Molecular Cell, Vol. 19, 223–234, July 22, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.molcel.2005.06.005

The Protein Kinase Kin4 Inhibits Exit from Mitosis in Response to Spindle Position Defects

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Summary

Accurate nuclear position is essential for each daughter cell to receive one DNA complement. In budding yeast, a surveillance mechanism known as the spindle position checkpoint ensures that exit from mitosis only occurs when the anaphase nucleus is positioned along the mother-bud axis. We identified the protein kinase Kin4 as a component of the spindle position checkpoint. *KIN4* prevents exit from mitosis in cells with mispositioned nuclei by inhibiting the mitotic exit network (MEN), a GTPase signaling cascade that promotes exit from mitosis. Kin4 is active in cells with mispositioned nuclei and predominantly localizes to mother cells, where it is ideally situated to inhibit MEN signaling at spindle pole bodies (SPBs) when anaphase spindle elongation occurs within the mother cell.

Introduction

Accurate chromosome segregation requires the orchestration of multiple cellular events. Surveillance mechanisms also known as checkpoints ensure this coordination. In the presence of unattached kinetochores, the spindle assembly checkpoint inhibits entry into anaphase by inhibiting a ubiquitin ligase, the anaphase-promoting complex or cyclosome, bound to the specificity factor Cdc20 (APC/C-Cdc20; reviewed in Lew and Burke [2003]). The APC/C-Cdc20 functions at the top of an inhibitory cascade that regulates entry into anaphase (reviewed in Nasmyth [2001]). The APC/C-Cdc20 promotes the degradation of Securin (Pds1 in yeast), which inhibits a protease known as Separase (Esp1 in yeast). Upon its liberation from Securin, Separase cleaves a component of the cohesin complex, Scc1/Mcd1, which holds sister chromatids together, thereby triggering anaphase chromosome movement.

A second surveillance mechanism, the spindle position checkpoint, ensures that exit from mitosis only occurs when the DNA is partitioned between the two daughter cells (reviewed in Lew and Burke [2003]). This checkpoint was discovered in budding yeast cells defective in guidance or capture of cytoplasmic microtubules at the cell cortex, which orients the mitotic spindle along the mother-daughter axis. Mutations in factors required for these processes lead to spindle orientation defects, causing anaphase to take place within the mother cell (Yeh et al., 1995). In such anaphase cells, exit from mitosis, that is the disassembly of the mitotic spindle, does not occur.

In budding yeast, the spindle position checkpoint inhibits exit from mitosis by preventing the activation of the protein phosphatase Cdc14, the trigger of exit from mitosis. The checkpoint does so by preventing the release of the phosphatase from its inhibitor Cfi1/Net1 in the nucleolus, which normally occurs during anaphase (reviewed in Stegmeier and Amon [2004]). The dissociation of Cdc14 from its inhibitor during anaphase is mediated by two regulatory networks, the Cdc14 early anaphase release (FEAR) network and the MEN. The MEN is a GTPase signaling cascade in which the activity of the GTPase Tem1 is thought to be positively regulated by the putative exchange factor (GEF) Lte1 and negatively regulated by the GAP complex Bub2-Bfa1 (Stegmeier and Amon, 2004). The activated form of Tem1, which is likely, but not proven, to be the GTP bound form, is thought to stimulate the protein kinase Cdc15 to activate the protein kinase Dbf2 and its associated factor Mob1.

Genetic evidence indicates that activation of the spindle position checkpoint prevents MEN activity. The first mechanism identified to help prevent MEN signaling in cells with a mispositioned mitotic spindle was the spatial segregation of MEN components until part of the nucleus has moved into the bud. The MEN activator Lte1 becomes sequestered at the bud cortex concomitant with bud formation, whereas Tem1 and other MEN components localize to the daughter bound SPB (Bardin et al., 2000; Pereira et al., 2000). Hence, Tem1 and Lte1 are only in the same cellular compartment when the daughter bound SPB moves into the bud during anaphase. Overexpressing *LTE1* or deleting the septin ring component *SHS1*, which causes Lte1 accumulation in the mother cell, allows cells with misoriented anaphase nuclei to exit from mitosis (Bardin et al., 2000; Castillon et al., 2003).

The spatial restriction of Lte1 and Tem1 is not the only mechanism that prevents cells with misaligned nuclei from exiting mitosis, because deletion of either *BUB2* or *BFA1* also allows cells with mispositioned anaphase nuclei to exit from mitosis (Bardin et al., 2000; Daum et al., 2000; Pereira et al., 2000; Wang et al., 2000). The identity of these additional mechanisms that inhibit the MEN in response to spindle position defects is not known. In this study, we describe the identification of the protein kinase Kin4 as a spindle position checkpoint component and show that Kin4 regulates MEN activity in an Lte1-independent manner. The finding that Kin4 predominantly localizes to mother cells leads us to propose that Kin4 establishes a domain of

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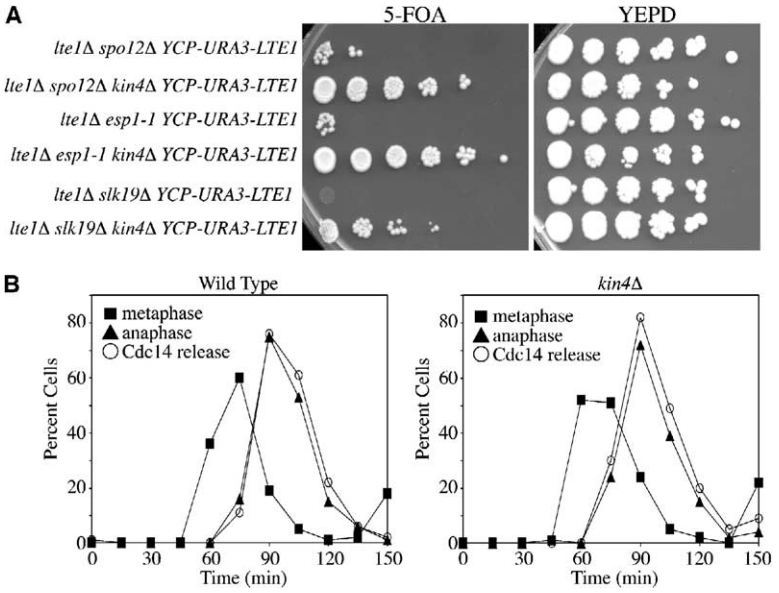

Figure 1. Deletion of *KIN4* Suppresses the Synthetic Lethality of *lte1Δ* FEAR Network Mutants, but *KIN4* Is Not Essential for Mitotic Exit

(A) *lte1Δ spo12Δ* ± *kin4Δ* (A4504 and A12342), *lte1Δ esp1-1* ± *kin4Δ* (A3722 and A12356), and *lte1Δ slk19Δ* ± *kin4Δ* (A4394 and A12357) cells carrying *LTE1* on a *CEN4-URA3* plasmid were spotted on plates containing 5-fluoroorotic acid (5-FOA; selects against the *URA3* plasmid) or YEPD plates.

(B) Wild-type (wt) (A1411) and *kin4Δ* (A8453) cells carrying a *CDC14-HA* fusion were arrested in G1 by using 5 μg/ml α factor followed by a release into medium lacking pheromone. The percentage of cells containing metaphase spindles (closed squares), anaphase spindles (closed triangles), and Cdc14-HA released from the nucleolus (open circles) was determined at the indicated times.

MEN inhibition within the mother cell, allowing MEN signaling and exit from mitosis only to occur when movement of the daughter bound SPB, which functions as the MEN signaling center, out of this domain and into the Lte1-containing bud takes place.

Results

A Genetic Selection Identifies a Negative Regulator of Exit from Mitosis

Many FEAR network and MEN components have been identified to date that function as positive regulators of exit from mitosis, but only three negative regulators, *BUB2*, *BFA1*, and *FOB1*, have been discovered (reviewed in Stegmeier and Amon [2004]). To identify additional negative regulators of either the MEN or the FEAR network, we took advantage of the fact that inactivation of the MEN activator *LTE1* is lethal in cells lacking the FEAR network component *SPO12* or *ESP1* or *SLK19* and that inactivation of *BUB2* or *BFA1*, both known negative regulators of the MEN, suppresses this synthetic lethality (Stegmeier et al., 2002). *lte1Δ spo12Δ* or *lte1Δ slk19Δ* cells bearing *LTE1* on a plasmid were mutagenized by using transposon mutagenesis, and colonies were isolated that could grow in the absence of the *LTE1* plasmid. Ten transposon insertions were identified in this manner, of which three were in *BUB2* and one in *BFA1*. Six insertions were identified in *KIN4*, which encodes a protein kinase. A complete deletion of *KIN4* also suppressed the proliferation defect of *lte1Δ spo12Δ* or *lte1Δ slk19Δ* cells and, in addition, that of *lte1Δ esp1-1* cells at the permissive temperature (Figure 1A).

