ for another 16 hr. The cells were lysed and in vivo MT co-sedimentation assays were performed with or without Taxol. After centrifugation, the lysates were separated into supernatant (S) and pellet (P) fractions and analyzed by western blotting using the indicated antibodies (a). Three independent experiments were performed. A representative blot is shown (a). Quantifications of GFP-CPAP-WT and mutant protein expression levels in the presence of Taxol also are shown (b).

(B) GST-A5N-S467A shows increased MT binding in vitro. Purified tubulins (15 μM) were incubated with GST-A5N-WT, GST-A5N-S467A, or GST-A5N-S467D recombinant proteins in the presence of Taxol, and then they were subjected to an in vitro MT co-sedimentation assay. After centrifugation, the S and P fractions were analyzed by western blotting using the indicated antibodies (a). Histograms illustrate the percentages of various GST-A5N recombinant proteins in the S or P fractions (b). Error bars represent means ± SD from three independent experiments. Quantification was performed using the ImageJ software (*p < 0.05 and **p < 0.01, unpaired t test).

(C) CPAP-S467A-myc is strongly associated with MTs in vivo. U2OS-based CPAP-WT-, CPAP-S467A-, and CPAP-S467D-myc-inducible cells were transfected with siCPAP as shown (b). The cells were then pre-extracted in PHEM buffer containing 0.5% Triton X-100 with 5 μM Taxol, fixed with formaldehyde, incubated with the indicated antibodies, and analyzed by confocal fluorescence microscopy (a). (c) U2OS-based CPAP-WT-myc-inducible cells were treated as shown in (b) in the presence of 50 nM MLN8237.

(D) Endogenous CPAP is detected at the mitotic poles and MTs, while phosphorylated CPAP is mainly restricted to the mitotic spindle poles. U2OS cells were pre-extracted and fixed as described in (C).

mimicked CPAP phosphorylated at S467; Figures 5C, e, and 5D, e), but not by CPAP-S467A-myc (Figures 5C, d, and 5D, d). Similar results were obtained in U2OS-derived inducible cell lines, in which the dispersion of pericentrin could be overcome by inducing the expression of CPAP-WT-myc or CPAP-S467D-myc (Figure S7). Together, these results show that CPAP can cohere PCM proteins at the focus poles and contribute to maintaining centrosome integrity during mitosis and that this is controlled by Aurora-A-mediated phosphorylation at S467.

CPAP-S467A Has a High Affinity for MTs during Mitosis

The Aurora-A phosphorylation site (S467) identified herein is located in the A5N polypeptide (residues 423–607) of CPAP (Figure 3D), which previously was reported to directly bind MTs (Hsu et al., 2008). To examine whether Aurora-A-mediated phosphorylation could influence the ability of CPAP to bind MTs, we transfected HEK293T cells with constructs encoding GFP-CPAP-WT, -S467A, or -S467D and performed in vivo MT co-sedimentation assays. Our results showed that CPAP-S467A had a stronger MT-binding ability than CPAP-WT or -S467D (Figure 6A), with

