A Specific Form of Phospho Protein Phosphatase 2 Regulates Anaphase-promoting Complex/Cyclosome Association with Spindle Poles

Jorge Z. Torres,*† Kenneth H. Ban,*‡ and Peter K. Jackson*†

*Department of Pathology and ‡Program in Cancer Biology, Stanford University School of Medicine, Stanford, CA 94305; and †Department of Cell Regulation, Genentech Inc., South San Francisco, CA 94080

In early mitosis, the END (Emi1/NuMA/Dynein-dynactin) network anchors the anaphase-promoting complex/cyclosome (APC/C) to the mitotic spindle and poles. Spindle anchoring restricts APC/C activity, thereby limiting the destruction of spindle-associated cyclin B and ensuring maintenance of spindle integrity. Emi1 binds directly to hypophosphorylated APC/C, linking the APC/C to the spindle via NuMA. However, whether the phosphorylation state of the APC/C is important for its association with the spindle and what kinases and phosphatases are necessary for regulating this event remain unknown. Here, we describe the regulation of APC/C-mitotic spindle pole association by phosphorylation. We find that only hypophosphorylated APC/C associates with microtubule asters, suggesting that phosphatases are important. Indeed, a specific form of PPP2 (CA/R1A/R2B) binds APC/C, and PPP2 activity is necessary for Cdc27 dephosphorylation. Screening by RNA interference, we find that inactivation of CA, R1A, or R2B leads to delocalization of APC/C from spindle poles, early mitotic spindle defects, a failure to congress chromosomes, and decreased levels of cyclin B on the spindle. Consistently, inhibition of cyclin B/Cdk1 activity increased APC/C binding to microtubules. Thus, cyclin B/Cdk1 and PPP2 regulate the dynamic association of APC/C with spindle poles in early mitosis, a step necessary for proper spindle formation.

INTRODUCTION

The family of okadaic acid (OA)–sensitive phosphatases has been proposed to have roles in organizing the cellular microtubule network and possibly the mitotic spindle (Tournebize et al., 1997). This family consists of phospho protein phosphatase 1–7 (PPP1–PPP7; Fernandez et al., 2002). PPP2 is a serine/threonine phosphatase that functions as a trimeric protein complex consisting of catalytic (C), structural (A), and regulatory subunits (B). The human genome encodes at least 12 B-subunits, which are divided into four gene families (B, B′, B″, and B‴) and many have multiple splice variants (Shi, 2009; Virshup and Shenolikar, 2009). Each B-subunit can assemble with C and A subunits to form over 100 different PPP2 holoenzymes (Janssens and Goris, 2001; Virshup and Shenolikar, 2009). The B-subunits function as specificity factors for substrate recognition and holoenzyme localization (Virshup and Shenolikar, 2009). In addition, PPP2 holoenzyme formation, activity, and subcellular localization can be regulated via methylation and phosphorylation (Janssens et al., 2008). Such diversity allows specific PPP2 complexes to have diverse roles in signal transduction, apoptosis, and cell division (Janssens and Goris, 2001).

Early in vitro experiments suggested that PPP2 was the major phosphatase activity that dephosphorylated Cdk1 substrates in vivo (Ferrigno et al., 1993). PPP2 activity is cell cycle regulated: highest in interphase and lowest in mitosis. However, nearly all of the PPP2 activity in mitosis is associated with the mitotic spindle (Sontag et al., 1995). Coincidently, some microtubule-stabilizing proteins rely on PPP2 phosphatase activity for microtubule binding (Sontag et al., 1996; Tournebize et al., 1997; Gong et al., 2000). PPP2 also counteracts PLK1-mediated phosphorylation and chromosomal removal of Shugoshin (Sgo1), a protein that protects centromeric cohesion from being inappropriately phosphorylated and removed (Tang et al., 2006). Depletion of the PPP2 structural subunit R1A, leads to an abnormal mitosis that includes chromosome segregation defects in humans and in flies (Tang et al., 2006; Chen et al., 2007). Additionally, immunofluorescence and biochemical purifications indicate that PPP2 associates with centrosomes and the mitotic spindle (Sontag et al., 1995; Andersen et al., 2003; Arachchige Don et al., 2006). Thus, PPP2 is likely to have a role in regulating mitotic spindle dynamics.

Previously, we described an essential APC/C regulatory pathway independent of the spindle checkpoint called the END (Emi1/NuMA/dynein-dynactin) network. This network inhibits the Cdh1 form of APC/C on mitotic spindle poles (Ban et al., 2007). The network provides a critical self-reinforcing mechanism that sustains cyclin B/Cdk1 activity at the poles to maintain the prometaphase spindle. We observed that Emi1 bridges the interaction between the APC/C and the END network by specifically associating with the c-terminal microtubule-binding domain of NuMA. In turn, Emi1 also binds preferentially to hypophosphorylated inactive Cdc27 (APC/C subunit). Genetic evidence suggests that APC/C and its targets, Securin and cyclin B, are regulated by phosphatases, but no specific mechanism has been described (Deak et al., 2003; Gil-Bernabe et al., 2006).

Here we test the hypothesis that an equilibrium of kinase-phosphatase activities regulates the phosphorylation state of

the APC/C and its association with spindle poles. Through RNA interference (RNAi) screening, in vitro microtubule-binding assays, protein phosphorylation studies, and immunofluorescence colocalization experiments we implicate PPP2 in regulating the phosphorylation state of Cdc27 and its localization to spindle poles. More importantly we identify the specific PPP2 complex composed of the catalytic CA, structural R1A, and regulatory R2B subunits that are necessary for these events in early spindle assembly.

MATERIALS AND METHODS

Cell Culture

Adherent HeLa cells were grown in logarithmic phase in F12:DMEM 50:50 medium (Invitrogen, Carlsbad, CA) with 5% CO_2 at 37°C. To obtain synchronized HeLa cells in mitosis, cycling cells were treated with 300 nM nocodazole (Sigma-Aldrich, St. Louis, MO) for 18 h. For in vivo Cdk inhibition, cells were incubated with 2.5 μM roscovitine (Rosco; Sigma-Aldrich) for 1 h before cell harvesting. For in vivo phosphatase inhibition, cells were incubated with 175 nM OA (Sigma-Aldrich) for 30 min before cell harvesting. For phosphatase gene knockdowns, cells were transfected with Dharmacon (Lafayette, CO) ON-TARGET*plus* SMART*pool* small interfering RNA (siRNA; NonTargeting cat. no. D-001810-10, PPP1CA cat. no. L-008927-00, PPP2CA cat. no. L-003598-00, PPP2R1A cat. no. L-010259-00, PPP2R2A cat. no. L-004824-00, PPP2R2B cat. no. L-003022-00, PPP2R2C cat. no. L-019167-00, PPP2R2D cat. no. L-0322298-00, PPP2R3A cat. no. L-017376−00, PPP2R3B cat. no. L-019459-00, PPP2R4 cat. no. L-005214-00, PPP2R5A cat. no. L-009352-00, PPP2R5B cat. no. L-009366-00, PPP2R5C cat. no. L-009433-00, PPP2R5D cat. no. L-009799-00, PPP2R5E cat. no. L-008531-00, PPP3CA cat. no. L-008300-00, PPP4C cat. no. L-008486-00, PPP5C cat. no. L-009259-00, PPP6C cat. no. L-009935-00) at 50 nM using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) for 48 h before harvesting or fixation.