Although deletion of *KIN4* efficiently suppressed the lethality of *lte1Δ* cells lacking a FEAR network component, it had little if any effect on cell cycle progression in otherwise wild-type (wt) cells (Figure 1B). Upon release from a pheromone-induced G1 arrest, cells lacking *KIN4* progressed through the cell cycle with similar kinetics as wt cells, and release of Cdc14 from the nucleolus was not affected (Figure 1B). Thus, *KIN4* plays little if any role in controlling exit from mitosis in an unperturbed cell cycle. However, under conditions where both the FEAR network and the MEN are impaired, deletion of *KIN4* allows cell survival, suggesting that *KIN4* can function as a negative regulator of exit from mitosis.

High Levels of Kin4 Delay Cdc14 Release from the Nucleolus and Exit from Mitosis by Antagonizing MEN Activity

To investigate how *KIN4* inhibits mitotic exit, we analyzed the consequences of overproducing Kin4. Cells overexpressing *KIN4* from the galactose-inducible *GAL1-10* were inviable in the presence of galactose (Figure 2D) and exhibited a severe delay in anaphase spindle disassembly and degradation of the mitotic cyclin Clb2 (Figure 2A). This defect in exit from mitosis was due to a failure to release Cdc14 from the nucleolus (Figure 2A).

Overproduced Kin4 could interfere with either FEAR network or MEN function or both to prevent Cdc14 release from its inhibitor. To distinguish among these possibilities, we first examined whether FEAR network function was impaired in cells overexpressing *KIN4*. Cdc14 released by the FEAR network, but not the MEN, promotes the relocalization of the passenger protein Sli15 from kinetochores to the spindle midzone during anaphase (Pereira and Schiebel, 2003). As previously reported, cells lacking *CDC14* function failed to localize Sli15 to anaphase spindles, but the protein did localize to anaphase spindles in cells mutated for the MEN component *CDC15* (Pereira and Schiebel, 2003; Figure 2C; Figure S1 available in the Supplemental Data with this article online). Overexpression of *KIN4* did not prevent Sli15 localization to anaphase spindles (Figure 2C), indicating that high levels of Kin4 do not interfere with FEAR network function.

To examine the effects of Kin4 on MEN function, we

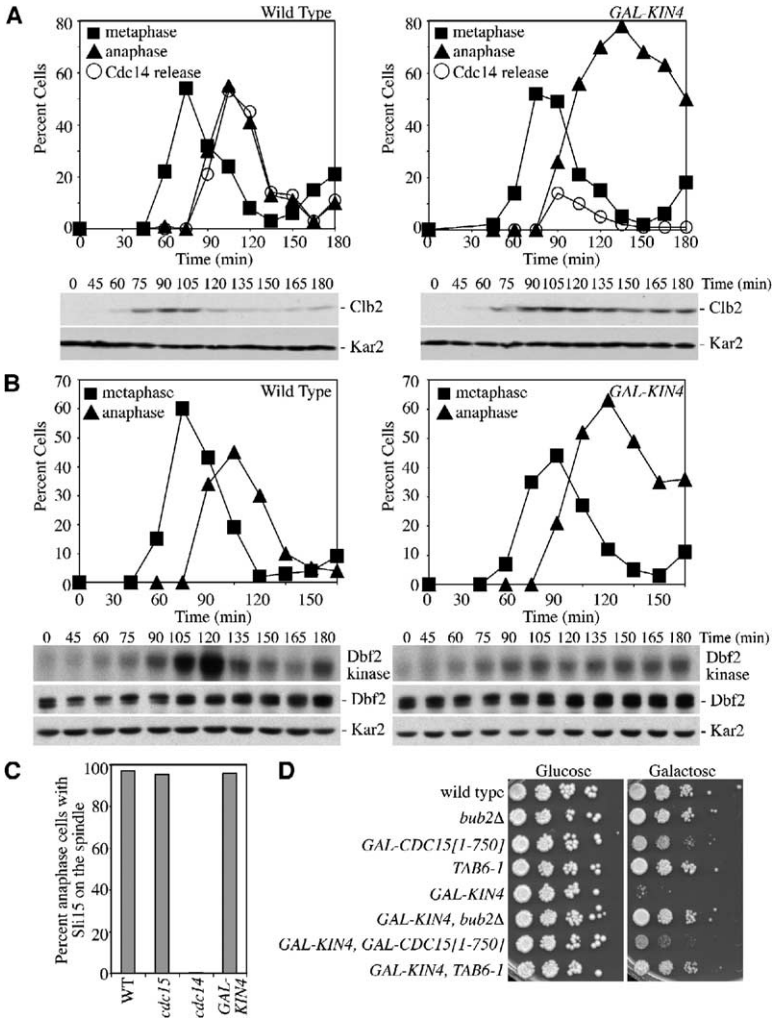

Figure 2. Overexpression of *KIN4* Delays Mitotic Exit

(A) Wt (A1411) and *GAL-KIN4* (A9249) cells carrying a *CDC14-HA* fusion were arrested in G1 in YEP-raffinose (YEPR) with 5 μg/ml α factor. *GAL-KIN4* transcription was induced for 1 hr by the addition of galactose (YEPRG) while in α factor. Cells were released into YEPRG lacking pheromone. The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed triangles), Cdc14 released from the nucleolus (open circles), and the amount of Clb2 protein was determined. Kar2 was used as a loading control in Western blots.

(B) Wt (A1931) and *GAL-KIN4* (A9281) cells carrying a *DBF2-MYC* fusion were grown as described in (A). The percentage of cells with metaphase (closed squares) and anaphase spindles (closed triangles) and the amount of Dbf2 protein and Dbf2 associated kinase activity were determined.

(C) Wt (A9726), *cdc15-2* (A9727), *cdc14-3* (A9728), and *GAL-KIN4* (A11778) strains carrying a *SLI15-MYC* fusion were arrested in G1 with α factor followed by release into medium lacking pheromone. The percentage of anaphase cells with Sli15 localized to the spindle was determined 90–105 min after release.

(D) Epistasis analysis using *GAL-KIN4* strains. Wt (A2587 and A9249), *bub2Δ* (A1901 and A9252), *GAL-CDC15[1–750]* (A5966 and A9284), and *TAB6-1* (A5617 and A11979) ± *GAL-KIN4* cells were spotted on plates containing glucose or galactose.

assessed the activity of the protein kinase Dbf2, which constitutes the most downstream component of the MEN. In wt cells, Dbf2 kinase activity was low during G1, S phase, and metaphase but was high during anaphase (Figure 2B). In contrast, Dbf2 kinase activity did not accumulate during anaphase in cells overexpressing *KIN4* (Figure 2B). Furthermore, the transient release of Cdc14 from the nucleolus observed in *GAL-KIN4* cells resembles the release observed in MEN mutants and not that observed in FEAR network mutants (Figure 2A; Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). Our results indicate that high levels of Kin4 inhibit MEN signaling.

Kin4 Prevents Bfa1 and Bub2 Hyperphosphorylation

To determine how high levels of *KIN4* antagonize MEN signaling, we first placed the gene within the MEN signaling cascade by using epistasis analysis. The lethality associated with high levels of Kin4 was efficiently suppressed by deleting the GAP complex component *BUB2* (Figure 2D). It was also suppressed by overexpression of a hyperactive allele of *CDC15* (*CDC15[1–750]*; Bardin et al., 2003) or expression of a dominant active allele of *CDC14*, *TAB6-1* (Shou et al., 1999; Figure 2D). Thus, *KIN4* antagonizes MEN signaling by inhibiting proteins near or at the top of the signaling cascade.

