The protein phosphatase 2A functions in the spindle position checkpoint by regulating the checkpoint kinase Kin4

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In budding yeast, a surveillance mechanism known as the spindle position checkpoint (SPOC) ensures accurate genome partitioning. In the event of spindle misposition, the checkpoint delays exit from mitosis by restraining the activity of the mitotic exit network (MEN). To date, the only component of the checkpoint to be identified is the protein kinase Kin4. Furthermore, how the kinase is regulated by spindle position is not known. Here, we identify the protein phosphatase 2A (PP2A) in complex with the regulatory subunit Rts1 as a component of the SPOC. Loss of PP2A-Rts1 function abrogates the SPOC but not other mitotic checkpoints. We further show that the protein phosphatase functions upstream of Kin4, regulating the kinase's phosphorylation and localization during an unperturbed cell cycle and during SPOC activation, thus defining the phosphatase as a key regulator of SPOC function.

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Many cell divisions are polarized, with intrinsic and/or extrinsic cues dictating the directionality of cell division. To ensure that the genetic material is segregated accurately during polarized division, the mitotic spindle must be positioned according to these polarization cues. There is mounting evidence to suggest that this process of spindle positioning is coupled to the cell cycle. In cultured rat kidney cells, a delay in anaphase onset occurs when the metaphase spindle is mispositioned (O'Connell and Wang 2000). In *Drosophila* male germline stem cells, cell cycle progression also appears delayed when the spindle is mispositioned (Cheng et al. 2008). Such coupling is suggestive of a feedback mechanism that delays the cell cycle in response to defects in spindle position.

A surveillance mechanism that delays the cell cycle in response to defects in spindle position has been described in the budding yeast *Saccharomyces cerevisiae*, and is termed the spindle position checkpoint (SPOC) (Yeh et al. 1995). Each cell division of budding yeast is asymmetric and thus inherently polarized. Budding yeast cells divide by forming a bud and hence determine the site of cytokinesis during entry into the cell cycle long before the mitotic spindle is formed. This manner of dividing requires the active positioning of the mitotic spindle along this predetermined division axis, which is known in yeast as the mother–bud axis. The SPOC ensures proper spindle position and thus chromosome segregation by delaying exit from mitosis—specifically anaphase spindle disassembly and cytokinesis—until the spindle is positioned correctly along the mother–bud axis (Yeh et al. 1995). The function of the SPOC is most apparent in cells deficient in cytoplasmic microtubule dynamics, guidance, and capture. Such cells fail to position the mitotic spindle along the division axis, resulting in anaphase spindle elongation in the mother cell (Yeh et al. 1995). Failure of this checkpoint results in the formation of anucleated and multinucleated cells.

The SPOC delays exit from mitosis by inhibiting the mitotic exit network (MEN). The MEN is a signal transduction pathway whose activity is controlled by the small GTPase Tem1 (Shirayama et al. 1994b). Tem1 regulates a kinase cascade whose activation ultimately leads to a sustained release of the phosphatase Cdc14 from its nucleolar inhibitor Cfi1/Net1 (Shirayama et al. 1994b; Shou et al. 1999; Visintin et al. 1999; Bardin et al. 2000; Lee et al. 2001; Luca et al. 2001; Mah et al. 2001). Once released, Cdc14 dephosphorylates key substrates to bring about exit from mitosis (Visintin et al. 1998; Zachariae et al. 1998; Jaspersen et al. 1999; Bardin et al. 2000).

Several proteins have been identified that regulate Tem1 during the cell cycle and in response to spindle position defects. Tem1 is controlled positively by the bud cortex restricted protein Lte1 (which resembles a guanine nucleotide exchange factor [GEF]) and negatively by the

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two-component GTPase-activating protein (GAP) Bub2–Bfa1 (Shirayama et al. 1994a; Lee et al. 1999; Li 1999; Bardin et al. 2000; Bloecher et al. 2000; Pereira et al. 2000; Geymonat et al. 2002). In the absence of *LTE1*, cells fail to activate the MEN and do not exit from mitosis at low temperatures (Shirayama et al. 1994a). The absence of the GAP leads to inappropriate MEN activation and mitotic exit in cells arrested in mitosis due to activation of the spindle assembly checkpoint (SAC) or the SPOC (Hoyt et al. 1991; Fesquet et al. 1999; Bardin et al. 2000; Bloecher et al. 2000; Pereira et al. 2000). A single SPOC-specific regulator of the MEN has also been identified. The protein kinase Kin4 is only required for MEN inhibition in response to spindle position defects (D'Aquino et al. 2005; Pereira and Schiebel 2005). Kin4 localizes to the mother cell cortex throughout most of the cell cycle. During anaphase, Kin4 also localizes to the spindle pole body (SPB) that remains in the mother cell (D'Aquino et al. 2005; Pereira and Schiebel 2005). In cells with mispositioned spindles, Kin4 associates with both SPBs where it phosphorylates Bfa1 (Pereira and Schiebel 2005; Maekawa et al. 2007). This phosphorylation protects the GAP from inhibitory phosphorylation by the Polo kinase Cdc5, effectively locking Bub2–Bfa1 in an active state, thus inhibiting the MEN (Hu et al. 2001; Geymonat et al. 2003; Maekawa et al. 2007). How Kin4 itself is controlled during the cell cycle or in response to spindle position defects is not understood.

Here we identify the protein phosphatase 2A (PP2A)-Rts1 as a regulator of Kin4 function. PP2A-Rts1 is required for the dephosphorylation of Kin4 during cell cycle entry and to maintain Kin4 in the dephosphorylated state during S phase and mitosis. Furthermore, the phosphatase controls the association of Kin4 with SPBs both during the cell cycle and in response to spindle position defects. The importance of this control is underlined by the finding that PP2A-Rts1 is essential for SPOC function but not for other mitotic checkpoints. We propose that PP2A-Rts1 is a SPOC component that facilitates Kin4 localization, thereby restraining MEN activity.

Results

PP2A-Rts1 regulates Kin4 phosphorylation

Kin4 is essential for SPOC function. To determine how the protein kinase is controlled, we focused on its previously observed cell cycle-regulated phosphorylation. Phosphorylated Kin4 can be detected as slower-migrating species by SDS-PAGE (D'Aquino et al. 2005). Kin4 is phosphorylated in G1, but upon release from a pheromone-induced G1 arrest, Kin4 phosphorylation is rapidly lost. The protein remains in a hypophosphorylated state throughout S phase and mitosis, but is rapidly rephosphorylated during exit from mitosis (Fig. 1A; D'Aquino et al. 2005).