Mitotic Microtubule Copelleting Assay

Mitotic microtubule copelleting assays were performed as previously described (Mack and Compton, 2001). Mitotic HeLa cells were harvested by mitotic shake-off, washed in PBS plus 20 μg/ml cytochalasin B (Sigma-Aldrich) twice, washed with KHMD (78 mM KCl, 50 mM HEPES, pH 7.0, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 20 μg/ml cytochalasin B) plus Halt phosphatase inhibitor (Thermo Scientific, Waltham, MA) once and resuspended in KHMD plus protease inhibitors leupeptin/pepstatin/chymostatin, 1 μg/ml, plus phosphatase inhibitors (as indicated). Cells were Dounce-homogenized, and the extract was cleared by ultracentrifugation at 38,000 rpm for 15 min. All steps were carried out at 4°C unless otherwise noted. Cleared lysates were supplemented with 5 μg/ml latrunculin B (Sigma-Aldrich) and 2.5 mM ATP. Microtubule polymerization reactions were carried out in the presence of control vehicle DMSO or 10 μM taxol (Sigma-Aldrich) at 33°C for 30 min. Polymerization reactions were layered onto a 50% wt/vol sucrose/KHMD cushion supplemented with 10 μM taxol for reactions with taxol-stabilized microtubules. Layered reactions were centrifuged for 2 h at 39,000 rpm in a TLS-55 (Beckman Instruments, Brea, CA) swinging bucket rotor. Samples from the supernatant were placed in an equal volume of 2× Laemmli sample buffer. The microtubule copelleting fractions were washed twice with KHMD buffer and resuspended in 1× Laemmli sample buffer. Supernatant (S) and pellet (P) samples were boiled for 5 min at 90°C, run on an 8% Tris-glycine gel, transferred onto Immobilon-P membrane (Millipore, Billerica, MA), and probed with indicated antibodies.

Mitotic Extract Treatments

For in vitro Cdk inhibition, extracts were incubated in 10 μM Rosco. For in vitro PPP2 phosphatase inhibition, extracts were incubated with 10 nM OA. For in vitro APC/C phosphorylation, cyclin B/Cdk1 (Promega, Madison, WI) was used as described by the manufacturer. For in vitro APC/C dephosphorylation, lambda phosphatase (NEB, Ipswich, MA) was used according to the manufacturer's instructions.

Immunofluorescence

HeLa cells were transfected with control or indicated siRNA (ON-TARGET-*plus* SMART*pool* siRNA, Dharmacon) for 48 h, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100/PBS, and stained with 0.5 μg/ml Hoechst 33342, rat anti-α-tubulin (Serotec, Oxford, United Kingdom) and either rabbit anti-Cdc27, Eg5, or cyclin B or mouse anti-centrin. For acute OA treatment, cells were treated with 175 nM OA for 13 min before fixation and staining. Slides were mounted with ProLong Gold anti-fade reagent (Invitrogen), and projection images (10-μm stacks captured every 0.5 μm) were captured with a Zeiss Axio Imager.Z1 microscope (Thornwood, NY) equipped with a CoolSNAP HQ camera (Photometrics, Tucson, AZ) and operated with SlideBook 4.2 (Intelligent Imaging, Denver, CO) at 63× (NA

1.4) at room temperature. One hundred cells were analyzed to determine the percentage of cells with Cdc27 spindle pole localization in control, indicated siRNA, and acute OA-treated cells. A 2 × 2-μm square was drawn around each of 20 spindle poles from control, indicated siRNA, or acute OA-treated cells, and the mean fluorescence intensity of Cdc27 or cyclin B spindle pole staining was plotted as arbitrary units (AU). Additionally, intensity measurements were taken along an axis intersecting the two spindle poles and the fluorescence intensity was graphed as arbitrary units (AU). Image processing was performed using Adobe Photoshop CS2 (version 9.0.2; San Jose, CA).

Antibodies

The following antibodies were used: mouse anti-Cdc27 (Western blot), Eg5 (BD Transduction Laboratories, Lexington, KY), mouse anti-Cdh1 (Neomarkers, Fremont, CA), rabbit anti-Cdc20, cyclin D, cyclin B (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-PPP1CA, PPP2CA, PPP2R1A, and PPP3CA (Upstate), rabbit anti-PPP2R2B (Bethyl Labs, Montgomery, TX), rabbit anti-PPP4C (Novus Biologicals, Littleton, CO), mouse anti-PPP5C (BD Transduction Laboratories), rabbit anti-PPP6C (Chemicon, Temecula, CA), rat anti-α-tubulin (Serotec), goat anti-GAPDH (Novus Biologicals), rabbit anti-pericentrin (Abnova), and rabbit anti-Emi1 (Zymed Laboratories, South San Francisco, CA). Rabbit anti-Apc8 was a gift from H. Yu (University of Texas Southwestern Medical Center, Dallas, TX). Rabbit anti-Cdc27 (immunofluorescence) was a gift from J. M. Peters (Research Institute of Molecular Pathology, Vienna, Austria). Mouse anti-centrin was a gift from J. L. Salisbury (Case Western Reserve University, Cleveland, OH). FITC and Cy3-conjugated secondary antibodies (AffiniPure) were from Jackson ImmunoResearch (West Grove, PA).

RESULTS

Identification of PPP2 Subunits Necessary for Proper Spindle Assembly and Chromosome Congression

In mitosis, the END network preferentially associates with the hypophosphorylated inactive form of the APC/C subunit Cdc27 and anchors the APC/C to the spindle and spindle poles. Disruption of the END network leads to an elongated spindle with disorganized spindle poles and defects in chromosome congression (Ban *et al.*, 2007). We considered PPP2 as a candidate for dephosphorylating the APC/C and regulating its association with the END network at spindle poles, based on its localization, purification with mitotic spindles, and activity toward Cdk1 substrates (Ferrigno *et al.*, 1993; Sontag *et al.*, 1995; Andersen *et al.*, 2003). To test this hypothesis, we used siRNAs to silence the expression of all known PPP2 subunits one by one. We then asked which depletions mimicked the phenotype observed upon perturbation of the END network (Table 1 and Figure 1A). Cells were transfected with siRNA for 48 h before fixation

Table 1. Mitotic phenotype of HeLa cells treated with siRNA directed against PPP2 subunits

|	Defective		
siRNA	Spindle	Chromosome	Centrosome
Control	−	−	−
CA	+	+	+/−
R1A	+	+	+/−
R2A	−	−	−
R2B	+	+	−
R2C	−	−	−
R2D	−	−	−
R3A	−	−	−
R3B	−	−	−
R4	−	−	−
R5A	+	+	+
R5B	−	−	−
R5C	+	+	+
R5D	−	−	−
R5E	−	−	−