Because the ability of overproduced Kin4 to inhibit MEN activity was suppressed by deletion of *BUB2*, we examined the effects of Kin4 on Bub2 and Bfa1 phosphorylation. Bfa1 is phosphorylated by Cdc5 during anaphase (Hu et al., 2001). This causes the GAP complex to dissociate from Tem1 and allows MEN signaling to occur (Hu et al., 2001). Phosphorylation of Bub2, which is in part dependent on *CDC5*, also correlates with inactivation of GAP activity during anaphase (Hu and Elledge, 2002). To examine Bub2 and Bfa1 hyperphosphorylation and to ensure that wt and *GAL-KIN4* cells were in the same cell cycle stage during the analysis, we conducted this analysis in a MEN mutant in which, like in *GAL-KIN4* strains, cells arrest in anaphase and in which Bfa1 and Bub2 phosphorylation is maximal (Hu et al., 2001; Hu and Elledge, 2002; Pereira et al., 2002). We employed a *CDC15* allele that can be inhibited by the ATP analog "PP1 analog 8" (*cdc15-as1*; Bishop et al., 2000) to inactivate the MEN. Addition of

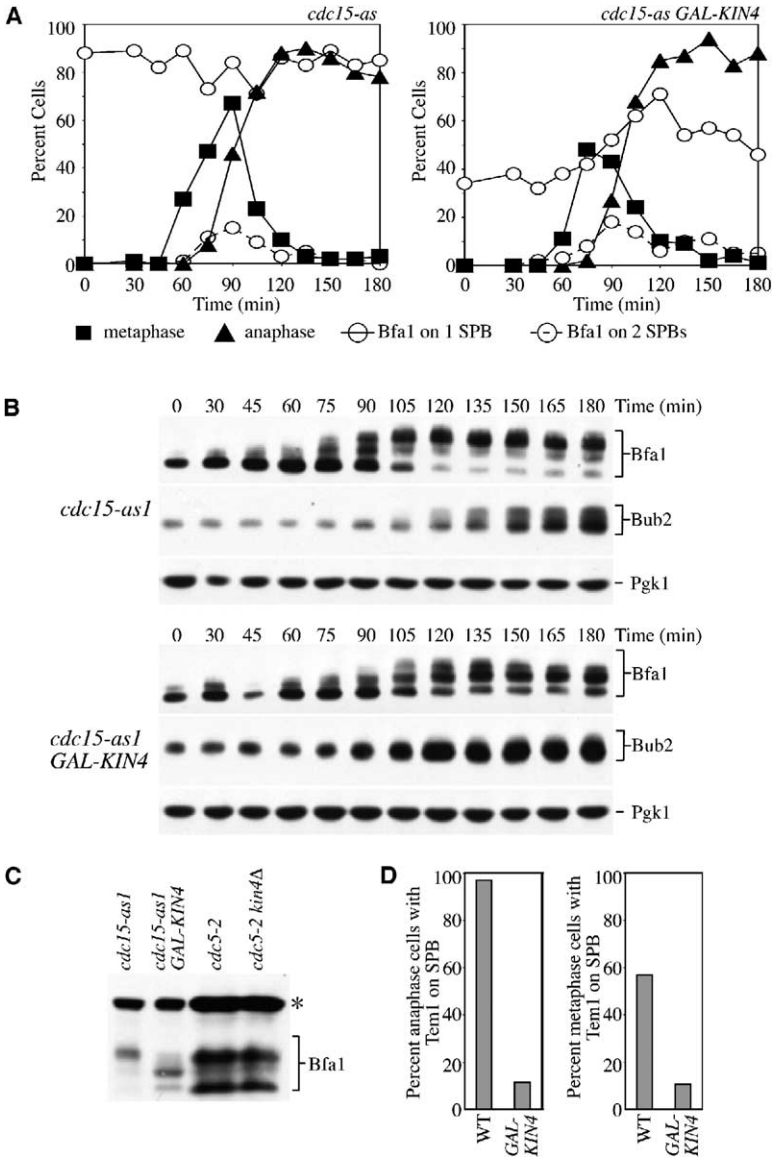

Figure 3. *KIN4* Overexpression Interferes with the Regulation of Tem1, Bub2, and Bfa1

(A–C) *cdc15-as1* (A11993) and *cdc15-as1 GAL-KIN4* (A11991) cells carrying a *BFA1-HA* allele and *cdc15-as1* (A11994) and *cdc15-as1 GAL-KIN4* (A11992) cells carrying a *BUB2-HA* allele were arrested in G1 as described in the legend to Figure 2. Cells were released into YEPRG containing 10 μM PP1 analog 8, the ATP analog that inhibits Cdc15-as1. (A) The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed triangles), and Bfa1 localized to one spindle pole body (open circle, straight line) or two spindle pole bodies (open circle, dashed line) was determined. (B) The phosphorylation status of Bfa1 and Bub2 was monitored by Western blot analysis. Pgk1 served as a loading control. The Western blot in (C) shows a comparison of Bfa1 migration between *cdc15-as1* mutants 180 min after release, *cdc15-as1 GAL-KIN4* mutants 180 min after release, and *cdc5-2* (A13313) and *cdc5-2 kin4Δ* (A13312) cells grown at 37°C for 2 hr. An asterisk indicates a crossreacting band that serves as a loading control.

(D) Wt (A2266) and *GAL-KIN4* (A11978) cells carrying a *TEM1-MYC* fusion were grown as described in Figure 2A. The percentage of metaphase or anaphase cells with Tem1 localized to the SPB was determined.

the drug to cells carrying the *cdc15-as1* allele caused a first cycle arrest in anaphase (Figure 3A), indicating that the allele was effectively inhibited by the ATP analog in vivo. Although cells carrying the *cdc15-as1* allele cannot grow in the presence of the drug, wt cells are unaffected (Bishop et al., 2000). Both Bfa1 and Bub2 accumulated in their hyperphosphorylated forms in *cdc15-as1* cells (Figure 3B), as expected of cells that arrest in anaphase. Furthermore, Bfa1 localization resembled that of wt cells, with the majority of cells exhibiting Bfa1 localization on the SPB that is present in the bud (Figure 3A). In *KIN4* overexpressing cells, the slowest migrating forms of Bfa1 and Bub2 observed in *cdc15-as1* cells did not accumulate (Figure 3B). The migration pattern of Bfa1 in *GAL-KIN4* cells differed from that observed in a *cdc5-2* mutant, which is partially impaired in Bfa1 phosphorylation (Hu et al., 2001; Figure 3C). Whereas Bfa1 was either hypo- or hyperphosphorylated in *cdc5-2* mutants, intermediate amounts of Bfa1 phosphorylation (as judged by migration in SDS-PAGE) were observed in cells overexpressing *KIN4*. This observation suggests that high levels of Kin4 either only partially affect Cdc5 activity or that Kin4 functions as an antagonist of Cdc5 promoting Bfa1 dephosphorylation.

Bfa1 association with SPBs was also somewhat reduced (Figure 3A), though the significance of this observation is at present unclear. Overexpression of *KIN4* also prevented the association of Tem1 with metaphase and anaphase SPBs (Figure 3D). Because Tem1 association with SPBs depends on *BUB2* and *BFA1* during metaphase and most of anaphase (Pereira et al., 2000), loss of Tem1 from SPBs could be a consequence of Kin4's effects on Bub2 and Bfa1. It is also possible that overproduced Kin4 affects Tem1 localization independently of Bub2 and Bfa1, an idea that is consistent with the finding that Tem1 association with SPBs during late anaphase is *BUB2-BFA1* independent (Gruneberg et al., 2000). Our results indicate that overexpressed *KIN4*

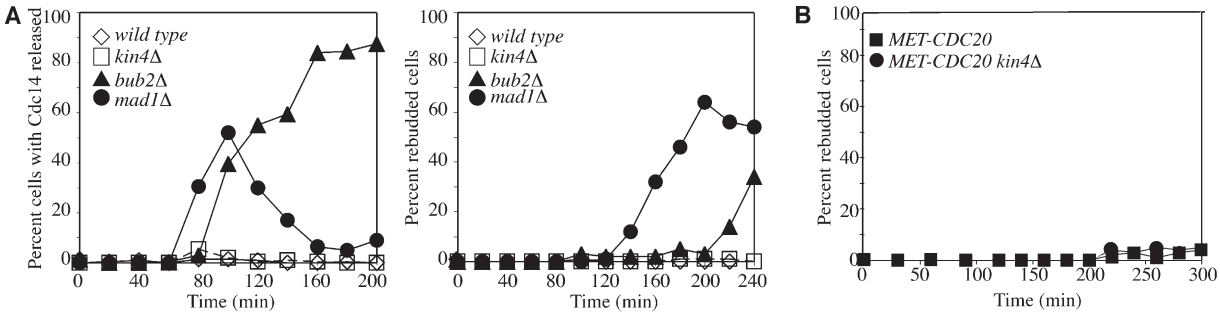

Figure 4. *KIN4* Is Not Required for the Spindle Assembly Checkpoint

(A) Wt (A1411, open diamonds), *kin4Δ* (A8453, open squares), *bub2Δ* (A1901, closed triangles), and *mad1Δ* (A2853, closed circles) cells carrying a *CDC14-HA* fusion were arrested with 5 μg/ml α factor and released into medium containing 15 μg/ml nocodazole. The percentage of cells with Cdc14 released from the nucleolus (left graph) and the percent of rebudded cells (right graph) were determined. Rebudding signifies that cells exited from the nocodazole block and began a new cell cycle.