Kin4 is in the dephosphorylated state during the stages of the cell cycle when Kin4 activity is needed to inhibit the MEN in response to potential spindle position defects. This correlation prompted us to investigate the significance of Kin4 phosphorylation by identifying phosphatases responsible for dephosphorylating Kin4. We screened mutants defective in known cell cycle-associated phosphatases for effects on Kin4 phosphorylation. Kin4 phosphorylation was examined in cells harboring temperature-sensitive alleles of *CDC14 (cdc14-3)*, *SIT4 (sit4-102)* (Wang et al. 2003), protein phosphatase 1 (PP1;

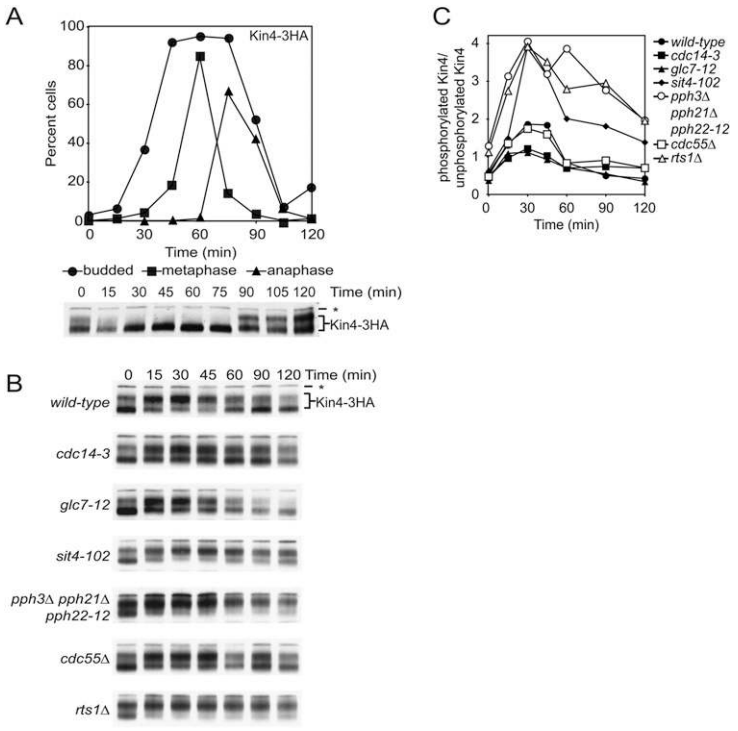

Figure 1. PP2A-Rts1 is necessary for Kin4 dephosphorylation. (*A*) Wild-type cells expressing a Kin4-3HA fusion (A11779) were arrested in G1 with 5 μg/mL α factor and released into pheromone-free media. After 70 min, 10 μg/mL α factor were added to prevent entry into a subsequent cell cycle. Cell cycle stage was determined by spindle and bud morphology. Kin4-3HA was monitored by Western blot. An asterisk indicates a cross-reacting band with the HA antibody. (*B*,*C*) Wild-type (A11779), *cdc14-3* (A19111), *glc7-12* (A19808), *sit4-102* (A20176), *pph3*Δ *pph21*Δ *pph22-12* (A20126), *cdc55*Δ (A19804), and *rts1*Δ (A20187) cells expressing a Kin4-3HA fusion were grown to exponential phase at room temperature and then shifted to 37°C ($t = 0$). Samples were taken at the indicated times to examine Kin4-3HA mobility. An asterisk indicates a cross-reacting band with the HA antibody. Quantification of *B* is shown in *C*.

glc7-12), and a combination of alleles that renders PP2A temperature-sensitive (*pph3Δ*, *pph21Δ*, *pph22-12*; henceforth temperature-sensitive PP2A) (Evans and Stark 1997). We grew the cells to mid-exponential phase at the permissive temperature (23°C) and followed Kin4 phosphorylation upon shift to the restrictive temperature (37°C). The temperature shift led to a transient accumulation of hyperphosphorylated Kin4 even in wild-type cells, for unknown reasons, which was particularly evident when the ratio of phosphorylated to unphosphorylated Kin4 was determined (Fig. 1B,C). After the transient accumulation of hyperphosphorylated Kin4, however, both hyper- and hypophosphorylated Kin4 were detected in wild-type and most phosphatase mutants. The *sit4-102* mutant displayed hyperphosphorylation after temperature shift, which was progressively lost during incubation at 37°C (Fig. 1B,C). Temperature-sensitive PP2A mutants showed a persistence of hyperphosphorylated Kin4 (Fig. 1B,C). Although other phosphatases, possibly Sit4, contribute to Kin4 dephosphorylation (see also Fig. 3B, below), we conclude that PP2A is required primarily for Kin4 dephosphorylation.

Budding yeast PP2A is a heterotrimeric enzyme composed of a single scaffolding subunit, Tpd3; a catalytic subunit, Pph21 or Pph22; and a regulatory subunit, Cdc55 (B-type PP2A) or Rts1 (B′-type PP2A) (for review, see Jiang 2006). To determine which regulatory subunit is required for Kin4 dephosphorylation, we examined the phosphorylation status of Kin4 in the *cdc55*Δ and *rts1*Δ mutants. In *cdc55*Δ cells, Kin4 phosphorylation status resembled that of wild type. In contrast, in the *rts1*Δ strain, Kin4 phosphorylation resembled that of the PP2A mutant (Fig. 1B,C). We conclude that PP2A-Rts1 is responsible primarily for Kin4 dephosphorylation.

PP2A-Rts1 is a component of the SPOC

If dephosphorylation of Kin4 is important for its checkpoint function, mutants in which Kin4 dephosphorylation is impaired should exhibit SPOC defects. To test this possibility, we examined how *PP2A-RTS1* mutants respond to spindle misposition. Cells lacking cytoplasmic dynein (*dyn1*Δ) exhibit spindle position defects particularly at low temperature (14°C) (Li et al. 1993). As a result, chromosome segregation frequently occurs within the mother cell. This in turn leads to activation of the SPOC, which causes inhibition of the MEN and cell cycle arrest in late anaphase ("arrested" morphology) (Fig. 2A). *dyn1*Δ cells with an impaired SPOC fail to delay mitotic exit, resulting in anucleated and multinucleated cells ("bypassed" morphology) (Fig. 2A).

After growth for 24 h at 14°C, 27% of *dyn1*Δ mutants exhibit the arrested morphology while only 10% exhibited the bypassed morphology (Fig. 2B), indicating that the SPOC is functional and that the MEN is inhibited. When the SPOC is inactivated by deletion of *KIN4*, 35% of *dyn1*Δ *kin4*Δ double mutant cells exhibit the bypassed morphology. Deletion of *RTS1* in the *dyn1*Δ mutant also lead to inactivation of the SPOC, with 42% of cells showing the bypassed morphology (Fig. 2B). Importantly, the *rts1*Δ single mutant did not exhibit spindle position defects, indicating that PP2A-Rts1 likely does not play a role in the actual positioning of the spindle. The SPOC defect was specific to PP2A-B′ as the *cdc55*Δ *dyn1*Δ mutant displayed no SPOC defect (Fig. 2B). We observed a defect in SPOC function in *dyn1*Δ *pph21*Δ *pph22-12 pph3*Δ quadruple mutants, although this defect was not as pronounced as in the *dyn1*Δ *rts1*Δ mutant—possibly due to the proliferation defect of the quadruple mutant (Fig. 2B). Lastly, we found that deletion of *KIN4* did not enhance the SPOC defect of *dyn1*Δ *rts1*Δ mutants. Taken together, these data indicate that PP2A-Rts1 is a component of the SPOC that likely functions in the same pathway as *KIN4*.