(C and D) The dispersed PCM signals in CPAP-depleted cells could be effectively rescued by exogenous expression of CPAP-WT-myc or CPAP-S467D-myc, but not CPAP-S467A-myc. U2OS cells were transfected with siControl (C and D, a) or siCPAP (C and D, b–e) for 24 hr and then transfected with siCPAP-resistant CPAP-WT-myc (C and D, c), CPAP-S467A-myc (C and D, d), or CPAP-S467D-myc (C and D, e) for an additional 48 hr. The cells were then fixed and analyzed with the indicated antibodies followed by visualization under confocal fluorescence microscopy.
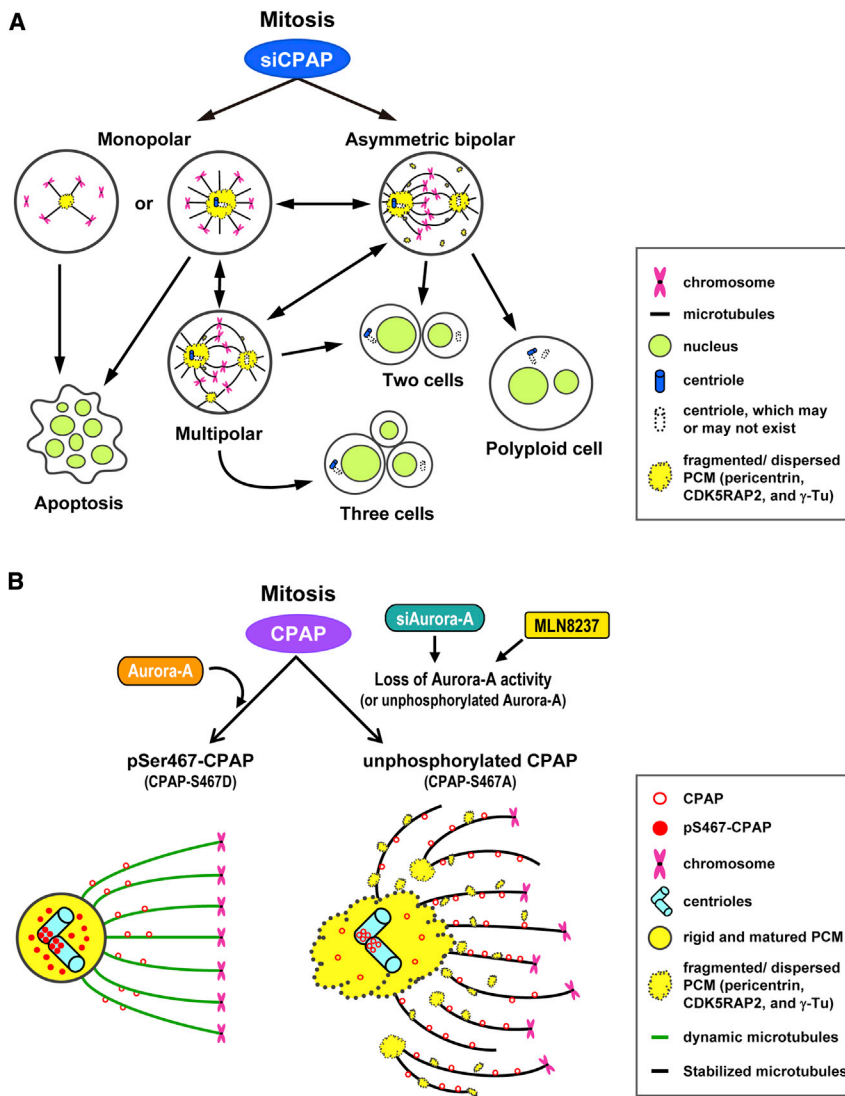See also Figure S7.

A

Mitosis

siCPAP

Monopolar ··· or ··· **Asymmetric bipolar**

Multipolar

Apoptosis

Two cells

Three cells

Polyploid cell

✗	chromosome
—	microtubules
●	nucleus
▮	centriole
⬚	centriole, which may or may not exist
☀	fragmented/ dispersed PCM (pericentrin, CDK5RAP2, and γ-Tu)

B

Mitosis

CPAP

Aurora-A

siAurora-A ··· MLN8237

Loss of Aurora-A activity
(or unphosphorylated Aurora-A)

pSer467-CPAP
(CPAP-S467D)

unphosphorylated CPAP
(CPAP-S467A)

○	CPAP
●	pS467-CPAP
✗	chromosome
◇	centrioles
●	rigid and matured PCM
☀	fragmented/ dispersed PCM (pericentrin, CDK5RAP2, and γ-Tu)
—	dynamic microtubules
—	Stabilized microtubules

Figure 7. Proposed Model of the Roles Played by CPAP during Mitosis

(A) CPAP-depleted cells can show dynamic transitions among monopolar, asymmetric bipolar, and multipolar spindles, and they can progress to different cell fates, including two or three cells, a large polyploid cell, or apoptosis.

(B) CPAP phosphorylated by Aurora-A at Ser467 is required for the integrity of the spindle pole during mitosis. Ser467-phosphorylated CPAP has a high affinity for PCM proteins and a low affinity for MTs, which may promote PCM protein cohesion and MT dynamics at the spindle poles during mitosis. Conversely, unphosphorylated CPAP or loss of Aurora-A activity has the opposite effects, causing dispersion of PCM proteins and centrosome fragmentation. For details, please see the Discussion.

in Figure 6C, a, CPAP-S467A-myc was more strongly associated with MTs during mitosis compared with CPAP-WT-myc and CPAP-S467D-myc. In agreement with this finding, we found that CPAP-WT-myc increases its binding to mitotic MTs in cells treated with MLN8237, an Aurora-A kinase inhibitor (Figure 6C, c).

To examine whether endogenous CPAP also is associated with MTs, U2OS cells were treated with PHEM and Taxol as described in Figure 6C. We found that a significant portion of endogenous CPAP (in addition to its known centrosomal localization) co-localized with mitotic MTs, including astral MTs and spindle MTs, while phosphorylated CPAP (pS467) was mainly restricted to the spindle poles (Figure 6D). Taken together, our results strongly support the notion that Aurora-A-mediated phosphorylation of CPAP at S467 negatively regulates the MT-binding activity of CPAP during mitosis.