(B) *MET-CDC20* (A6808) and *MET-CDC20 kin4Δ* (A12440) cells were arrested with α factor in medium lacking methionine. Cells were released into medium containing 8 mM methionine to shut off *MET-CDC20* transcription, and the percentage of rebudded cells was determined.

interferes with the inactivation of the Bub2-Bfa1 complex and Tem1 localization to SPBs during anaphase.

The Spindle Assembly Checkpoint Is Intact in Cells Lacking *KIN4*

The observation that deletion of *KIN4* had little effect on cell cycle progression, yet the protein inhibited MEN activity when overproduced, raised the possibility that the primary role of the protein kinase is to function in a surveillance mechanism that affects MEN signaling. Two surveillance mechanisms, the spindle assembly checkpoint and the spindle position checkpoint, have been shown to inhibit exit from mitosis (reviewed in Lew and Burke [2003]). The spindle assembly checkpoint is activated in response to unattached kinetochores and causes cell cycle arrest in metaphase through the simultaneous inhibition of the APC/C-Cdc20 and the MEN. Inactivation of *BUB2* or *BFA1* causes inappropriate exit from mitosis when the checkpoint is activated. To determine whether *KIN4* was required to restrain MEN activity when the spindle assembly checkpoint was activated, we examined the consequences of deleting *KIN4* on cells treated with the microtubule poison nocodazole, which causes activation of the spindle assembly checkpoint. As observed previously, deletion of *BUB2* or the spindle assembly checkpoint component *MAD1* led to Cdc14 release from the nucleolus, exit from mitosis, and entry into a new cell cycle as judged by the formation of a new bud in nocodazole-treated cells (rebudding, Figure 4A; Stegmeier et al., 2002; Yoshida et al., 2002). In contrast, cells lacking *KIN4* arrested in the presence of nocodazole and did not exit from mitosis (Figure 4A). Next, we considered the possibility that an intact microtubule cytoskeleton was needed in *kin4Δ* cells to observe a bypass of the spindle assembly checkpoint arrest. To this end, we examined whether deletion of *KIN4* allowed cells depleted for the APC/C activator and spindle assembly checkpoint target Cdc20 to exit the metaphase arrest. Inactivation of *KIN4* did not allow Cdc20-depleted cells to escape the metaphase arrest (Figure 4B), excluding the possibility that a bypass of the checkpoint arrest required an intact microtubule cytoskeleton. Our results show that *KIN4* is not required to prevent exit from mitosis in response to activation of the spindle assembly checkpoint.

kin4Δ Cells Fail to Arrest in Anaphase in Response to Spindle Position Checkpoint Activation

The spindle position checkpoint prevents Cdc14 release from the nucleolus and exit from mitosis when the anaphase nucleus is not correctly aligned along the mother-bud axis (reviewed in Lew and Burke [2003]). To determine whether *KIN4* plays a role in restraining exit from mitosis when the anaphase nucleus is mispositioned, we examined the effects of deleting *KIN4* in cells lacking cytoplasmic dynein (*DYN1*). *dyn1Δ* mutants are impaired in sliding of microtubules along the cell cortex, which is necessary for accurate nuclear position, leading to the accumulation of cells in which anaphase occurred within the mother cell particularly at low temperatures (Yeh et al., 1995; Figure 5A). At 16°C, the percentage of *dyn1Δ* cells with mispositioned spindles is around 10% (Figure 5A), and *kin4Δ* cells also exhibited a slight spindle position defect (3.1% ± 1.96%), and 1.2% ± 0.6% of cells contained either no nucleus or multiple nuclei (Figure 5A). It was nevertheless obvious that deletion of *KIN4* allowed *dyn1Δ* cells with mispositioned spindles to exit from mitosis, leading to the accumulation of multinucleate and anucleate cells both in the presence and absence of an intact microtubule cytoskeleton (Figure 5A and Figure S2; data not shown). The extent to which multinucleate and anucleate cells accumulated was similar in *dyn1Δkin4Δ*, *dyn1Δbub2Δ*, and *dyn1Δbfa1Δ* cells and triple and quadruple mutant combinations (Figure 5A), indicating that *BUB2*, *BFA1*, and *KIN4* function in the same pathway.

kar9Δ act5ts cells also accumulate cells with mispositioned anaphase nuclei due to a defect in anchoring cytoplasmic microtubules at the bud cortex (reviewed in Pearson and Bloom [2004]; Figure 5A). Deletion of *KIN4* also affected the ability of *kar9Δ act5ts* cells with mispositioned spindles to arrest in anaphase, though