PP2A-Rts1 functions in the SPOC by controlling Kin4 localization to SPBs

Having established that PP2A-Rts1 is required for SPOC function, we wished to determine whether PP2A-Rts1

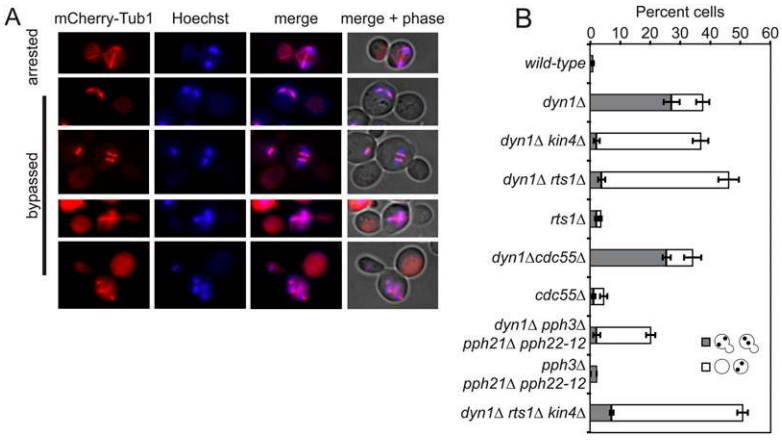

Figure 2. PP2A-Rts1 functions in the SPOC. (*A*) A *dyn1*Δ *rts1*Δ mutant (A21725) carrying a mCherry-Tub1 marker (red) was grown for 24 h at 14°C. The DNA was visualized by Hoechst staining (blue). Cells with the arrested morphology are budded and have two DNA masses spanned by an anaphase spindle in the mother cell. Cells with the bypassed morphology have no DNA masses or multiple DNA masses but with disassembled spindles or spindles indicative of cell cycle progression after improper mitotic exit. (*B*) Wild-type (A2587), *dyn1*Δ (A17349), *dyn1*Δ *kin4*Δ (A17351), *dyn1*Δ *rts1*Δ (A20310), *rts1*Δ (A20312), *dyn1*Δ *cdc55*Δ (A21520), *cdc55*Δ (A15396), *dyn1*Δ *pph3*Δ *pph21*Δ *pph22-12* (A21574), *pph3*Δ *pph21*Δ *pph22-12* (A19130), and *dyn1*Δ *rts1*Δ *kin4*Δ (A21538) were grown as described in *A*. Cells were stained for tubulin by indirect immunofluorescence and the DNA was stained with DAPI. $n \geq 100$ cells per sample. Gray bars represent the percentage of cells with the arrested morphology and white bars represent the percentage of cells with the bypassed morphology. Error bars represent SEM.

acts through Kin4. To this end, we examined the effects of deleting *RTS1* on the phenotype associated with *KIN4* overexpression. Overexpression of *KIN4* from the *GAL1–10* promoter terminally arrests cells in anaphase (D'Aquino et al. 2005). Deletion of *RTS1* suppressed the lethality caused by *GAL-KIN4* (Fig. 3A), indicating that overexpressed *KIN4* requires PP2A-Rts1 function to exert its inhibitory effects on mitotic exit. Importantly, deletion of *RTS1* did not suppress the lethal anaphase arrest induced by *GAL-BFA1* (Lee et al. 1999; Li 1999), suggesting that *rts1*Δ is not a general suppressor of anaphase arrest but instead shows specificity toward *KIN4*. The rescue of the *GAL-KIN4* lethality by the deletion of *RTS1* was not as complete as that brought about by the deletion of *BUB2*, a gene known to function downstream from *KIN4*, suggesting that the mechanisms of suppression may not be the same (Fig. 3A; D'Aquino et al. 2005).

To determine the relationship between PP2A-Rts1 and Kin4, we examined the effects of deleting *RTS1* on Kin4 phosphorylation, activity, and localization. We found that Kin4 is hyperphosphorylated throughout the cell cycle in the *rts1*Δ mutant (Fig. 3B). While we observed a consistent 10- to 15-min delay in cell cycle progression prior to metaphase spindle assembly, inactivation of *RTS1* did not affect the phosphorylation state of the Bub2–Bfa1 complex, the MEN GTPase Tem1, or the Tem1 activator Lte1, as judged by changes in electrophoretic mobility (Fig. 3C–F). These findings suggest that not all SPOC components and their targets are substrates of PP2A-Rts1.

Next we examined whether PP2A-Rts1 affects Kin4 activity, which is essential for checkpoint function (Supplemental Fig. 1A). Kin4 kinase activity was not decreased when isolated from *rts1*Δ mutant cells (Supplemental Fig. 1B,C), indicating that PP2A-Rts1 does not affect Kin4's in vitro enzymatic activity.

An intact SPOC requires not only Kin4 kinase activity but also binding of the protein kinase to SPBs (Maekawa et al. 2007). During an unperturbed cell cycle, Kin4 localizes to the mother cell cortex. During anaphase, Kin4 also associates with the SPB that remains in the mother cell (mSPB). In cells with mispositioned anaphase spindles, Kin4 associates with both SPBs. Kin4 mutants that fail to localize to SPBs are checkpoint-defective (Maekawa et al. 2007). Furthermore, ectopically targeting