+, penetrant mitotic phenotype.
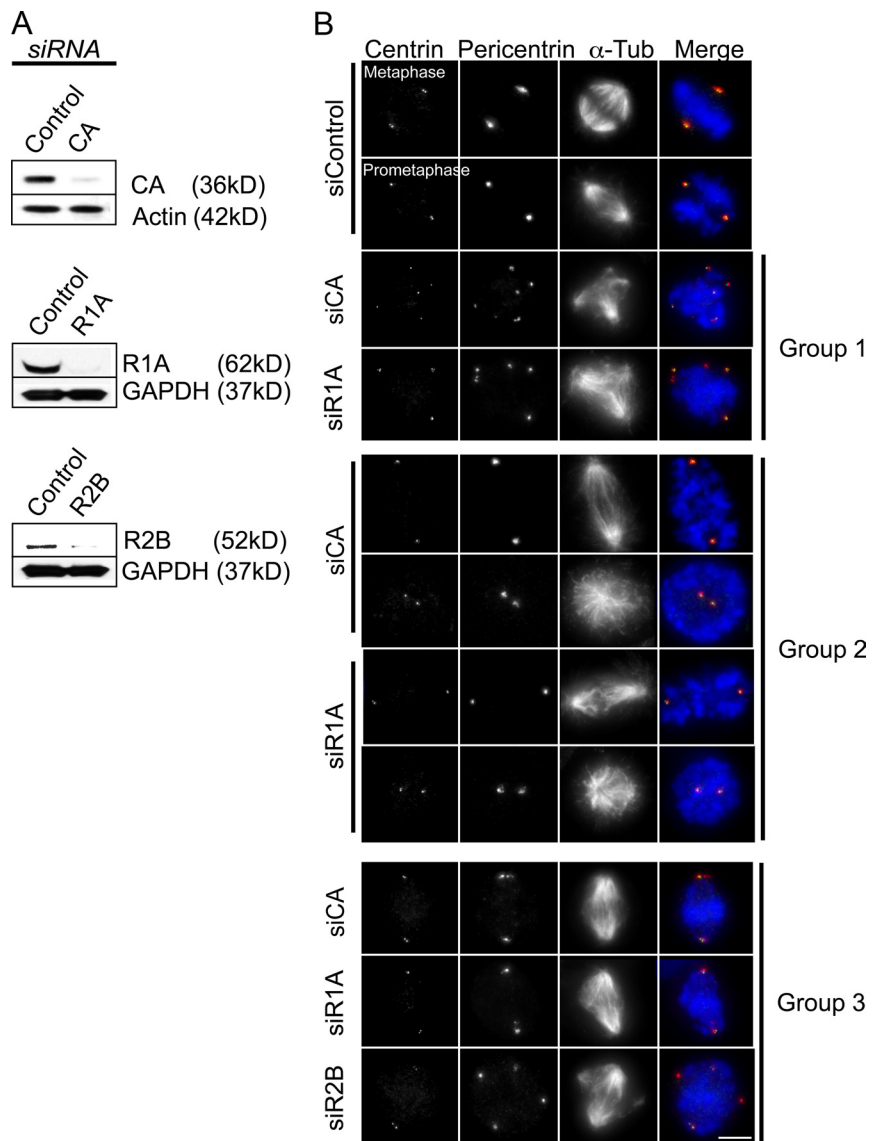
Figure 1. Depletion of the PPP2 subunits CA, R1A, or R2B leads to defective spindle formation and chromosome congression. (A) HeLa cells were transfected with siRNA targeting CA, R1A, or R2B for 48 h. The extent of gene knockdown was assessed by immunoblotting with antibodies directed against CA, R1A, R2B, and actin or GAPDH as loading controls. (B) Depletion of CA, R1A, or R2B leads to centrosome, mitotic spindle assembly, and chromosome congression defects. RNAi-treated cells in A were fixed and stained with Hoechst 33342, anti-α-tubulin, anti-pericentrin, and anti-centrin antibodies to visualize DNA, the mitotic spindle, the pericentriolar material, and the centrioles, respectively. Three groups of cell phenotypes were observed upon depletion of PPP2 subunits and are designated as groups 1 through 3. Group 1, has centrosome amplification and chromosome congression defects along with multipolar spindle formation. Group 2, contains chromosome cohesion defects and defective spindles. Group 3, exhibits defective spindle formation, spindle pole fragmentation, and a failure to congress chromosomes properly. Bar, 5 μm.

and staining for DNA, the mitotic spindle, the pericentriolar material, and centrosomes with Hoechst 33342, anti-α-tubulin, anti-pericentrin, and anti-centrin antibodies (Table 1). Three groups of phenotypes were observed upon depletion of PPP2 subunits: group 1: centrosome overduplication, spindle assembly defects, and chromosome congression defects (Table 1, Figure 1B, and Figure S1); group 2: spindle assembly and chromosome cohesion defects (Table 1, Figure 1B, and Figure S1); and group 3: spindle assembly and chromosome congression defects, but no centrosomal amplification (Table 1 and Figure 1B). The first and second groups of phenotypes were observed upon depletion of the PPP2 subunits CA, R1A, R5A, or R5C. The third phenotypic group was observed upon depletion of R2B, CA, or R1A. Because this phenotype was similar to mitotic abnormalities previously observed upon disruption of the END network, subunits R2B, CA, and R1A were chosen for more detailed characterization.

The PPP2 Subunits CA, R1A, and R2B Are Required for Cdc27 Spindle Pole Localization

Most APC/C subunits exist in a hypophosphorylated state during G1/S and several subunits are phosphorylated in