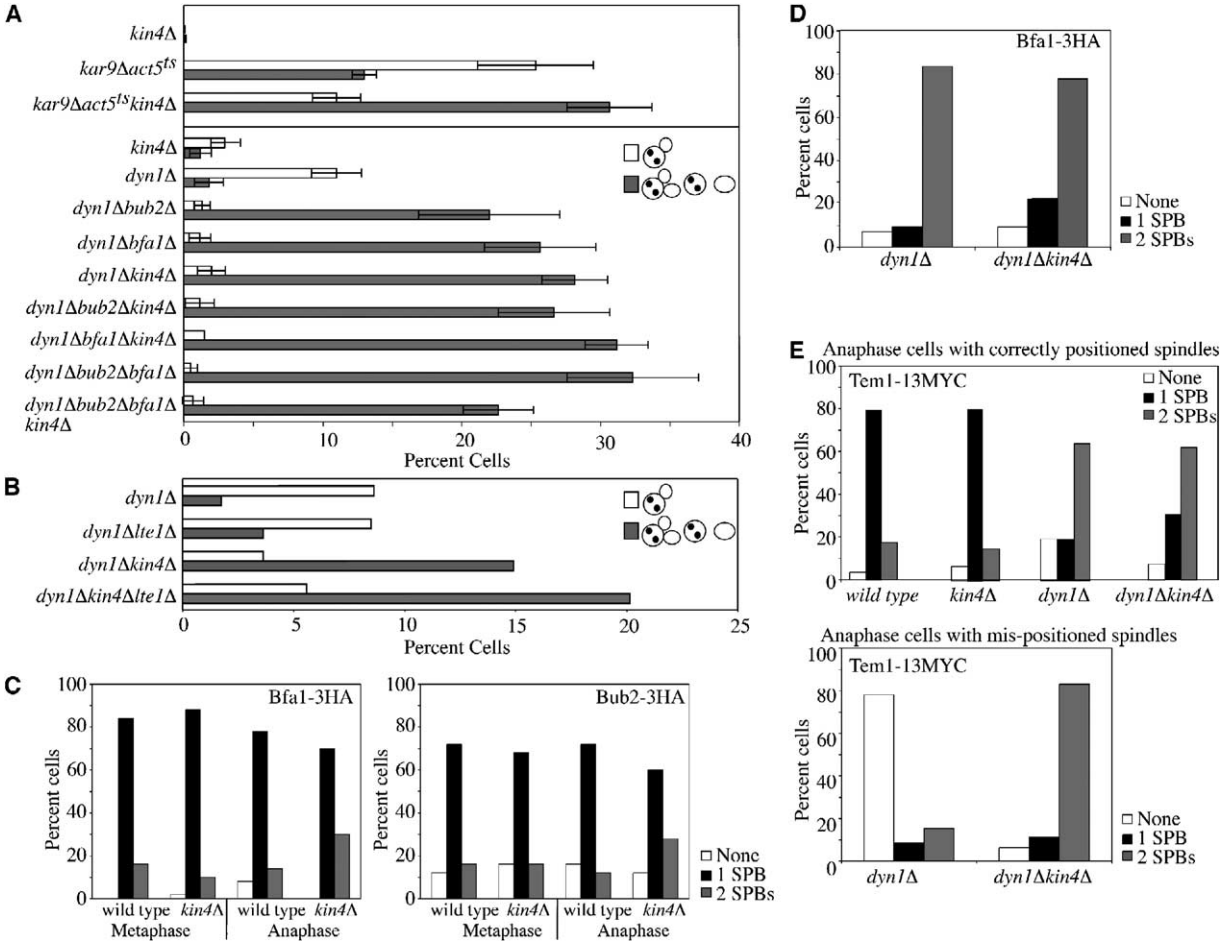

Figure 5. Kin4 Functions in the Spindle Position Checkpoint

(A) *kin4Δ* (A8453), *kar9Δ act5ts* (A2025), and *kar9Δ act5ts kin4Δ* (A12004) cells were grown for 3 hr at 37°C; *kin4Δ* (A8453), *dyn1Δ* (A2077), *dyn1Δ bub2Δ* (A2270), *dyn1Δ bfa1Δ* (A11775), *dyn1Δ kin4Δ* (A9290), *dyn1Δ bub2Δ kin4Δ* (A9292), *dyn1Δ bfa1Δ kin4Δ* (A11769), *dyn1Δ bub2Δ bfa1Δ* (A11990), and *dyn1Δ bub2Δ bfa1Δ kin4Δ* (A11987) cells were grown for 24 hr at 16°C. The percentage of cells with mispositioned anaphase nuclei (white bars) and the percentage of cells with more than one bud, multiple nuclei, or no nucleus (gray bars) were determined. Error bars indicate SD.

(B) *dyn1Δ* (A11982), *dyn1Δ lte1Δ* (A11999), *dyn1Δ kin4Δ* (A12000), and *dyn1Δ kin4Δ lte1Δ* (A11985) cells were grown and analyzed as described in (A).

(C) Bfa1 and Bub2 localization was analyzed in exponentially growing wt (A4378 and A12597) and *kin4Δ* (A11772 and A12119) cells. The percentage of metaphase and anaphase cells with Bfa1 or Bub2 not localized, on one SPB, or on two SPBs was determined.

(D) *dyn1Δ* (A5079) and *dyn1Δ kin4Δ* (A11771) cells were grown at 16°C for 24 hr, and the percentage of cells with Bfa1 on no, one, or two SPB(s) in cells with misaligned anaphase spindles was determined.

(E) Wt (A2266), *kin4Δ* (A12121), *dyn1Δ* (A12123), and *dyn1Δ kin4Δ* (A12122) cells containing a *TEM1-13MYC* allele were arrested in α factor at 25°C and released into medium at 16°C. Samples were collected at 5, 5.5, 6, and 6.5 hr after release to analyze Tem1 localization at SPBs.

to a lesser extent than observed in *dyn1Δ* cells. Our findings show that *KIN4* is required to inhibit exit from mitosis when the anaphase spindle is misaligned, irrespective of whether the microtubule cytoskeleton is intact or not.

Tem1 Associates with SPBs in *dyn1Δ kin4Δ*, but Not in *dyn1Δ* Cells with Mispositioned Anaphase Spindles

To begin to address how *KIN4* inhibits MEN signaling in cells with mispositioned anaphase spindles, we asked whether localization of MEN components was affected in cells lacking *KIN4*. *KIN4* could be required in the spindle position checkpoint in a manner similar to the septin ring component Shs1 in that it prevents the diffusion of Lte1 into the mother cells (Castillon et al., 2003). However, *dyn1Δkin4Δlte1Δ* accumulated anucleate and multinucleate cells to the same extent as *dyn1Δkin4Δ* cells (Figure 5B), and deletion of *KIN4* did not affect the localization of Lte1 (data not shown). Together these results show that *KIN4* prevents exit from mitosis in cells with misaligned anaphase spindles in an *LTE1*-independent manner. Because our epistasis analyses indicated that *KIN4* functions near or at the top of the MEN to inhibit signaling through this pathway (Figure 2D), we next examined the localization of Bub2 and

Bfa1 in *kin4Δ* and *dyn1Δkin4Δ* cells. Inactivation of *KIN4* did not significantly affect the localization of Bub2 and Bfa1 in cells with correctly positioned spindles (Figure 5C) or Bfa1 localization in cells with misaligned anaphase spindles (Figure 5D). Thus, the localization of the GAP complex is not affected by *KIN4*.

During metaphase and anaphase of an unperturbed cell cycle, Tem1 localizes to the SPB destined to migrate into the bud (Bardin et al., 2000; Pereira et al., 2000; Figure 5E). In *dyn1Δ* mutants, localization is impaired in that Tem1 localizes to both SPBs in the majority of cells with correctly positioned anaphase spindles and not at all in the majority of cells with mispositioned anaphase spindles (Figure 5E, Figure S3). The reasons for these localization defects are at present unclear. It is, however, interesting to note that deletion of *KIN4* restores localization of Tem1 to both SPBs in *dyn1Δ* mutants with mispositioned spindles (Figure 5E, Figure S3). It is possible that Kin4 masks the epitope tag on Tem1 on SPBs in *dyn1Δ* cells, and deletion of the gene allows detection of Tem1. However, because localization of Bfa1 and Bub2, which form a complex with Tem1, is not affected, we favor the conclusion that Kin4 prevents the association of Tem1 with SPBs in cells with mispositioned spindles.

Kin4 Is Active in Metaphase-Arrested Cells and Anaphase Cells with Mispositioned Spindles

To gain insight into how Kin4 regulates MEN activity, we examined the regulation of Kin4 itself. Kin4 is present throughout the cell cycle with little or no fluctuation in protein levels (Figure 6A), though its mobility in PAGE changes. The slower migrating species of Kin4 culminated during exit from mitosis and G1 but were absent during S phase and metaphase (Figure 6A). Immunoprecipitation of Kin4 led to a dramatic loss of the slower migrating species of Kin4 in the precipitate (Figure S4), indicating that we either failed to immunoprecipitate these Kin4 species and/or that they were highly unstable. However, treatment of immunoprecipitated Kin4 led to a small but reproducible reduction in slower migrating forms of Kin4, indicating that they were due to phosphorylation (Figure S4). Our results suggest that phosphorylation of Kin4 changes during the cell cycle.

Although Kin4 protein is readily detectable, Kin4 kinase activity was barely if not undetectable in exponentially growing cells when measured with several artificial substrates in vitro (Figure 6B; data not shown). Even when the protein was overproduced from the galactose-inducible *GAL1-10* promoter did we detect little kinase activity in cells progressing through the cell cycle in a synchronous manner (Figure 6C). However, by using myelin basic protein (MBP) as a substrate, we did detect kinase activity associated with endogenous (Figure 6B) and overproduced Kin4 (Figure 6C) in nocodazole-arrested and Cdc20-depleted cells (data not shown) at room temperature. Despite difficulties in detecting Kin4 activity, the low levels of activity detected in Kin4 precipitates were nevertheless due to Kin4, because MBP phosphorylation was reduced to background levels in cells carrying a kinase-defective allele of *KIN4* (Figure 6D). These findings indicate that Kin4 is active during a metaphase arrest irrespective of whether the microtubule cytoskeleton is intact or not and irrespective of whether the spindle is positioned correctly (Cdc20 depletion) or not (nocodazole).

Next, we examined Kin4 activity during anaphase. *cdc15-2* mutants arrest in anaphase with correctly positioned spindles. In these cells, Kin4 activity was detectable but was very low with a specific activity of 1.96 AU, which is similar to that of exponenitally growing cells (1.44 AU; Figure 6B). The specific activity was increased to 4.57 AU in *kar9Δ act5ts* cultures, in which 20% of cells contained misaligned anaphase spindles (Figure 6B). This difference in Kin4 activity between *cdc15-2* and *kar9Δ act5ts* mutants was neither due to less Kin4 protein being present in *cdc15-2* mutants than in *kar9Δ act5ts* mutants nor a decreased ability to immunoprecipitate Kin4 from *cdc15-2* mutant extracts (Figure 6B). Our results suggest that Kin4 kinase activity is low in cells with correctly positioned anaphase nuclei and slightly increased in cells with mispositioned anaphase nuclei.