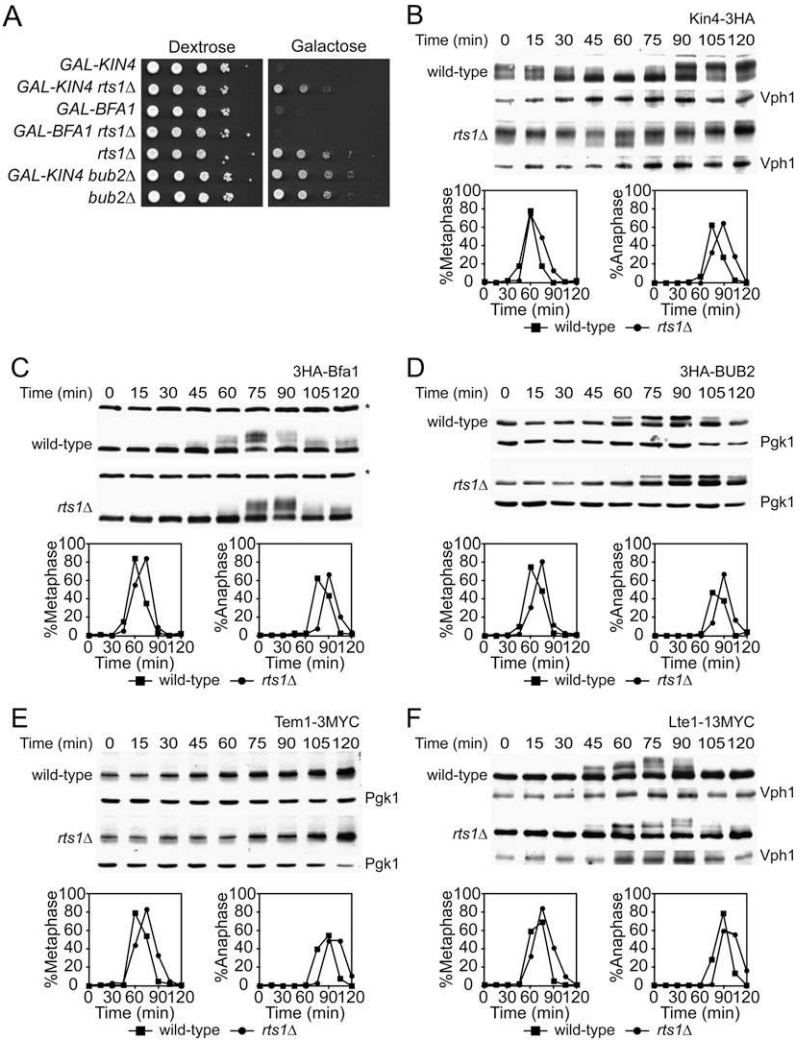

Figure 3. PP2A-Rts1 affects Kin4 phosphorylation but not other MEN components. (*A*) *GAL-GFP-KIN4* (A11997), *GAL-GFP-KIN4 rts1*Δ (A20893), *GAL-GFP-BFA1* (A3487), *GAL-GFP-BFA1 rts1*Δ (A20892), *rts1*Δ (A20312), *GAL-GFP-KIN4 bub2*Δ (A18792), and *bub2*Δ (A1863) cells were spotted on plates containing either dextrose or galactose and raffinose. The first spot represents growth of $\sim 3 \times 10^4$ cells and each subsequent spot is a 10-fold serial dilution. (*B–F*) Wild-type and *rts1*Δ cells expressing Kin4-3HA (A11779 and A20187) (*B*), 3HA-Bfa1 (A4378 and A21540) (*C*), 3HA-Bub2 (A21921 and A22064) (*D*), Tem1-3MYC (A1828 and A21539) (*E*), or Lte1-13MYC (A22668 and A22669) (*F*) were analyzed as described in Figure 1A. Vph1, Pgk1, and a cross-reacting band with the HA antibody (labeled with an asterisk) were used as loading controls.

Kin4 to SPBs suffices to delay mitotic exit (Maekawa et al. 2007). We monitored the ability of Kin4-GFP to associate with the mSPB during anaphase and observed a strong reduction in mSPB localization in *rts1Δ* cells (Fig. 4). Whereas Kin4-GFP localized to the mSPB during anaphase in 54% of wild-type cells, Kin4-GFP was found on mSPBs of only 11% of *rts1*Δ anaphase cells (Fig. 4C). Furthermore, in the *rts1*Δ mutant cells that showed Kin4 localization at the mSPB, the signal was weaker (Fig. 4A). The loss of Kin4 localization to SPBs was not due to a reduction in *KIN4-GFP* expression in the *rts1*Δ mutant, as levels of Kin4-GFP in these cells are similar to those observed in wild-type cells (Fig. 4B). The cortical localization and asymmetry of Kin4 was also reduced in *rts1*Δ cells, which was most pronounced in small budded cells (Figs. 4A, 5A,D). The basis of this loss in asymmetric localization of Kin4 is currently unclear. Localization of Bub2, Bfa1, and Tem1 was not affected by the deletion of *RTS1* (Supplemental Fig. 2A–C), indicating that loss of *RTS1* function does not alter the overall structure of SPBs nor does it affect the localization of other SPB-associated MEN proteins. Bud-restricted cortical localization of Lte1 was also not affected by deletion of *RTS1*, suggesting that overall cell polarity and integrity of the bud neck is maintained in the mutant (Supplemental Fig. 2D). We conclude that PP2A-Rts1 is required for Kin4 function and for the association of Kin4 with the mSPB during anaphase.

RTS1 *is required for efficient SPB loading of Kin4 in response to SPOC activation*

Having determined that *RTS1* was required for Kin4 localization during an unperturbed cell cycle, we next investigated whether *RTS1* also affected Kin4 loading onto SPBs when the spindle is depolymerized or mispositioned—situations in which the SPOC is active and *KIN4* function would be most important. We first examined cells treated with the spindle depolymerizing drug nocodazole. Under these conditions, Kin4 associates with SPBs (Fig. 5A,B; Pereira and Schiebel 2005). In contrast, Kin4 association with SPBs was significantly reduced in *rts1*Δ mutants (Fig. 5A,B). In the few cells where Kin4 was detected on SPBs, the signal intensity was reduced. This loss of SPB association could not be explained by changes in Kin4 expression, as Kin4-GFP protein levels were similar in the two strains (Fig. 5C). Similar results were obtained in *dyn1*Δ cells with mispositioned spindles. In such cells, Kin4 localizes to both SPBs (Fig. 5D,E; Pereira and Schiebel 2005). In the absence of *RTS1*, Kin4 localization to SPBs was severely impaired (Fig. 5D,E).

Overexpression of *KIN4* only partially suppressed the Kin4 SPB loading defect of *rts1*Δ mutants, providing a possible explanation for why deleting *RTS1* suppressed the lethality associated with high levels of Kin4 (Fig. 3A). Overexpression of *KIN4* from the *MET25* promoter, which inhibits proliferation (Fig. 5F), allowed some Kin4 to associate with SPBs in *rts1*Δ mutants, but the number of cells that exhibit Kin4 localization to SPBs was reduced compared with wild-type cells (Fig. 5G). Thus, in all situations in which Kin4 is known to load onto SPBs (correctly positioned anaphase spindles, spindle depolymerization, mispositioned anaphase spindles, and overexpressed *KIN4*), we observe a defect in SPB loading in the *rts1*Δ mutant.

In cells with a mispositioned spindle and an intact SPOC, both Bub2 and Bfa1 localize to both SPBs but Tem1 fails to load onto SPBs. When *KIN4* is deleted, Tem1 loads onto SPBs and thus, presumably, allows for premature exit from mitosis (Fig. 5J; D'Aquino et al. 2005). Consistent with previously observed effects of loss of *KIN4* function on the localization of Tem1 complex components, both Bub2 and Bfa1 localization were unaffected by deletion of *RTS1*, but Tem1 was observed to improperly load onto SPBs of cells with mispositioned spindles (Fig.