mitosis by the activities of cyclin B/Cdk1 and Plk1 (Kraft *et al.*, 2003; Pines, 2006). Previous immunofluorescence data indicated that phosphorylation might regulate APC/C subcellular localization, with phospho-APC/C (Cdc20 subset) associating with centrosomes and hypophosphorylated APC/C (Cdh1 subset) associating with the spindle, spindle poles, kinetochores, and chromosome arms (Tugendreich *et al.*, 1995; Topper *et al.*, 2002; Kraft *et al.*, 2003). Thus, we hypothesized that the ability of the APC/C to associate with spindle poles was regulated through phosphorylation. To test this hypothesis, we used a mitotic microtubule aster copelleting assay (Mack and Compton, 2001) to monitor APC/C binding to the mitotic spindle. Mitotic HeLa cell extracts were prepared and treated with the microtubule-stabilizing drug, taxol, to induce microtubule polymerization. Polymerization of microtubule asters was monitored by fluorescence microscopy using anti-α-tubulin antibodies. Polymerized microtubules and their associated proteins were then sedimented through a sucrose cushion. Protein samples from the supernatant (S) and the pelleted microtubule-binding proteins (P) were probed with antibodies specific for Eg5, a kinesin necessary for proper mitotic spindle
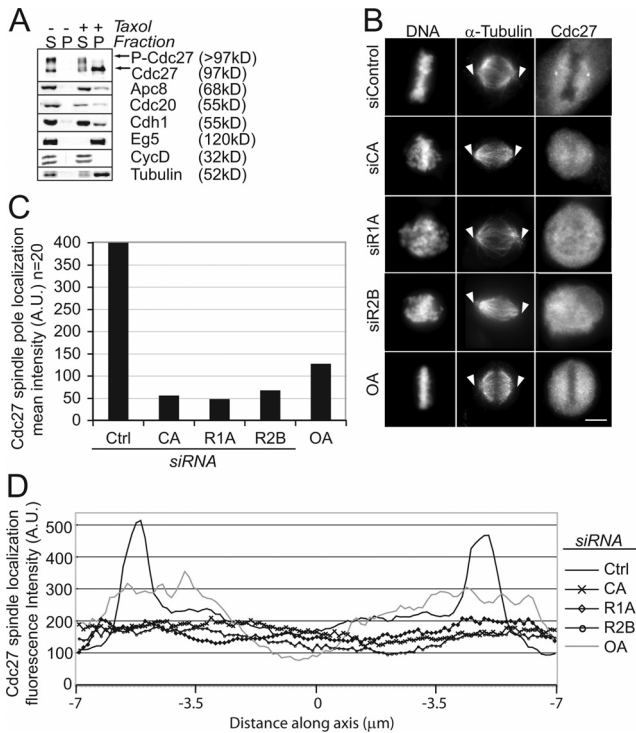
Figure 2. Association of Cdc27 with spindle poles requires the PPP2 subunits CA, R1A, and R2B. (A) Dephosphorylated APC/C preferentially associates with microtubule asters in vitro. Mitotic HeLa cell extracts were used for in vitro microtubule polymerization reactions in the presence or absence of taxol. Polymerized microtubules were subjected to centrifugation, and samples from the supernatant (S) and pelleted (P) microtubules were analyzed by immunoblot. Immunoblotting was used to detect the association of the APC/C subunits Cdc27, Apc8, Cdc20, and Cdh1, and positive (mitotic kinesin Eg5) and negative (CycD) controls with microtubules. The upper and lower arrows indicate phosphorylated and dephosphorylated forms of Cdc27, respectively. Tubulin polymerization was confirmed by staining the membrane with Coomassie blue. (B) Depletion of CA, R1A, or R2B by siRNA leads to delocalization of Cdc27 from spindle poles. RNAi-treated cells (as in Figure 1) or cells treated acutely with 175 nM OA for 13 min were fixed and stained with Hoechst 33342 and anti-α-tubulin and anti-Cdc27 antibodies to visualize DNA, the mitotic spindle, and Cdc27, respectively. Bar, 5 μm. (C) Quantitation of mean intensity of Cdc27 at spindle poles. A 2-μm square was drawn around each of 20 spindle poles from control, CA, R1A, or R2B siRNA-treated cells or cells treated acutely with OA, and the mean fluorescence intensity of Cdc27 spindle pole staining was quantified and presented as arbitrary units (AU). (D) Spindle associated Cdc27 is reduced in mitotic cells depleted of CA, R1A, or R2B or treated acutely with OA. Line intensity measurements taken along axis intersecting the two poles (marked by arrows in A) were quantified and graphed. X-axis, distance in micrometers from the center of the two poles. Y-axis, fluorescence intensities along the axis in arbitrary units (AU).

assembly, and cyclin D, a protein that does not bind the mitotic spindle (Sawin *et al.*, 1992; Figure 2A). Eg5 kinesin associated with the taxol-stabilized microtubule pellet, whereas cyclin D remained in the supernatant (Figure 2A). Neither protein pelleted in the absence of taxol, indicating minimal nonspecific pelleting (Figure 2A).

In the absence of taxol, APC/C subunits remained in the supernatant (Figure 2A). On taxol addition, APC/C subunits were localized to both supernatant and microtubule pellets (Figure 2A). However, only the hypophosphorylated form of Cdc27 associated with microtubules (Figure 2A, lower

arrow). Consistently, immunofluorescence using anti-Cdc27 antibodies stains the spindle poles, the mitotic spindle, and the cytokinetic bridge (Kraft *et al.*, 2003; Figure S2A).

In addition to mitotic spindle defects, perturbation of the END network also leads to the displacement of APC/C from the minus ends of microtubules (spindle poles; Ban *et al.*, 2007). Because depletion of the PPP2 subunits CA, R1A, or R2B showed similar mitotic defects, we asked if these subunits were also necessary for localizing APC/C to spindle poles. For CA and R1A depletions, only group 3 (see Figure 1) cells were analyzed to focus on the class of events correlating with R2B depletion. RNAi knockdown of CA, R1A, or R2B each resulted in a reduced percentage of cells with Cdc27 spindle pole localization by immunofluorescence (Figure 2B and Figure S3). These results were confirmed by measuring the mean fluorescence intensity of Cdc27 spindle pole staining in control, CA, R1A, or R2B siRNA-treated cells (n = 20 each; Figure 2C). Similarly, a line scan of fluorescence intensity taken along the axis intersecting the two poles (marked by arrows in Figure 2B) indicated that Cdc27 levels were decreased on the spindle poles (Figure 2D). As an independent test, we sought to inhibit PPP2 activity acutely in vivo and determine if Cdc27 was delocalized from spindle poles. Okadaic acid is a known inhibitor of the PPP2-like family of phosphatases and acute (13 min) inhibition of PPP2 activity in vivo with OA also led to rapid displacement of Cdc27 from spindle poles (Figure 2, B–D, and Figure S3). In contrast, localization of the Eg5 kinesin was largely unaffected in CA RNAi or OA-treated cells (Figure S4), indicating that knockdown of PPP2 function did not nonspecifically disrupt localization of other spindle pole associated proteins. Additionally, depletion of other PPP2 subunits that showed no mitotic phenotype had no effect on Cdc27 localization (data not shown). These results indicate that the PPP2 holoenzyme composed of CA/R1A/R2B plays a role in regulating the APC/C association with spindle poles.

Cyclin B/Cdk1 and PPP2 Activities Regulate the Association of APC/C with Mitotic Aster Microtubules

The displacement of Cdc27 from spindle poles upon acute OA treatment indicated that this association was likely dynamic, which suggests it could be counteracted by a mitotic kinase. Cyclin B/Cdk1 is the predominant mitotic kinase that regulates APC/C activity and phosphorylates several APC/C subunits in vitro, including Cdc27 (Kraft *et al.*, 2003). Thus, we analyzed whether cyclin B/Cdk1 could directly regulate APC/C-microtubule association. First, we inactivated Cdks with the small molecule inhibitor Rosco. Mitotic HeLa cells were treated in vivo with Rosco before extract preparation and in vitro microtubule aster polymerization reactions were performed in the continued presence of Rosco. Inhibition of Cdk activity led to an accumulation of hypophosphorylated Cdc27 with an increased binding to microtubules (Figure 3A). This is in agreement with previous in vitro results showing that cyclin B/Cdk1 phosphorylates Cdc27 in mitosis and that phosphorylated Cdc27 localizes to centrosomes and not the mitotic microtubule spindle (Kraft *et al.*, 2003). Another APC/C subunit, Apc8 and the substrate adaptor proteins Cdc20 and Cdh1 also increased in microtubule binding upon Rosco treatment (Figure 3A).