DISCUSSION

Our group and others previously showed that CPAP plays an essential role in controlling centriole length during S/G2 phase (Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). Here we demonstrate that Aurora-A-phosphorylated CPAP could maintain spindle pole integrity during mitosis (Figure 7). Two major findings are reported here. First, we show that CPAP interacts with Aurora-A kinase, which phosphorylates it at S467 (Figures 3 and S4A), and that the phosphorylation of CPAP is essential for maintaining spindle pole integrity during mitosis. Second, we reveal that depletion of CPAP not only causes centriole duplication defects but also produces multiple mitotic abnormalities, including monopolar, asymmetric bipolar, and multipolar spindles that can dynamically co-exist in a single

the majority of CPAP-S467A proteins co-precipitating with MTs in the pellet (1:9 ratio of supernatant to pellet). Consistent with this, in vitro MT co-sedimentation experiments using purified recombinant proteins showed that GST-A5N-S467A had a high affinity for MT binding (Figure 6B). Unexpectedly, GST-A5N-WT had a similarly high MT-binding activity (Figure 6B). This may reflect that we did not include recombinant Aurora-A protein in the reaction solution; thus, the recombinant GST-A5N-WT protein was presumably the unphosphorylated form. Collectively, our results strongly support the notion that Aurora-A-mediated phosphorylation of CPAP at S467 negatively regulates the MT-binding activity of CPAP.

To confirm and further examine the stable association of CPAP-S467A with MTs, we treated CPAP-inducible cells (CPAP-WT-myc, -S467A-myc, and -S467D-myc) as described in Figure 6C, b, pre-extracted the cells with PHEM buffer in the presence of Taxol to stabilize the MTs, and analyzed by confocal fluorescence microscopy using indicated antibodies. As shown

living cell during mitosis (Figure 1). We thus propose that the variable mitotic abnormalities observed in CPAP-depleted cells may be due to the production of abnormal centrosomes with three or fewer centrioles (Figures 7A and S2B). In our model, a monopolar spindle could be derived from a single-pole centrosome that contains either no centrioles or one/two centrioles (∼49%, Figure 1A, f), whereas an asymmetric bipolar spindle (∼68%, Figure 1A, f) would most likely be generated by an uneven distribution of centrioles between two separate poles (i.e., ratios of 2:1, 1:1, 1:0, or 2:0) (Figure 7A).

Interestingly, we found that a mono-pole could dynamically transit into asymmetric bi-poles or multi-poles. Moreover, we observed that such mitotic abnormalities in a single CPAP-depleted cell could drive the cell toward different fates, including the production of two or three separate cells, the production of a single polyploid cell, or apoptosis (Figures 1A, f, and 7A). Thus, it is very likely that the abnormal centrosomes generated by CPAP depletion might create imbalanced MT forces derived from the centrosome-kinetochore or centrosome-chromosome during chromosome segregation in early mitosis, thereby triggering the production of various mitotic abnormalities. Furthermore, the multiple mitotic abnormalities in the CPAP-depleted cells appear to be generated by abnormal centrosomes containing zero, one, two, or three centrioles during cell division. We speculate that this may reflect a differential efficiency of CPAP knockdown. Our present findings may explain the variation of mitotic abnormalities observed by different laboratories working with siRNA-mediated CPAP knockdown cells (Cho et al., 2006; Kitagawa et al., 2011; Lee et al., 2014).

Careful examination by confocal immunofluorescence microscopy revealed that mitotic CPAP-depleted cells had loosely ordered and non-compact PCMs, which frequently were accompanied by significant dispersion of PCM proteins (e.g., pericentrin, CDK5RAP2, and γ-tubulin) at the mitotic spindle poles (Figure 2B). This is consistent with the previous report that *Drosophila* cells lacking functional dSAS-4 exhibited dispersion of the pericentriolar proteins D-PLP, centrosomin, and γ-tubulin (Lecland et al., 2013). In our study, some CPAP-depleted cells also showed severe mitotic abnormalities, including large fragments of the centrosome mass and multipolar spindles wherein one pole had one or two centrioles while the other poles lacked any centrioles (Figures S2B and S2C). Together, these findings strongly suggest that CPAP helps cohere PCM proteins and maintain spindle pole (centrosome) integrity during mitosis.

A possible model for CPAP-mediated PCM coherence and maintenance of spindle pole integrity is illustrated in Figure 7B. We propose that, during early mitosis, CPAP Ser467 is phosphorylated by Aurora-A kinase at the mitotic spindle poles (Figures 3 and 4). This phosphorylated CPAP (CPAP-pSer467) has increased affinity for PCM proteins, including pericentrin and CDK5RAP2 (Figures 5A and 5B), but decreased affinity for MTs (Figure 6). This opposing effect may cohere PCM proteins to the mitotic spindle poles and promote MT dynamics at the poles during early mitosis. In contrast, loss of Aurora-A activity (via siAurora-A or MLN8237 treatment) may lead to the accumulation of unphosphorylated CPAP-Ser467 (which has a high affinity for MTs but a low affinity for PCM proteins) at the mitotic spindle