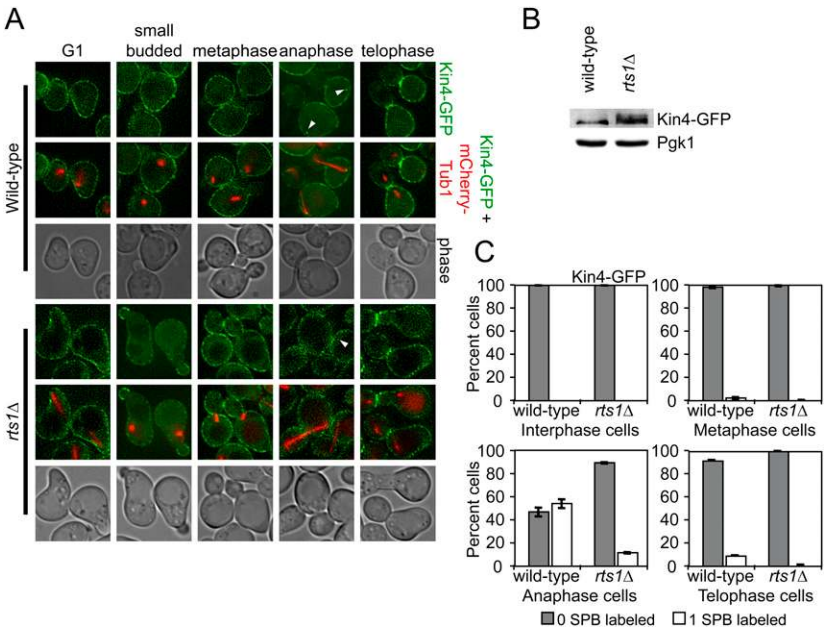

Figure 4. Localization of Kin4 to the mother SPB during anaphase requires *RTS1*. (*A–C*) Wild-type (A19900) and *rts1*Δ (A20918) cells expressing Kin4-GFP and mCherry-Tub1 fusion proteins were treated as described in Figure 1A. Samples were taken every 15 min for 2 h after release and the cells were imaged live. Serial sections spanning the entire cell were collected to ensure imaging of all spindle poles. (*A*) Deconvolved images from 20 serial sections. Kin4-GFP is in green and mCherry-Tub1 is in red. Levels of Kin4-GFP for equal ODs of culture are shown in *B*. Pgk1 was used as a loading control. Quantification of Kin4-GFP colocalization with spindle poles is shown in *C*. $n \geq 100$ for interphase, metaphase, and anaphase cells. $n \geq 40$ for the rarer telophase cells. Error bars represent SEM.

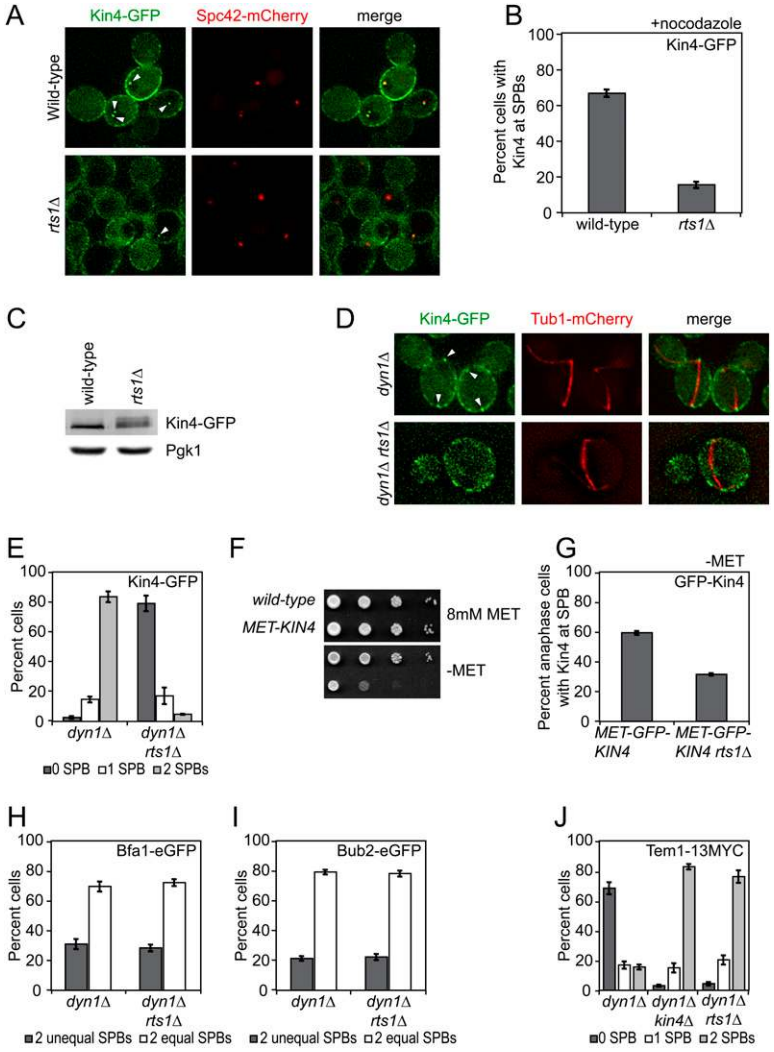

Figure 5. SPOC-induced Kin4 localization to spindle poles requires *RTS1*. (*A–C*) Wild-type (A19902) and *rts1*Δ (A21732) cells expressing Kin4-GFP and Spc42-mCherry fusion proteins were treated with 1.5 μg/mL nocodazole for 90 min. Cells were analyzed as described in Figure 4A. Kin4-GFP is in green and Spc42-mCherry is in red. Quantification of Kin4-GFP colocalization with the Spc42-mCherry marker is shown in *B*. $n \geq 50$ for each strain. Error bars represent SEM. Levels of Kin4-GFP for equal ODs of culture are shown in *C*. Pgk1 was used as a loading control. (*D,E*) *dyn1*Δ (A21720) and *dyn1*Δ *rts1*Δ (A22878) cells expressing Kin4-GFP and mCherry-Tub1 proteins were grown to exponential phase and then shifted to 14°C for 24 h. Cells were collected and analyzed as described in Figure 4A. Quantification of Kin4-GFP colocalization with spindle poles is shown in *E*. $n \geq 50$. Error bars represent SEM. (*F*) Wild-type (A2587) and *MET-GFP-KIN4* (A23232) were spotted on SC medium supplemented with 8 mM methonine or SC medium lacking methonine and incubated for 24 h at 30°C. The first spot represents growth of $\sim 3 \times 10^4$ cells and each subsequent spot is a 10-fold serial dilution. This overexpression allele was used instead of *GAL-GFP-KIN4* for single-cell analysis of the effects of *KIN4* overexpression due to the low viability of *rts1*Δ mutants when grown in raffinose. (*G*) *MET-GFP-KIN4* (A23358) and *MET-GFP-KIN4 rts1*Δ (A23357) cells expressing a mCherry-Tub1 fusion protein were grown and arrested with α factor inYePAD + 8 mM methonine. After 2.5 h of arrest, cells were washed and resuspended in SC-MET + α factor media and the arrest was held for an additional 30 min. Cells were then released into pheromone-free SC-MET media. Anaphase cells were counted for colocalization of GFP-Kin4 with the ends of the spindle. $n \geq 100$. Error bars represent SEM. (*H,I*) *dyn1*Δ and *dyn1*Δ *rts1*Δ cells expressing mCherry-Tub1 and Bfa1-eGFP (A21723 and A21722) (*H*) or Bub2-eGFP (A21724 and A21725) (*I*) were analyzed as described in *D* and *E*. $n \geq 50$. Error bars represent SEM. (*J*) *dyn1*Δ (A12123), *dyn1*Δ *kin4*Δ (A12122), and *dyn1*Δ *rts1*Δ (A22636) cells expressing a Tem1-13MYC fusion protein were grown as described in *D* and stained for tubulin and Tem1-13MYC by indirect immunofluorescence. $n \geq 50$. Error bars represent SEM.