To substantiate our in vivo results implicating PPP2 in regulating APC/C-microtubule association, we first inhibited PPP2 with a low concentration of OA that selectively inhibits PPP2. This treatment led to an accumulation of hyperphosphorylated Cdc27 and a failure of phospho-
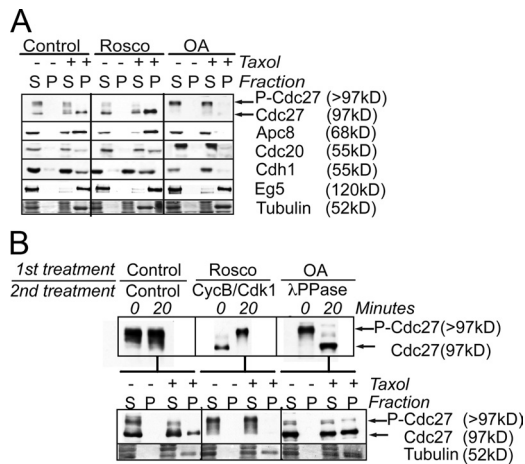
Figure 3. Dynamic association of the APC/C with mitotic microtubule asters is regulated by a kinase-phosphatase equilibrium. (A) Inhibition of Cdk activity and an OA-sensitive phosphatase in vivo perturbs the association of APC/C with microtubules. Mitotic HeLa cells were treated with control vehicle, the Cdk inhibitor roscovitine (Rosco; 1 h), or the phosphatase inhibitor okadaic acid (OA; 30 min) before extract preparation. Mitotic microtubule copelleting reactions (as in Figure 2) were performed in the presence of control vehicle, Rosco, or OA. Supernatant and pellet fractions were immunoblotted with antibodies against APC/C subunits/coactivators (Cdc27, Apc8, Cdc20, and Cdh1) or Eg5 to detect microtubule association. Cdc27 phosphorylation status, association with microtubules, and tubulin polymerization was monitored, as described in Figure 2B. For A and B, arrows indicate phosphorylated and dephosphorylated forms of Cdc27. (B) Phosphorylation and dephosphorylation of APC/C in vitro perturbs APC/C microtubule association. Mitotic HeLa cells were treated with control vehicle, Rosco, or OA as described in A before extract preparation. Extracts from Rosco-treated cells were incubated with cyclin B/Cdk1 for 20 min at 30°C. Conversely, extracts from OA-treated cells were incubated with lambda phosphatase (λPPase) for 20 min at 30°C. All extracts were subjected to mitotic microtubule copelleting reactions and the status of Cdc27 phosphorylation, its association with microtubules, and tubulin polymerization was detected as described in Figure 2B.

Cdc27 to associate with microtubules (Figure 3A). Similarly, other APC/C subunits (Apc8, Cdc20, and Cdh1) failed to associate with microtubules upon OA treatment (Figure 3A). Interestingly, OA also strongly enhanced Cdc20 phosphorylation (Figure 3A). These results indicate that the mitotic kinase cyclin B/Cdk1 and an OA-sensitive phosphatase, likely PPP2, regulate the association of APC/C with mitotic microtubule asters.

To further test whether APC/C-microtubule association is regulated by the activities of cyclin B/Cdk1 and PPP2, we asked whether addition of purified cyclin B/Cdk1 or lambda phosphatase to mitotic extracts would allow APC/C-microtubule binding. In extracts prepared from Rosco-treated cells, a hypophosphorylated form of APC/C associated with microtubules. However, when cyclin B/Cdk1 was added in vitro before performing microtubule aster polymerization reactions, Cdc27 became hyperphosphorylated rapidly (20 min) and phospho-Cdc27 was unable to associate with microtubules (Figure 3B, bottom). Conversely, extracts prepared from OA-treated cells showed that phospho-Cdc27 was not competent to interact with microtubules, but dephosphorylation of Cdc27 with lambda phosphatase (λPPase) strongly increased its ability to associate with microtubules (Figure 3B). These results indicate that a kinase-phosphatase equilibrium regulates the APC/C-microtubule association and that perturbation of this equilibrium leads to
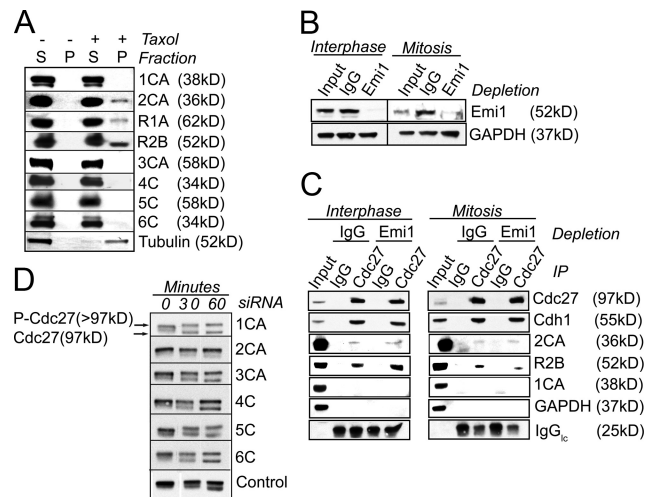
Figure 4. PPP2 associates with mitotic microtubules, binds Cdc27, and is required for Cdc27 dephosphorylation. (A) PPP2 associates with microtubules. Mitotic HeLa cell extracts were used for in vitro microtubule polymerization reactions in the presence or absence of taxol. Polymerized microtubules were subjected to centrifugation and samples from the supernatant (S) and pelleted (P) microtubules were analyzed by Western blot. Antibodies directed against the catalytic subunit of OA-sensitive phosphatases (PPP1–PPP6) and the PPP2 regulatory subunits R1A and R2B were used to detect their association with microtubules. Tubulin polymerization was detected using rat anti-α-tubulin antibody. (B) Emi1 is efficiently immunodepleted from HeLa cell extracts. G1/S (Interphase) or G2/M (Mitosis) HeLa cell extracts, 500 μg, were incubated with 10 μg of control IgG (Ig) or anti-Emi1 antibodies. Depleted extracts were probed with anti-Emi1 and GAPDH antibodies. (C) Cdc27 associates with PPP2. G1/S and G2/M extracts depleted of Emi1, from B, were incubated with 10 μg of control IgG or anti-Cdc27 antibodies. Immunoprecipitations were probed with anti-Cdc27, -Cdh1, -PPP1CA, -PPP2CA, -PPP2R2B, and -GAPDH antibodies. IgG_lc indicates the IgG light chain cross-reacting band. (D) PPP2 knockdown inhibits Cdc27 dephosphorylation. Mitotic HeLa cell extracts were prepared from siRNA-treated cells targeting the catalytic subunit of each member of the family of OA-sensitive phosphatases. Extracts were incubated at 37°C and samples taken at 0-, 30-, and 60-min time points. The extent of Cdc27 dephosphorylation was monitored by immunoblot using anti-Cdc27 antibodies. The upper and lower arrows indicate phosphorylated and dephosphorylated forms of Cdc27, respectively.

an increase or decrease of APC/C association with microtubule asters.