Kin4 Localizes to the Mother Cell Cortex

Next, we examined the localization of Kin4 within cells. The only tagged version of Kin4 that was weakly detectable within cells at endogenous levels was a Kin4-GFP fusion. Three-dimensional deconvolution microscopy of exponentially growing cells showed that Kin4-GFP localized to the cell cortex during G1 (Figure 7A). As buds started to form, Kin4 remained at the cortex of the mother cell and did not enter the bud. Kin4 was present in the mother cell throughout most of the cell cycle. In large-budded cells, however, Kin4 was also sometimes seen at the mother-bud neck, and restriction to the mother cell cortex was less pronounced (Figure 7A). Kin4-GFP was also detected transiently on the mother cell SPB during anaphase (Pereira and Schiebel, 2005). Similar results were obtained when Kin4-GFP was overproduced (Figure S5).

To determine when exactly during mitosis Kin4 diminished from the mother cell cortex and associated with the mother-bud neck, we marked SPBs by using an Spc42-CFP fusion. The CFP signal exhibited weak emission in the GFP emission spectrum, allowing us to examine Kin4-GFP and Spc42-CFP simultaneously by using a GFP filter set. This analysis revealed that in budded cells with an SPB distance between 0 and 2 μm, which represents cells in S phase and metaphase, Kin4-GFP localized exclusively to mother cells (Figure 7A, S phase; n = 12). In 60% of cells with an SPB distance of 3–7 μm, which represents cells in anaphase, Kin4-GFP localized to the mother cell cortex and the mother-bud neck (Figure 7A, early anaphase; n = 22). In the remaining 40%, Kin4-GFP was only associated with the mother cell cortex (data not shown). In most cells with an SPB distance of 7 μm or more, which represents cells in late anaphase that either exit from mitosis or have just completed this transition, Kin4 localization at the mother cell cortex diminished (Figure 7A, late anaphase; n = 8). These results indicate that during S phase and early stages of mitosis, Kin4 is almost exclusively found in the mother cell. During anaphase, Kin4-GFP appears to spread first to the bud neck and

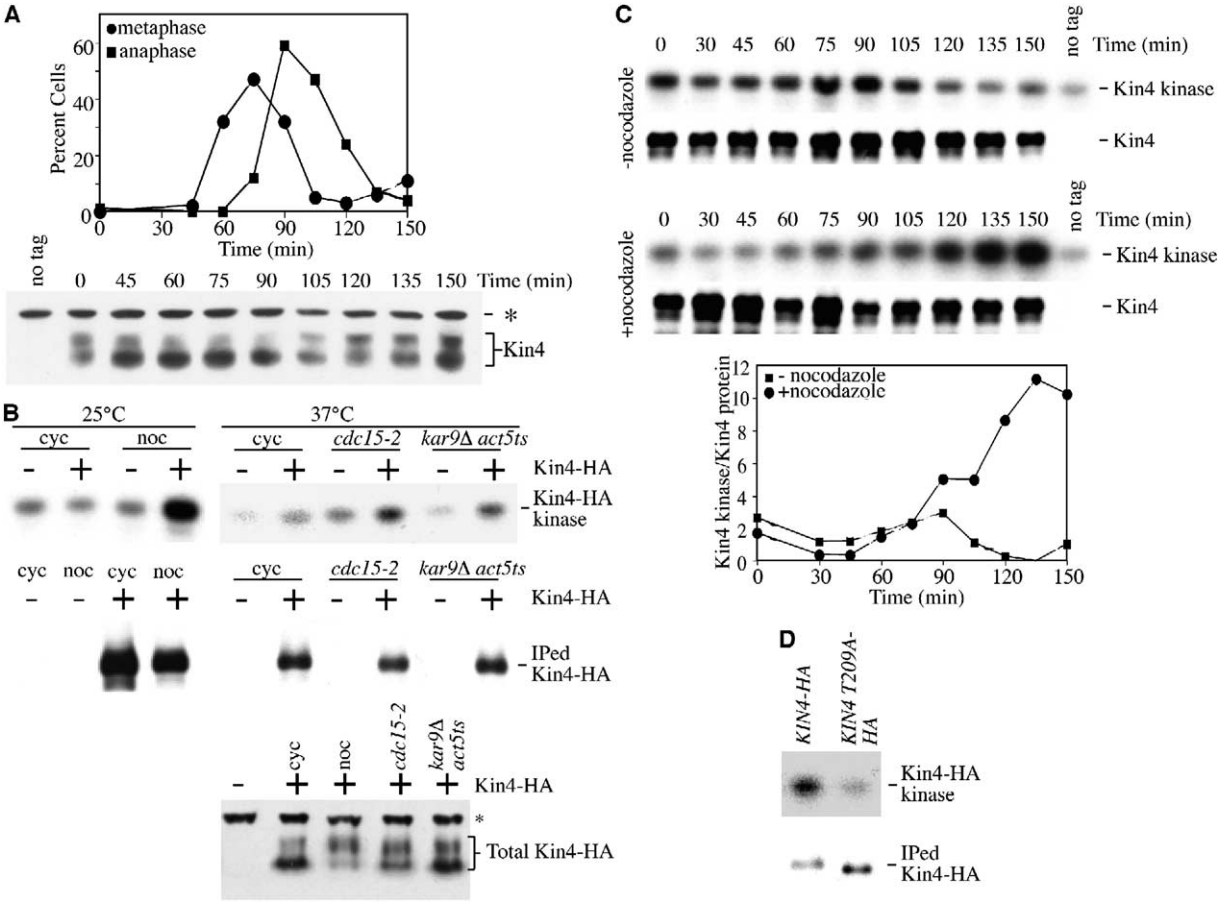

Figure 6. Characterization of Kin4 during the Cell Cycle

(A) Wt cells carrying a *KIN4-HA* fusion (A9233) were treated as described in Figure 1B. Kin4 protein was monitored throughout the cell cycle by Western blot analysis.

(B) Wt cells carrying a *KIN4-HA* fusion (A9233) or lacking the fusion (A2587) were grown to exponential phase (cyc) or arrested with nocodazole (noc) either at 25°C or 37°C. *cdc15-2* (A2596), *cdc15-2 KIN4-HA* (A11781), *kar9Δ act5^{ts}* (A2025), and *kar9Δ act5^{ts} KIN4-HA* (A12003) cells were grown at 37°C for 2 hr. Kin4-associated kinase activity (top, Kin4 kinase), the amount of immunoprecipitated Kin4-HA (middle, IPed Kin4), and the total amount of Kin4-HA in cell extracts (bottom, total Kin4-HA) were determined. An asterisk indicates a crossreacting band that serves as a loading control.

(C) *GAL-HA-KIN4* (A11998) cells were arrested with α factor as described in the legend to Figure 2A. Transcription of *KIN4* was shut off by glucose addition upon release from the block (squares, top) or containing 15 μg/ml nocodazole (circles, bottom). Kinase activity and immunoprecipitated Kin4 were measured at the indicated times. The graph illustrates the amount of kinase activity/immunoprecipitated Kin4 protein.

(D) Kin4-associated kinase activity in nocodazole-arrested cells carrying a *KIN4-HA* fusion (A13474) or a *KIN4-HA* fusion in which Thr209 in the activation loop was mutated to alanine (A13476). The analogous mutation in Snf1, the closest characterized homolog of Kin4, has been shown to lead to loss of Snf1 activity (McCartney and Schmidt, 2001).

then during late stages of anaphase diminishes from the mother cell cortex.

We also analyzed Kin4 localization in *dyn1Δ* cells in which the anaphase spindle was mispositioned (Figure 7B; n = 8). This analysis revealed that Kin4 associated with the mother cell cortex in these cells and was not found at the mother-bud neck. The signal at the mother cell cortex was lower than that seen in wt cells that are in early anaphase but comparable to that observed in late anaphase wt cells. However, in contrast to wt late anaphase cells, some Kin4-GFP accumulated in the vacuole. Our results indicate that in cells with mispositioned spindles, Kin4 localizes predominantly to the mother cell, where it is ideally situated to inhibit MEN signaling at SPBs when anaphase occurred within the mother cell.