5H–J). This improper loading of Tem1 onto SPBs of cells with mispositioned spindles in the *rts1*Δ mutant indicates that *RTS1* is required for normal *KIN4* function.

RTS1 *inhibition of the MEN is SPOC-specific*

Our data show that PP2A-Rts1 inhibits the MEN when the SPOC is active. We next wished to determine whether this inhibition was SPOC-specific or whether PP2A-Rts1 was a general MEN inhibitor. To address this question, we first examined the importance of MEN activity on SPOC and SAC activity. We constructed a series of hypermorphic alleles of *TEM1* and tested their behavior in response to challenges to both spindle assembly and spindle positioning. We isolated a hypermorphic allele of *TEM1*, *TEM1-eGFP*. *TEM1-eGFP* suppresses the growth defect of *lte1*Δ cells at 16°C. Other C-terminal-tagged alleles of *TEM1* do not display this suppression (Fig. 6A). This allele produces approximately twofold more protein than wild-type *TEM1* (Fig. 6B). In addition, we examined two overexpression constructs of *TEM1*. *TEM1* expressed from a multicopy 2μ plasmid expressed approximately sixfold more than wild-type *TEM1*, and *TEM1* expressed from the *GAL1–10* promoter produced ~50-fold more protein (Fig. 6B). We then tested the ability of these *TEM1* alleles to hyperactivate the MEN and thus bypass the cell cycle arrests caused by the SAC and the SPOC. All three hypermorphic alleles of *TEM1* bypassed the cell cycle arrest caused by spindle position defects to a similar degree (Fig. 6C). Cells treated with the spindle poison nocodazole activate the SAC, which in turn prevents both entry into anaphase and MEN activation in response to microtubule–kinetochore attachment defects (for review, see Lew and Burke 2003). Indeed, deletion of *BUB2* leads to inappropriate activation of the MEN and exit from mitosis in cells treated with nocodazole (Fig. 6D; Fraschini et al. 1999). This is evident from examining the ability of cells to form small buds (termed

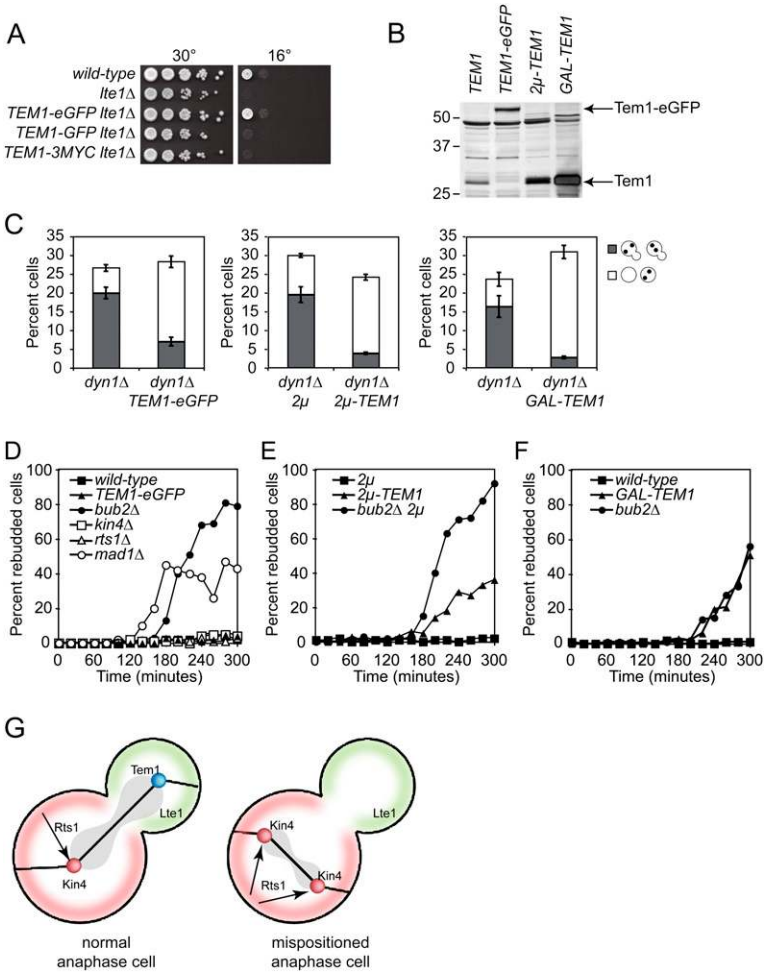

Figure 6. The SPOC and SAC are bypassed by differing levels of MEN activation. (*A*) Wild-type (A2587), *lte1*Δ (A18591), *lte1*Δ *TEM1-eGFP* (A21483), *lte1*Δ *TEM1-GFP* (A22567), and *lte1*Δ *TEM1-3MYC* (A4365) cells were spotted on YePAD plates and incubated at 30°C and 16°C. Pictures shown represent growth from 2 d for the 30°C condition and 4 d for 16°C. The first spot represents growth of ~3 × 10^4 cells and each subsequent spot is a 10-fold serial dilution. (*B*) Whole-cell lysates from 0.13 ODs of cells were analyzed by Western blot for Tem1 levels in the wild-type (A2587), *TEM1-eGFP* (A21089), YEp13-*TEM1* (A23122), and *GAL-TEM1* (A2441) strains. A23122 was grown in SC-LEU for plasmid maintenance, and A2441 was pregrown in YePA + 2% raffinose and induced with addition of 2% galactose for 2 h. (*C*) *dyn1*Δ (A17349), *dyn1*Δ *TEM1-eGFP* (A22811), *dyn1*Δ YEp13 (A23125), *dyn1*Δ YEp13-*TEM1* (A19104), and *dyn1*Δ *GAL-TEM1* (A23117) were grown and analyzed as described in Figure 2, A and B, with the following modifications: Strains carrying YEp13 plasmids were pregrown in SC-LEU and then transferred to YePAD medium prior to temperature shift, and strains A17349 and A23117 for the experiment in the third panel were pregrown in YePA + 2% raffinose and the GAL promoter was induced 2 h prior to temperature shift with the addition of 2% galactose. $n \geq 100$. Error bars represent SEM. (*D*) Wild-type (A2587), *mad1*Δ (A928), *bub2*Δ (A1863), *kin4*Δ (A17865), *rts1*Δ (A20312), and *TEM1-eGFP* (A21089) cells were grown and arrested in G1 as described in Figure 1A and subsequently released into media containing 1.5 μg/mL nocodazole. An additional 0.75 μg/mL nocodazole was added at 220 min after release to maintain the metaphase block. Samples were taken every 20 min for microscopic analysis. $n \geq 100$. (*E*) YEp13 (A23121), YEp13-*TEM1* (A23122), and YEp13 *bub2*Δ (A23120) cells were grown and analyzed as in Figures 1A and 6D with the following modification: Strains were pregrown in SC-LEU to maintain the plasmid and then transferred to YePAD medium prior to pheromone arrest. (*F*) Wild-type (A2587), *GAL-TEM1* (A2441), and *bub2*Δ (A1863) cells were grown and analyzed as in Figures 1A and 6D with the following modifications: Strains were grown and arrested in YePA + 2% raffinose and the GAL promoter was induced half an hour prior to release with the addition of 2% galactose. (*G*) A model for the role of *RTS1* in the control of *KIN4*. See the Discussion for further details.

rebudding) in the presence of nocodazole (Fig. 6D; Hoyt et al. 1991; Li and Murray 1991; Fraschini et al. 1999). Tem1-eGFP failed to bypass the nocodazole-induced cell cycle arrest (Fig. 6D), whereas the intermediate-strength allele, 2μ-*TEM1*, displayed an intermediate level of bypass (Fig. 6E). The strongest allele, *GAL-TEM1*, displayed the greatest degree of bypass of the nocodazole-induced arrest (Fig.6F).