PPP2 Associates with Mitotic Aster Microtubules and Is Required for Cdc27 Dephosphorylation

If PPP2 is important for dephosphorylating APC/C to allow its association with spindle poles, we predict that PPP2 (CA/R1A/R2B) would also localize to the spindle during mitosis. Therefore, we tested PPP2 and other OA-sensitive phosphatases for their ability to associate with microtubules. The PPP2 subunits CA, R1A, and R2B copelleted with microtubules and minor amounts of PPP1CA, PPP5C, and PPP6C could be seen at long exposures (Figure 4A). This is in agreement with published results indicating that the catalytic subunit of PPP2 (CA) associates with the mitotic spindle by microtubule aster pelleting assay and immunofluorescence (Sontag et al., 1995; Arachchige Don et al., 2006). By immunofluorescence, the PPP2 regulatory B subunit (R2B) localized to the spindle poles, the spindle, and the cytokinetic bridge (Figure S2B). These data indicate that PPP2

localizes to the spindle and spindle poles in mitosis and supports its role in dephosphorylating APC/C to allow its association with spindle poles.

Next, we asked whether PPP2 was able to interact with the APC/C and if this interaction was dependent on Emi1, given that Emi1 bridges the END network-APC/C interaction (Ban *et al.*, 2007). Interphase (thymidine arrested) and mitotic (nocodazole arrested) extracts were depleted of Emi1 with anti-Emi1 antibodies or mock depleted with preimmune IgG (Figure 4B). These extracts were used to test for PPP2 coimmunoprecipitation with anti-Cdc27 antibodies (Figure 4C). Consistent with our demonstration that PPP2 affects APC/C dephosphorylation, we found that the catalytic subunit (CA) and to a greater extent the regulatory subunit (R2B) of PPP2 associated with Cdc27 in interphase and mitotic extracts (Figure 4C). As a specificity control, another phosphatase, PPP1, did not interact with Cdc27 (Figure 4C). In both interphase and mitotic extracts, immunodepletion of Emi1 did not abolish the coimmunoprecipitation of PPP2 subunits with Cdc27, indicating that the PPP2-APC/C association was not dependent on Emi1. Indeed, Emi1 itself only associated weakly with the catalytic subunit of PPP2 (CA; Figure S5).

To confirm that PPP2 was the OA-sensitive phosphatase regulating APC/C phosphorylation and its association with microtubules, we knocked down, one by one, the catalytic subunit of each OA-sensitive phosphatase by siRNA and asked if phospho-Cdc27 was stabilized (Figure 4D and Fig-

ure S5B). Mitotic extracts, prepared from siRNA knockdown and control cultures, were incubated at 37°C for 0, 30, and 60 min to allow dephosphorylation, and the phosphorylation of Cdc27 was determined by immunoblot. Knockdown of the catalytic subunit of PPP2 (CA) led to a stabilization of phospho-Cdc27 in vitro, whereas knockdown of other family members had no effect (Figure 4D). This indicates that PPP2 is the OA-sensitive phosphatase responsible for regulating the APC/C-microtubule interaction.

PPP2 Activity Is Required to Maintain Proper Cyclin B Levels on the Spindle

Inhibition of PPP2 activity leads to accumulation of hyperphosphorylated APC/C. A possible consequence is that APC/C will be unable to interact with the END network at the minus ends of microtubules and will remain active near spindle poles leading to the premature destruction of cyclin B on the spindle in early mitosis. To test this, we analyzed the levels of spindle pole associated cyclin B in CA, R1A, and R2B siRNA-treated cells. Consistent with our model, knockdown of CA, R1A, or R2B showed decreased levels of spindle and spindle pole–associated cyclin B by immunofluorescence (Figure 5A). This was further confirmed by measuring the mean fluorescence intensity of cyclin B spindle pole staining in 20 spindle poles from control, CA, R1A, or R2B siRNA-treated cells (Figure 5B). Additionally, fluorescence intensity measurements taken along an axis intersecting the two poles (marked by arrows in Figure 5A) indicated that cyclin B levels were decreased along the spindle (Figure 5C). These data indicate that depletion of CA, R1A, or R2B arrests cells in prometaphase with reduced spindle associated cyclin B levels.

DISCUSSION

Early studies in *Xenopus* indicated that microtubule dynamics during mitosis were likely controlled by a balance
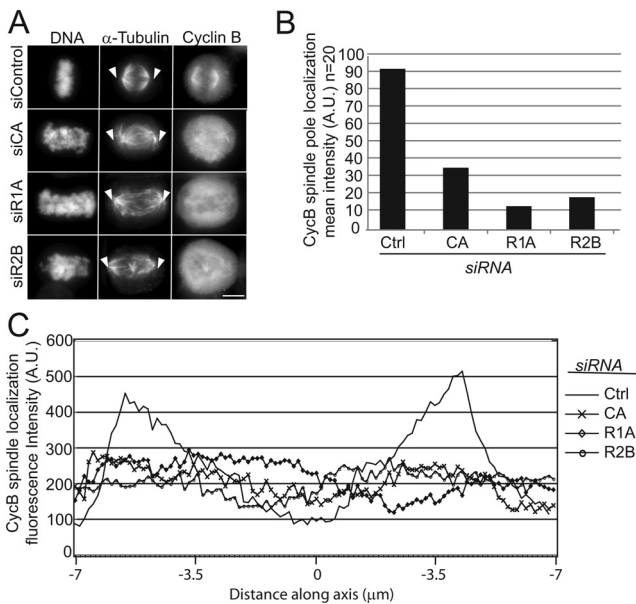
Figure 5. PPP2 activity is required to maintain proper cyclin B levels on the spindle. (A) Knockdown of PPP2 subunits, (CA, R1A, or R2B), reduces pole-associated cyclin B levels. HeLa cells were transfected with control, CA, R1A, or R2B siRNA for 48 h before fixation. Staining with Hoechst 33342, anti-α-tubulin, and anti-cyclin B visualized DNA, the mitotic spindle, and cyclin B localization, respectively. Bar, 5 μm. (B) Quantitation of mean fluorescence intensity of cyclin B at spindle poles as described in Figure 2C for control, CA, R1A, and R2B siRNA-treated cells. (C) Spindle associated cyclin B is reduced in mitotic cells depleted of CA, R1A, or R2B. Line intensity measurements taken along an axis intersecting the two poles (marked by arrows in A) were quantified and graphed. X-axis, distance, in micrometers, from the center of the two poles. Y-axis, fluorescence intensities along the axis in arbitrary units (AU).
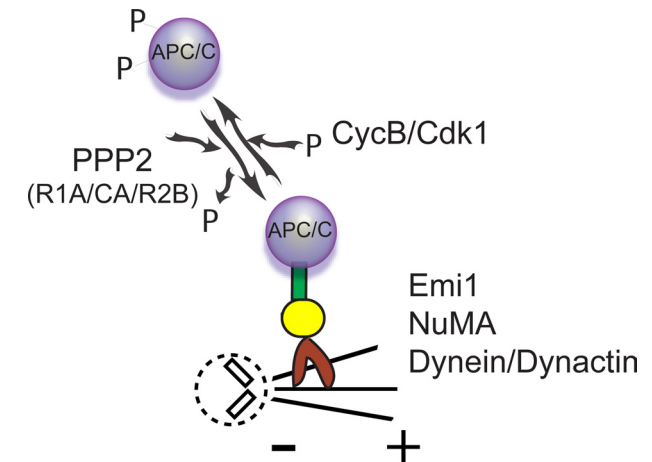
Figure 6. Model for APC/C association with mitotic spindle poles. Mitotic kinases and phosphatases control the dynamic association of the APC/C with mitotic spindle poles. The PPP2 (CA/R1A/R2B) phosphatase is necessary for APC/C dephosphorylation and regulates its association with the minus ends of microtubules (spindle poles), possibly via the END network. Local inhibition of APC/C activity at spindle poles in early mitosis allows for stabilization of cyclin B/Cdk1 activity near poles, an activity necessary for mitotic spindle formation. Phosphorylation of the APC/C by cyclin B/Cdk1 inhibits its association with spindle poles. Perturbation of this equilibrium alters APC/C localization and results in mitotic spindle defects and a failure to congress chromosomes.