poles. Under this condition, the spindle forces erected by the MTs that tether the centrosome-kinetochore, centrosome-chromosome, centrosome-cortex, or inter-poles might tear apart intact centrosomes, particularly during the process of chromosome segregation in early mitosis. Consistent with this model, CPAP-S467A (which mimics unphosphorylated CPAP) shows high affinity for MTs but low affinity for PCM proteins, while CPAP-S467D (which mimics phosphorylated CPAP) has the opposite effects (Figures 5 and 6). Moreover, only CPAP-S467D could rescue the PCM dispersion phenotype (Figures 5C, e, 5D, e, S7C, and S7D). Overall, we propose that Aurora-A-phosphorylated CPAP might keep centrosomal architecture rigid enough to endure the forces generated by MTs during chromosome separation and prevent the PCM from fragmenting/dissociating from the mitotic spindle poles.

The depletion of various proteins known to regulate spindle pole integrity (e.g., CLASP, Kizuna, ASAP, Cep72, and Cep90) reportedly induce similar phenotypes during mitosis, including PCM dispersion and multipolar spindles with fragmented PCMs (Logarinho et al., 2012; Oshimori et al., 2006, 2009; Venoux et al., 2008; Eot-Houllier et al., 2010; Kim and Rhee 2011). For example, the depletion of Kizuna or Cep72 (two important regulators of centrosome integrity) significantly increased the proportion of fragmented spindle poles; moreover, Cep72 depletion dramatically decreased the centrosome localization of Kizuna, suggesting that Cep72 is required for the centrosome targeting of Kizuna (Oshimori et al., 2006, 2009). Intriguingly, we found that CPAP-S467A had a higher affinity than CPAP-WT or CPAP-S467D for Cep72 (E.-J.C., unpublished data). Future work is warranted to elucidate whether Aurora-A-phosphorylated CPAP is functionally linked to the ability of Cep72, Kizuna, or other regulators to maintain spindle pole integrity.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Recombinant Proteins

The CPAP-S467A, CPAP-S467D, Aurora-A-T288D, and Aurora-A-K162M mutants were generated by site-directed mutagenesis using a QuikChange kit (Stratagene), according to the manufacturer's protocol. The sequences of all plasmids were confirmed. The cDNA clones and GST-recombinant proteins were produced as previously described (Hung et al., 2004; Hsu et al., 2008; Tang et al., 2009). For further experimental details, see the Supplemental Experimental Procedures.

Cell Culture, Transfection, and siRNA Experiments

U2OS and HEK293T cells were transiently transfected with various cDNA constructs using Lipofectamine 2000 (Invitrogen). The U2OS-T-REx-derived doxycycline-inducible CPAP-myc cell line was as described previously (Tang et al., 2009). The utilized CPAP-targeting siRNA was as described previously (Tang et al., 2009). For further experimental details, see the Supplemental Experimental Procedures.

Antibodies and Immunofluorescence Confocal Microscopy

Rabbit polyclonal antibodies against phosphor-Ser467-CPAP were raised against the peptide [461]KCSNRKpSLSPSGLK[474] (GeneTex International). The other antibodies were as described previously (Tang et al., 2009) or purchased from commercial companies. For fluorescence confocal microscopy, the samples were examined on a confocal system (LSM 700, Carl Zeiss). Images were acquired with the ZEN software (Carl Zeiss). For further details, see the Supplemental Experimental Procedures.

Live-Cell Imaging

HeLa-T-REx-derived mCherry-α-tubulin-expressing cells were transfected with either siControl or siCPAP for 60 hr. Time-lapse movies were taken with an LSM780 (Carl Zeiss) confocal system with incubation at 37°C under 5% CO_2.

In Vivo and In Vitro MT Co-sedimentation Assays

For in vivo MT co-sedimentation assays, HEK293T cells were transfected with plasmids encoding GFP-CPAP-WT, -S467A, or -S467D. For in vitro MT co-sedimentation assays, purified bovine brain α/β-tubulin was incubated with GST-A5N-WT, GST-A5N-S467A, or GST-A5N-S467D recombinant proteins. A detailed description of the experiments is given in the Supplemental Experimental Procedures.

Statistical Analyses

To determine the significance among the two or three experimental conditions, an unpaired two-tailed Student's t test or one-way ANOVA with Tukey's multiple comparison test (GraphPad Prism 5 software) was used as indicated in the figure legends (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ were considered statistically significant).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.085.

AUTHOR CONTRIBUTIONS

E.J.C., a PhD student at the Graduate Institute of Life Sciences at the National Defense Medical Center, performed most of the experiments, designed the study, and wrote the manuscript. L.Y.H., C.J.C.T., W.B.H., H.Y.W., and P.C.L. conducted the experiments. T.K.T. conceived the project, designed the experiments, and wrote the manuscript.

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