Having established this series of hyperactive *TEM1* alleles, we then compared these alleles with mutants of known SPOC components. Cells lacking *RTS1* or *KIN4* resembled *TEM1-eGFP* cells. The SAC was intact in these cells but the SPOC was not (Fig. 6D; D'Aquino et al. 2005; Pereira and Schiebel 2005). In contrast, *bub2*Δ cells most resembled *GAL-TEM1* cells, exhibiting both SAC and SPOC defects (Fig. 6F). This finding indicates that like *KIN4* and unlike *BUB2*, *RTS1* is not a general inhibitor of the MEN but only exerts its inhibitory function in the SPOC. Additionally, our results suggest that higher levels of MEN activity are required to bypass the SAC than the SPOC.

Discussion

PP2A-Rts1 is a component of the SPOC

Previous studies identified the protein kinase Kin4 as an inhibitor of MEN activity in response to spindle position defects. How the protein kinase was controlled, however, was not understood. Based on the correlation between phosphorylation status and presumptive time of *KIN4* function during the cell cycle, we hypothesized that dephosphorylated Kin4 was active and that phosphatases that promoted accumulation of this form would be required for Kin4 function. To test this hypothesis, we screened known phosphatases implicated in cell cycle control and identified PP2A and its regulatory subunit Rts1 as being required for Kin4 dephosphorylation. Consistent with the

idea that dephosphorylation of Kin4 is indeed required for the protein's function, we found that cells lacking PP2A-Rts1 failed to delay exit from mitosis in response to spindle position defects. Our studies also shed light on the mechanism whereby PP2A-Rts1 restrains the MEN when spindles are mispositioned. The phosphatase does not appear to affect Kin4 kinase activity but instead promotes its association with SPBs, which was shown previously to be essential for SPOC activity (Maekawa et al. 2007).

Several lines of evidence indicate that PP2A-Rts1 is not a general inhibitor of the MEN but specifically functions in the SPOC by regulating Kin4 localization. Deletion of *RTS1*, like deletion of *KIN4*, leads to a bypass of the SPOC arrest but not the nocodazole-induced cell cycle arrest. In contrast, deletion of the MEN inhibitor and target of Kin4, Bub2–Bfa1, leads to a bypass of both checkpoint arrests. The specificity of PP2A-Rts1 toward Kin4 regulation is revealed by the observations that (1) deletion of *KIN4* does not enhance the SPOC defect of the *dyn1*Δ *rts1*Δ mutant, (2) loss of *RTS1* function only alters the phosphorylation state and localization of Kin4 and not other MEN components, and (3) loss of *RTS1* function suppresses the lethality of overexpression of *KIN4* but not *BFA1*. Attempts to test whether targeting Kin4 to SPBs was the sole function of PP2A-Rts1 in the SPOC failed because a *KIN4* allele described previously to constitutively localize to SPBs [*KIN4-SPC72(177–622)*] (Maekawa et al. 2007) is not functional in the checkpoint (Supplemental Fig. 3).

PP2A-Rts1 regulation of Kin4—an additional layer of control in the SPOC

Based on the localization patterns of the MEN activator Lte1 and MEN inhibitor Kin4, we and others proposed previously that bud-restricted Lte1 creates a zone of MEN activation in the bud and that mother cell-restricted Kin4 generates a zone of MEN inhibition in the mother cell (Fig. 6G; Bardin et al. 2000; Pereira et al. 2000; D'Aquino et al. 2005). Because components of the MEN localize to SPBs, movement of the MEN-bearing SPB into the bud would lead to MEN activation and exit from mitosis. In principle, this division of the cell into mitotic exit-restrictive and -permissive zones would be sufficient to ensure that exit from mitosis occurs only when one SPB, and hence half of the nucleus, moves into the bud. Our studies described here show that an additional layer of control exists, where PP2A-Rts1 controls the association of Kin4 with SPBs that are located in the mother cell. This additional layer of control could provide temporal control over Kin4, restricting access of the kinase to its target to the time when a spindle is present in cells—specifically mitosis. This hypothesis would be consistent with the observed cell cycle regulation of Kin4 phosphorylation.

We do not yet understand how *RTS1* promotes Kin4 loading onto SPBs. PP2A-Rts1 could mobilize Kin4 at the cortex and facilitate its association with SPBs (Fig. 6G). Whether the phosphatase does so by dephosphorylating Kin4 or a Kin4 receptor at SPBs is also not known. PP2A-Rts1 affects not only the ability of Kin4 to associate with SPBs in the mother cell, but also its association with the mother cell cortex. In wild-type cells, Kin4 is restricted to the mother cell cortex during S phase and early mitosis. In cells lacking *RTS1*, less Kin4 is observed at the cortex and localization is not restricted to the mother cell. It is therefore also possible that PP2A-Rts1 plays an important role in the establishment or maintenance of specific Kin4 localization to the mother cell cortex and that perhaps this localization is a prerequisite for Kin4 to load onto SPBs (Fig. 6G). Indeed, we observe that Kin4 mutants that fail to associate with the mother cell cortex also fail to associate with SPBs during anaphase (L.Y. Chan, unpubl.).

A key question raised by the observation that Kin4 phosphorylation changes during the cell cycle is whether PP2A-Rts1 itself is cell cycle- and SPOC-regulated or whether it is a yet-to-be-identified kinase(s) whose ability to phosphorylate Kin4 is regulated. Despite intense efforts, we have been unsuccessful in identifying kinases that affect phosphorylation and function of Kin4. Regardless of whether the Kin4 kinase(s) is cell cycle regulated, is there evidence that PP2A-Rts1 activity changes during the cell cycle? We do not detect any changes in Rts1 binding to the scaffolding subunit of PP2A, Tpd3, during the cell cycle (Supplemental Fig. 4), arguing against cell cycle-regulated changes in PP2A subunit composition. There is, however, evidence that PP2A-Rts1 localization is cell cycle-regulated. The protein localizes to centromeric DNA upon cell cycle entry and remains there until anaphase entry. The phosphatase also localizes to the bud neck during late mitosis where it regulates septin dynamics (Gentry and Hallberg 2002; Dobbelaere et al. 2003; L.Y. Chan, unpubl.). Rts1 is not detected on SPBs (Gentry and Hallberg 2002; L.Y. Chan, unpubl.) arguing against a model in which Rts1 association with its targets at the SPB is part of SPOC control. Rather, we favor the model in which Rts1 is required for robust localization of Kin4 to the mother cell cortex, which is itself a requirement for SPB localization.