between the cyclin B/cdc2 kinase and an OA-sensitive phosphatase, likely PPP2 (Belmont *et al.,* 1990; Verde *et al.,* 1990, 1992; Tournebize *et al.,* 1997). In this study, we demonstrate that a balance of kinase (cyclin B/Cdk1) and phosphatase (PPP2) activities regulate the phosphorylation state of the APC/C and early spindle formation. We describe a novel regulatory role for a specific PPP2 complex (CA/R1A/R2B) in dephosphorylating APC/C to regulate its association with spindle poles. Perturbation of this kinase-phosphatase equilibrium causes deficiencies in APC/C spindle pole localization, spindle formation, chromosome congression, and cyclin B stabilization on the spindle. To account for these deficiencies, we propose a model where as cells enter mitosis, cyclin B/Cdk1 activity phosphorylates the APC/C on several of its subunits. The APC/C near the poles is actively dephosphorylated by PPP2 (CA/R1A/R2B) activity. Hypophosphorylated APC/C is competent to interact with the END network at the spindle poles (Figure 6). Local inhibition of APC/C activity provides a self-reinforcing mechanism that sustains cyclin B/Cdk1 activity near the poles to maintain the prometaphase spindle.

The coprecipitation of CA, R1A, and R2B subunits with Cdc27 immunoprecipitations and the requirement of the PPP2 catalytic subunit for Cdc27 dephosphorylation indicate that dephosphorylation of APC/C by PPP2 is likely direct. However, it remains to be determined which of the cyclin B/Cdk1 mitotic phosphorylated APC/C subunits, in addition to Cdc27, are directly dephosphorylated by PPP2 and which are necessary for interaction with the END network. The displacement of Cdc27 from properly formed spindle poles with acute OA treatment suggests that spindle pole–associated PPP2 activity dynamically maintains a pool of hypophosphorylated APC/C at spindle poles. At the metaphase to anaphase transition, cyclin B destruction initiates from the spindle poles, and localization of the APC/C to spindle poles is likely necessary for cyclin B degradation (Huang and Raff, 1999).

A recent survey of protein phosphatases in *Drosophila* S2 cells revealed that depletion of the catalytic subunit, *mts*, and the structural subunit, *Pp2A-29B*, of *Drosophila* PPP2 had defects in mitotic spindle formation, chromosome cohesion, and spindles nucleated from a centrosomal mass (Chen *et al.,* 2007). In addition, previous results showed that *Drosophila* PPP2 mutant embryos exhibited cells with multiple centrosomes in interphase and mitosis (Snaith *et al.,* 1996). These results are in agreement with the three groups of phenotypes that we observed upon depletion of CA and R1A in human cells (Table 1 and Figure 1B). Depletion of the B' subunit, *wdb*, in *Drosophila* leads to centromere cohesion defects and the scattering of chromosomes during mitosis, a phenotype that is recapitulated by depleting the human B' subunits, R5A and R5C (Chen *et al.,* 2007; Table 1 and Figure S1). Finally, depletion of the *Drosophila* B subunit, *tws*, leads to increased lagging chromosomes during anaphase and an anaphase-like arrest (Deak *et al.,* 2003; Chen *et al.,* 2007). However, in human cells we observe that depletion of the B subunit, R2B, leads to defects in chromosome congression and spindle pole fragmentation (Table 1 and Figure 1B). Thus, it is likely that compared with human cells, *Drosophila* cells depleted of *tws* are able transition further into mitosis before arresting.

Interestingly, mutations in *tws*, reduced the ability of PPP2 to dephosphorylate Cdk1 substrates (Mayer-Jaekel *et al.,* 1994). Similarly, our results implicate the PPP2 holoenzyme, containing the B subunit R2B, in dephosphorylating Cdc27, a Cdk1 substrate (Kraft *et al.,* 2003). The interaction between Cdc27 and *tws*, has been explored in *Drosophila*,

where mutation in *makos* (Cdc27) arrests cells in mitosis with elevated levels of cyclin B on the spindle (Deak *et al.,* 2003). Conversely, only residual cyclin B levels were observed on the spindles of mutant *tws* cells (Deak *et al.,* 2003). This parallels our observations where depletion of R2B leads to a decrease in cyclin B localization to the spindle (Figure 5). Thus, it is likely that in *Drosophila* a PPP2 holoenzyme (containing a B subunit) has a role in stabilizing cyclin B levels at mitotic spindle poles, as we propose for human cells.

Misregulation of PPP2 has been implicated in the development of cancer (Wang *et al.,* 1998; Schonthal, 2001). Mutations in PPP2 are found in several cancers including those of lung, colon, and breast (Wang *et al.,* 1998; Schonthal, 2001). There is mounting evidence that PPP2 acts as a tumor suppressor and that suppression of PPP2 activity in immortalized cell lines leads to tumorigenic transformation (Rangarajan *et al.,* 2004; Janssens *et al.,* 2005; Mumby, 2007). It will be of interest to determine whether the defects in mitotic spindle formation caused by PPP2 inactivation are a trigger leading to tumor development.

ACKNOWLEDGMENTS

REFERENCES

Andersen, J. S., Wilkinson, C. J., Mayor, T., Mortensen, P., Nigg, E. A., and Mann, M. (2003). Proteomic characterization of the human centrosome by protein correlation profiling. Nature *426*, 570–574.

Arachchige Don, A. S., Dallapiazza, R. F., Bennin, D. A., Brake, T., Cowan, C. E., and Horne, M. C. (2006). Cyclin G2 is a centrosome-associated nucleo-cytoplasmic shuttling protein that influences microtubule stability and induces a p53-dependent cell cycle arrest. Exp. Cell Res. *312*, 4181–4204.