Discussion

We have identified the protein kinase Kin4 as a component of the spindle position checkpoint. Our data indicate that Kin4 prevents exit from mitosis in cells with mispositioned spindles by inhibiting the MEN. The observation that Kin4 kinase activity is detectable in cells with mispositioned anaphase spindles and the fact that deletion of *KIN4* does not cause defects during an unperturbed cell cycle indicate that the protein kinase's main function is in the spindle position checkpoint. The

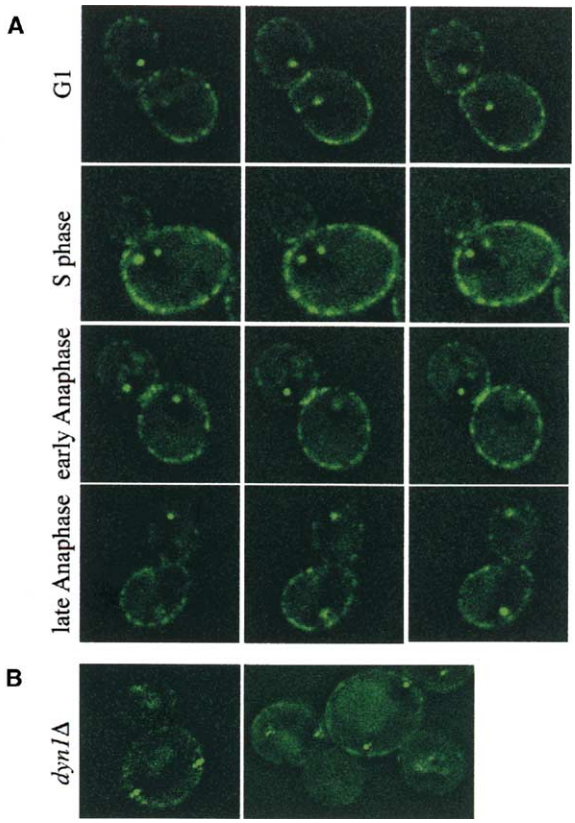

Figure 7. Kin4 Localizes to the Mother Cell Cortex during Most of the Cell Cycle

(A) The top panel shows two cells carrying a *KIN4-GFP* and an *SPC42-YFP* fusion (A12388) that just completed cytokinesis (G1). The second panel shows a cell in S phase as judged by the distance between SPBs. The third and fourth panels show a cell in early and late anaphase, respectively. SPBs are identified as green foci in the cell. Each panel shows deconvolved images of three serial sections.

(B) *dyn1::URA3* cells carrying a *KIN4-GFP* fusion (A12582) were analyzed as described in (A), and cells with mispositioned spindles were examined.

finding that Kin4 localizes predominantly to the mother cell furthermore raises the interesting possibility that Kin4 establishes a domain of MEN inhibition within the mother cell.

A Genetic Selection Identifies *KIN4* as a Negative Regulator of the MEN

Our genetic selection was designed to identify negative regulators of both the FEAR network and the MEN. Subsequent studies showed that *KIN4* functions to inhibit MEN activity rather than FEAR network function. Although *KIN4* can function as an inhibitor of the MEN, the question arises as to whether it does so during every cell cycle. The observation that deletion of *KIN4* suppresses the synthetic lethality of *lte1Δ spo12Δ*, *lte1Δ slk19Δ* cells, and *lte1Δ esp1-1* double mutants suggests so. However, during an unperturbed cell cycle, deleting *KIN4* has no effect on cell cycle progression in general and exit from mitosis in particular. Even under sensitizing conditions, in a nocodazole arrest when the MEN inhibitory effects of *BUB2* and *BFA1* are readily detectable (Stegmeier et al., 2002; Yoshida et al., 2002; Figure 4A), *KIN4* didn't reveal itself as a negative regulator of MEN activity. It is possible that a role for *KIN4* in an unperturbed cell cycle is masked by a redundant factor present in the yeast genome. However, inactivation of the closest homolog of *KIN4*, *YPL141c* (the two protein kinases are 43% identical and 56% similar), in a *kin4Δ* background did not expose a defect in cell cycle progression (K.E.D., unpublished data). Thus, we conclude that *KIN4* serves a minor role in inhibiting the MEN during an unperturbed cell cycle but is essential for restraining MEN activity in response to activation of the spindle position checkpoint.

How Does Kin4 Inhibit MEN Activity?

Our analysis of the posttranslational modifications occurring on Bfa1 and Bub2 sheds light on this question. Neither Bub2 nor Bfa1 were fully phosphorylated in cells overexpressing *KIN4*, suggesting that Kin4 inhibits MEN activity by preventing the dissociation of the GAP from Tem1. Kin4 could regulate Cdc5 function, either prohibiting access to its substrates at SPBs or inhibiting its kinase activity. The observation that inactivation of *KIN4* did not change the phosphorylation pattern of Bfa1 in *cdc5-2* mutants (Figure 3C) would be consistent with this idea. However, deletion or overexpression of *KIN4* affected neither Cdc5 localization nor its kinase activity (K.E.D., unpublished data), suggesting that Kin4 functions in parallel to Cdc5, perhaps inducing a phosphatase that dephosphorylates Bfa1 and Bub2.

Although we do not know how, Kin4 appears to restrain exit from mitosis in part by maintaining Bub2-Bfa1 in its active form. But why then do *kin4Δ* cells with mispositioned spindles exit from mitosis but nocodazole-arrested *kin4Δ* cells do not? It is possible that Kin4 and Bub2-Bfa1 exert different degrees of inhibition on Tem1, such that the GAP complex retains some activity in the absence of *KIN4*. However, the fact that deletion of *BUB2* or *BFA1* does not enhance the phenotype of *dyn1Δ kin4Δ* double mutants argues against a quantitative difference in GAP activity between cells deleted for *KIN4* and a GAP complex component. We therefore favor the idea that the different effects of *KIN4* and *BUB2-BFA1* on a nocodazole and *dyn1Δ* arrest are due to the fact that in *dyn1Δ* cells with mispositioned spindles, only the spindle position checkpoint restrains Cdc14 activation, whereas Cdc14 activation is prevented by both the spindle position and the spindle assembly checkpoint in nocodazole-treated cells. Thus, in *dyn1Δ* cells, deletion of the spindle position checkpoint component *KIN4* causes bypass of the arrest. In nocodazole-arrested cells, deleting *KIN4* only alleviates the inhibitory effects of the spindle position checkpoint, but not that of the spindle assembly checkpoint on MEN activity, which we speculate affects Bub2-Bfa1 in a *KIN4*-independent manner. Thus, nocodazole-treated *kin4Δ* cells would remain arrested.

High levels of Kin4 not only affected the phosphorylation status of the Bub2-Bfa1 complex but also led to loss of Bfa1 from SPBs in 50% of cells. We do not know whether this loss of Bfa1 from SPBs reflects an

additional mode whereby *KIN4* regulates Bub2-Bfa1 or whether it is an artifact of overexpressing *KIN4*. Given that deletion of *KIN4* did not affect Bfa1 localization, the latter interpretation is a distinct possibility. Though the effects of Kin4 on Bfa1 localization are questionable, the effects of the protein kinase on Tem1 localization are not. Overexpression of *KIN4* led to loss of Tem1 from SPBs during metaphase and anaphase. Conversely, deletion of *KIN4* allowed Tem1 to associate with SPBs in cells with misaligned anaphase spindles, when the protein is normally not found on SPBs. We do not know how Kin4 affects Tem1 localization. The loss of Tem1 from SPBs could be an indirect consequence of Kin4 affecting Bub2-Bfa1 activity. However, given that Bfa1 and Bub2 localization is not affected by deleting *KIN4*, and given that Tem1 can associate with SPBs in the absence of the GAP (Gruneberg et al., 2000; Pereira et al., 2000; this study), it is likely that Kin4's effect on Tem1 localization represents an additional level of Kin4 control on MEN activity.