PP2A—a key regulator of chromosome segregation fidelity

PP2A has been implicated previously in mitotic checkpoint control. The phosphatase together with its targeting subunit Cdc55 is required for cell cycle arrest upon triggering the SAC (Minshull et al. 1996). Our studies now show that the same phosphatase but with a different targeting subunit is essential for SPOC-induced cell cycle arrest. PP2A, however, has many other roles in mitotic progression. In controlling chromosome segregation, PP2A-Cdc55 controls the activity of the protein phosphatase Cdc14 during early anaphase as part of the FEAR network, and also potentially controls the activity of the APC/C at the metaphase-to-anaphase transition (Wang and Burke 1997; Queralt et al. 2006; Tang and Wang 2006; Yellman and Burke 2006; Chiroli et al. 2007). During meiosis, PP2A-Rts1 controls the chromosome segregation factor Sgo1 (Riedel et al. 2006). In all instances in which mechanisms are at least partly understood, PP2A

control involves the targeted action of the phosphatase at specific locations in the cell. In the case of the SPOC, it is possibly the cell cortex, during meiosis the kinetochore, and in the case of the FEAR network the nucleolus. It thus appears that the recurring theme in PP2A control of cell division is regulation by targeted localization.

Materials and methods

Yeast strains and growth conditions

All strains are derivatives of W303 (A2587) with the following exceptions: A20126 and A21574 are derivatives of DEY100 (A19130) and A20176 is a derivative of Y398 (A19926). All strains are listed in Supplemental Table 1. Lte1-GFP, Tem1-GFP, Rts1-3HA, *rts1*Δ, Spc42-mCherry, and *MET-GFP-KIN4* were constructed by standard PCR-based methods (Longtine et al. 1998; Hentges et al. 2005; Snaith et al. 2005). *kin4*^{T209A} was constructed by a two-step gene replacement using the *URA3* gene from *Kluyveromyces lactis*. Growth conditions are described in the figure legends.

Plasmid construction

The plasmid used to construct the *HIS3MX6:MET-GFP-KIN4* allele, pFA6a-*HIS3MX6:pMET25-GFP* (A1758), was constructed by digesting pFA6a-*HIS3MX6:pGAL-GFP* (Longtine et al. 1998) and a PCR product containing the *MET25* promoter with BglII and PacI and ligating the two fragments together.

Immunoblot analysis

Immunoblot analysis to determine total amounts of Kin4-3HA, 3HA-Bfa1, Tem1-3MYC, and Lte1-13MYC was performed as described (Bardin et al. 2000; Seshan et al. 2002; D'Aquino et al. 2005). For immunoblot analysis of Kin4-GFP, 3HA-Bub2, and Tem1, cells were incubated for a minimum of 10 min in 5% trichloroacetic acid. The acid was washed away with acetone and cells were pulverized with glass beads in 100 μL of lysis buffer (50 mM Tris-Cl at pH 7.5, 1 mM EDTA, 2.75 mM DTT, complete protease inhibitor cocktail [Roche]) using a bead mill. Sample buffer was added and the cell homogenates were boiled. Kin4-GFP was detected using an anti-GFP antibody (Clontech, JL-8) at 1:1000, 3HA-Bub2 was detected using an anti-HA antibody (Covance, HA.11) at 1:1000, and Tem1 was detected with an anti-Tem1 antibody at 1:1500. Semiquantitative measurements of relative protein levels of Tem1 and levels of phosphorylated and unphosphorylated Kin4-3HA were made using ECL Plus (GE Healthcare) and fluorescence imaging. Quantification was performed using NIH Image Quant software. Rts1-3HA was detected using the same anti-HA antibody at 1:1000, and Tpd3 was detected with a rabbit anti-Tpd3 antibody at 1:2000.

Antibody generation

An anti-Tem1 antibody was raised in rabbits against the peptide CKKLTIPEINEIGDPLLIYKHL. The antibody was then affinity-purified using immobilized antigen peptides (Covance).

Fluorescence microscopy

Indirect in situ immunofluorescence methods and antibody concentrations for Tub1 and Tem1-13MYC were as described previously (Kilmartin and Adams 1984; D'Aquino et al. 2005). For simultaneous visualization of nuclei and the mCherry-Tub1 fusion protein, cells were prepared as described (Monje-Casas et al. 2007) with the following modifications: Cells were permeabilized with 1% Triton X-100 for 5 min and the cells were resuspended in 1 μg/mL Hoechst 3342. For live cell microscopy, cells were grown in YePAD, harvested, resuspended in SC, and immediately imaged using a Zeiss Axioplan 2 microscope and a Hamamatsu OCRA-ER digital camera. Deconvolution was performed with Openlab 4.0.2 software. For quantifications, Z-stacks were taken and the localization was determined.

SPOC assay

Cells were grown to mid-exponential phase and then incubated for 24 h at 14°C. Cells were fixed, stained for the nuclei and spindle, and examined for endpoint morphology. Cells that were anucleated, multinucleated, or budded with two nuclei in the mother cell body but with a disassembled spindle were counted as bypassed. Budded cells with two nuclei in the mother cell body with an intact anaphase spindle were counted as arrested.

Kin4 kinase assays

Kin4 kinase assays were performed as described previously (D'Aquino et al. 2005) with the following modifications: Thirty microliters of anti-HA affinity matrix bead slurry (Roche) were used, 3.5 mg of total protein were used per immunoprecipitation, kinase reactions were allowed to run for 1 h, and the substrate was a mix of 1 μg of myelin basic protein and ~5 μg of recombinant MBP-BFA1. MBP-BFA1 was purified as described previously (Geymonat et al. 2002). Kinase signal was determined by PhosphorImaging, and immunoblot signal was determined using ECL Plus (GE Healthcare) and fluorescence imaging. Quantification was performed using NIH Image Quant software.

Co-immunoprecipitation assays

Approximately 12 OD units of cells were collected, washed once with 10 mM Tris-Cl (pH 7.5), and then lysed with glass beads in a bead mill with lysis buffer (50 mM Tris-Cl at pH 7.5, 150 mM NaCl, 1% NP-40, 60 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 15 mM para-nitrophenlylphosphate, 1 mM DTT, complete protease inhibitor cocktail [Roche]). One microgram of anti-HA antibody (Covance, HA.11) was added to 900 μg of total protein and incubated for 1 h. Ten microliters of preswelled protein-G bead slurry (Pierce) were then added to each sample and agitated for 2 h at 4°C. Beads were then washed six times with wash buffer (50 mM Tris-Cl at pH 7.5, 150 mM NaCl, 1% NP-40). Sample buffer was added to the beads, which were then boiled.

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The protein phosphatase 2A functions in the spindle position checkpoint by regulating the checkpoint kinase Kin4

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