Ban, K. H., Torres, J. Z., Miller, J. J., Mikhailov, A., Nachury, M. V., Tung, J. J., Rieder, C. L., and Jackson, P. K. (2007). The END network couples spindle pole assembly to inhibition of the anaphase-promoting complex/cyclosome in early mitosis. Dev. Cell *13*, 29–42.

Belmont, L. D., Hyman, A. A., Sawin, K. E., and Mitchison, T. J. (1990). Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. Cell *62*, 579–589.

Chen, F., *et al.* (2007). Multiple protein phosphatases are required for mitosis in *Drosophila.* Curr. Biol. *17*, 293–303.

Deak, P., Donaldson, M., and Glover, D. M. (2003). Mutations in makos, a *Drosophila* gene encoding the Cdc27 subunit of the anaphase promoting complex, enhance centrosomal defects in polo and are suppressed by mutations in twins/aar, which encodes a regulatory subunit of PP2A. J. Cell Sci. *116*, 4147–4158.

Fernandez, J. J., Candenas, M. L., Souto, M. L., Trujillo, M. M., and Norte, M. (2002). Okadaic acid, useful tool for studying cellular processes. Curr. Med. Chem. *9*, 229–262.

Ferrigno, P., Langan, T. A., and Cohen, P. (1993). Protein phosphatase 2A1 is the major enzyme in vertebrate cell extracts that dephosphorylates several physiological substrates for cyclin-dependent protein kinases. Mol. Biol. Cell *4*, 669–677.

Gil-Bernabe, A. M., Romero, F., Limon-Mortes, M. C., and Tortolero, M. (2006). Protein phosphatase 2A stabilizes human securin, whose phosphorylated forms are degraded via the SCF ubiquitin ligase. Mol. Cell. Biol. *26*, 4017–4027.

Gong, C. X., Wegiel, J., Lidsky, T., Zuck, L., Avila, J., Wisniewski, H. M., Grundke-Iqbal, I., and Iqbal, K. (2000). Regulation of phosphorylation of neuronal microtubule-associated proteins MAP1b and MAP2 by protein phosphatase-2A and -2B in rat brain. Brain Res. *853*, 299–309.

Huang, J., and Raff, J. W. (1999). The disappearance of cyclin B at the end of mitosis is regulated spatially in *Drosophila* cells. EMBO J. *18*, 2184–2195.

Janssens, V., and Goris, J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem. J. *353*, 417–439.

Janssens, V., Goris, J., and Van Hoof, C. (2005). PP2A: the expected tumor suppressor. Curr. Opin. Genet. Dev. *15*, 34–41.

Janssens, V., Longin, S., and Goris, J. (2008). PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail). Trends Biochem. Sci. *33*, 113–121.

Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Hagting, A., Pines, J., and Peters, J. M. (2003). Mitotic regulation of the human anaphase-promoting complex by phosphorylation. EMBO J. *22*, 6598–6609.

Mack, G. J., and Compton, D. A. (2001). Analysis of mitotic microtubule-associated proteins using mass spectrometry identifies astrin, a spindle-associated protein. Proc. Natl. Acad. Sci. USA *98*, 14434–14439.

Mayer-Jaekel, R.E., Ohkura, H., Ferrigno, P., Andjelkovic, N., Shiomi, K., Uemura, T., Glover, D.M., and Hemmings, B.A. (1994). *Drosophila* mutants in the 55 kDa regulatory subunit of protein phosphatase 2A show strongly reduced ability to dephosphorylate substrates of p34cdc2. J. Cell Sci. *107*(Pt 9), 2609–2616.

Mumby, M. (2007). PP2A: unveiling a reluctant tumor suppressor. Cell *130*, 21–24.

Pines, J. (2006). Mitosis: a matter of getting rid of the right protein at the right time. Trends Cell Biol. *16*, 55–63.

Rangarajan, A., Hong, S. J., Gifford, A., and Weinberg, R. A. (2004). Species- and cell type-specific requirements for cellular transformation. Cancer Cell *6*, 171–183.

Sawin, K. E., LeGuellec, K., Philippe, M., and Mitchison, T. J. (1992). Mitotic spindle organization by a plus-end-directed microtubule motor. Nature *359*, 540–543.

Schonthal, A. H. (2001). Role of serine/threonine protein phosphatase 2A in cancer. Cancer Lett. *170*, 1–13.

Shi, Y. (2009). Serine/threonine phosphatases: mechanism through structure. Cell *139*, 468–484.

Snaith, H.A., Armstrong, C.G., Guo, Y., Kaiser, K., and Cohen, P.T. (1996). Deficiency of protein phosphatase 2A uncouples the nuclear and centrosome cycles and prevents attachment of microtubules to the kinetochore in *Drosophila* microtubule star (mts) embryos. J. Cell Sci. *109*(Pt 13), 3001–3012.

Sontag, E., Nunbhakdi-Craig, V., Bloom, G. S., and Mumby, M. C. (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J. Cell Biol. *128*, 1131–1144.

Sontag, E., Nunbhakdi-Craig, V., Lee, G., Bloom, G. S., and Mumby, M. C. (1996). Regulation of the phosphorylation state and microtubule-binding activity of Tau by protein phosphatase 2A. Neuron *17*, 1201–1207.

Tang, Z., Shu, H., Qi, W., Mahmood, N. A., Mumby, M. C., and Yu, H. (2006). PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation. Dev. Cell *10*, 575–585.

Topper, L. M., Campbell, M. S., Tugendreich, S., Daum, J. R., Burke, D. J., Hieter, P., and Gorbsky, G. J. (2002). The dephosphorylated form of the anaphase-promoting complex protein Cdc27/Apc3 concentrates on kineto-chores and chromosome arms in mitosis. Cell Cycle *1*, 282–292.

Tournebize, R., Andersen, S. S., Verde, F., Doree, M., Karsenti, E., and Hyman, A. A. (1997). Distinct roles of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis. EMBO J. *16*, 5537–5549.

Tugendreich, S., Tomkiel, J., Earnshaw, W., and Hieter, P. (1995). CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. Cell *81*, 261–268.

Verde, F., Dogterom, M., Stelzer, E., Karsenti, E., and Leibler, S. (1992). Control of microtubule dynamics and length by cyclin A- and cyclin B-dependent kinases in *Xenopus* egg extracts. J. Cell Biol. *118*, 1097–1108.

Verde, F., Labbe, J. C., Doree, M., and Karsenti, E. (1990). Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of *Xenopus* eggs. Nature *343*, 233–238.

Virshup, D. M., and Shenolikar, S. (2009). From promiscuity to precision: protein phosphatases get a makeover. Mol. Cell *33*, 537–545.

Wang, S. S., Esplin, E. D., Li, J. L., Huang, L., Gazdar, A., Minna, J., and Evans, G. A. (1998). Alterations of the PPP2R1B gene in human lung and colon cancer. Science *282*, 284–287.