Regulation of Kin4 Activity and Localization

Kin4 encodes a protein kinase, with the protein kinase domain in the N terminus of the protein and an extended C-terminal domain that bears no homology to known proteins or protein motifs. Our analysis of Kin4 protein revealed that the protein kinase is regulated at at least two levels: (1) the migration of Kin4 in SDS PAGE, which is likely to be due to changes in Kin4 phosphorylation, changes during the cell cycle, and (2) Kin4 localizes to distinct regions of the cell in a dynamic manner. Are these events causally related, in that one is a consequence of the other? We observe a correlation between the phosphorylation state of Kin4 and its localization. Kin4 appears to be localized throughout the mother cell cortex during S phase and early mitosis, when the protein is hypophosphorylated. The protein begins to spread to the bud neck and diminishes at the mother cell cortex when the protein becomes phosphorylated. It is thus tempting to speculate that an unknown protein kinase that is active during anaphase causes this change in localization.

We know of no protein that shares Kin4's exact localization pattern, though some resemblance exists with Stt4, Sfk1, and Num1 (Audhya and Emr, 2002; Heil-Chapdelaine et al., 2000). Stt4 encodes 1-phosphatidylinositol 4-kinase and Sfk1, a transmembrane protein that anchors Stt4 at the plasma membrane. In cells with small buds, both proteins are enriched at the mother cell cortex, and in large-budded cells, the protein is also detected in the bud (Audhya and Emr, 2002). However, the two proteins are not enriched at the mother-bud neck during anaphase. Num1 is required for dynein-dependent sliding of microtubules along the mother cell cortex into the bud (Heil-Chapdelaine et al., 2000). The protein, like Kin4, localizes to the mother cells in budded cells. However, Num1 does not localize to the mother-bud neck but is instead found at the bud tip in large-budded cells (Heil-Chapdelaine et al., 2000). Any of the three proteins could function to promote Kin4 localization at the mother cell cortex, but a dependence on Num1 would be particularly attractive. Num1 and dynein function together to slide microtubules along the cell cortex, which is required for nuclear position. Deletion of *KIN4* causes a slight spindle position defect at low temperatures, raising the possibility that the protein serves an accessory function in the Num1-Dyn1 microtubule sliding mechanism. The lack of a putative interaction between Num1 and Dyn1 could thus function as a signal to activate the spindle position checkpoint functions of Kin4. The fact that Kin4 activity is slightly increased in anaphase cells with mispositioned spindles compared to anaphase cells with correctly positioned spindles, even though only 20% of *kar9Δ ac5ts* cells have mispositioned anaphase spindles whereas all *cdc15-2* cells have correctly positioned anaphase spindles furthermore, raises the interesting possibility that Kin4's kinase activity is stimulated by spindle misposition.

A Model for How Exit from Mitosis Is Inhibited in Cells with Mispositioned Anaphase Nuclei

Our results suggest the following model for how exit from mitosis is inhibited when anaphase spindle elongation occurs in the mother cell. The checkpoint system is defined by an activator of the MEN, Lte1, in the bud and an inhibitor of the MEN, Kin4, enriched in the mother cell. Whereas Lte1 creates a domain of MEN activation in the bud, Kin4 establishes a domain of MEN inhibition within the mother cell. MEN signaling and exit from mitosis would thus only occur when movement of the daughter bound SPB, which functions as the MEN signaling center, out of the Kin4 domain into the Lte1-containing bud takes place. Thus, MEN signaling would be coupled to spindle position.

How does Kin4 inhibit activation of the MEN? We propose that Kin4 activates mechanisms that promote dephosphorylation of the GAP complex. Inhibition of Bfa1 and Bub2 phosphorylation in turn prevents its dissociation from Tem1 and perhaps inactivation of its GAP activity. Kin4 also affects the association of Tem1 with SPBs either through regulating Bub2/Bfa1 phosphorylation or through an independent mechanism. The spindle position checkpoint also appears to function in *S. pombe* and Rat epithelial cells (Oliferenko and Balasubramanian, 2002; O'Connell and Wang, 2000). It will be interesting to determine whether homologs of Kin4 in these species are involved in surveying spindle position and signaling defects in this process to the cell cycle machinery.

Experimental Procedures

Yeast Strains and Growth Conditions

All strains are derivatives of W303 (A2587) and are listed in Table S1. The *KIN4-HA*, *kin4::kanMX*, *KIN4-GFP*, *GAL-HA-KIN4*, *GAL-GFP-KIN4*, and *SPC42-CFP* strains were constructed by a PCR-based method (Longtine et al., 1998). All tagged version of *KIN4* were functional as judged by a failure of the tagged proteins to suppress the synthetic lethality of *lte1Δ spo12Δ* mutants. To generate a *GAL-KIN4* fusion, the *KIN4* open reading frame was ligated under the control of the *GAL1-10* promoter fragment. A kinase-dead version of Kin4 was created by mutating Thr209 to alanine by using the QuikChange XL Site-Directed Mutagenesis Kit. *cdc15-as1* (L99G) was generated by two-step gene replacement at the *CDC15* locus. Growth conditions for individual experiments are described in the figure legends.

Isolation of Second-Site Suppressors of *lte1Δ spo12Δ* and *lte1Δ slk19Δ* Cells

lte1Δ spo12Δ and *lte1Δ slk19Δ* cells were mutagenized by using transposon mutagenesis (Burns et al., 1994). Linkage analysis revealed four insertions in 66,000 mutagenized *lte1Δ slk19Δ* colonies and six insertions in 48,000 mutagenized *lte1Δ spo12Δ* colonies that suppressed the synthetic lethality. In the *lte1Δ slk19Δ* strain, two insertions in *BUB2* and two in *KIN4* were identified. In the *lte1Δ spo12Δ* strain, we found one insertion in *BUB2*, one in *BFA1*, and four in *KIN4*.

Immunoblot Analysis

Immunoblot analysis to determine the total amount of Clb2, Dbf2-MYC, and Kar2 was performed as described in Cohen-Fix et al. (1996). For Western blot analysis of Kin4-HA, Bub2-HA, and Bfa1-HA, cells were incubated for 10 min in 5% trichlor acetic acid. Cells were broken in 100 μl lysis buffer (50 mM TRIS [pH 7.5], 1 mM EDTA, 50 mM DTT, and complete EDTA-free protease inhibitor cocktail [Roche]) with glass beads for 40 min and boiled in sample buffer.

Fluorescence Microscopy

Indirect in situ immunofluorescence methods and antibody concentrations for Cdc14-3HA were as described previously (Visintin et al., 1999). Primary anti-MYC 9E10 antibodies (Covance) were used at 1:1000 for Sli15-13MYC and 1:750 for Tem1-13MYC. Primary anti-HA mouse antibodies (Covance) were used at 1:500 for Bub2-HA and Bfa1-HA. Secondary anti-mouse antibodies (Jackson Laboratories) were used at 1:500 for Sli15-13MYC, 1:1000 for Tem1-13 MYC, and 1:2000 for Bub2-HA and Bfa1-HA. At least 100 cells were analyzed per time point. GFP fusions were analyzed on an Axiovert 200 M inverted microscope. Slidebook Software version 4 was used for deconvolution processing of the images.

Kin4 Kinase Assays

Cell extracts were made by using IP buffer (1% NP40, 150 mM NaCl. 50 mM Tris [pH 7.5], 60 mM β-glycerophosphate, 0.1 mM $NaVO_3$, 15 mM para-Nitro-phenyl phosphate, complete EDTA-free protease inhibitor cocktail [Roche], and 1 mM DTT). Kin4-HA was immunoprecipitated by using anti-HA antibodies and protein G-coupled beads. Kinase assays were performed in 20 mM HEPES (pH 7.4), 0.5 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$, and 50 μCi γP^{32}-ATP, and 2 μg MBP were added to each sample and allowed to incubate for 15 min at room temperature.

Supplemental Data

Supplemental Data include five figures and one table and are available with this article online at http://www.molecule.org/cgi/content/full/19/2/223/DC1/.

Acknowledgments

We are grateful to Pedro Carvalho and David Pellman for help with Kin4-GFP analysis and helpful discussions, to Frank Stegmeier for Figure S1, Elmar Schiebel and Gislene Pereira for communicating results prior to publication, and Frank Solomon and members of the Amon Lab for critically reading the manuscript. K.E.D. is a predoctoral fellow of the Howard Hughes Medical Institute. This work was supported by grant AI/CA44009 to K.M.S. J.P. is funded by a National Science Foundation predoctoral fellowship and a National Institutes of Health grant GM 56800 to A.A., who is also an investigator of the Howard Hughes Medical Institute.

Received: January 31, 2005
Revised: April 25, 2005
Accepted: June 1, 2005
Published: July 21, 2005

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