Identification of Novel Regulators of Mitotic Exit in

Saccharomyces cerevisiae

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by

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Abstract

The division of a eukaryotic cell into two daughter cells is controlled by cyclin dependent kinase (CDK). Entry into mitosis is promoted by the activity of CDK complexed with mitotic cyclins. Upon faithful segregation of a full complement of DNA between each daughter cell, exit from mitosis proceeds. In order for cells to exit from mitosis and enter into G1, mitotic CDKs must be inactivated. In *Saccharomyces cerevisiae*, mitotic exit is regulated by two signaling networks, the mitotic exit network (MEN) and the Cdc14 early anaphase release (FEAR) network. In this budding yeast, coordination of nuclear migration and mitotic exit is critical to prevent aneuploidy. 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. The work presented here describes two screens that have isolated novel regulators of mitotic exit. A model for the regulation of mitotic exit by the gene *KIN4* is proposed. This work identifies the protein kinase Kin4 as a component of the spindle position checkpoint.
Dedicated to my parents
for all their love, support,
and encouragement
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Background

Results and Discussion

Inhibition of mitotic exit through overexpressioin of KIN4
  is dependent on BUB2

Kin4 does not regulate Cdc5 kinase activity or localization

Kin4 kinase activity is required for the spindle position checkpoint

KIN4 interacts genetically with MEN components TEM1, CDC5, and NUD1

YPL141c, a kinase with similarity to KIN4, is not an inhibitor of
  mitotic exit

Experimental Procedures

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Appendix II: The stress-activated MAP kinase signaling cascade
  promotes exit from mitosis

Results and Discussion

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Chapter I:

Introduction
Summary

A cell must duplicate its DNA, segregate the two copies of the DNA from each other, and then divide to produce two daughter cells. Preserving the sequence of these events in the cell division cycle is instrumental in maintaining faithful chromosome segregation and thus cell survival. Therefore mechanisms are in place to assure that exit from mitosis and cytokinesis do not occur before chromosome segregation has been completed. In the budding yeast *Saccharomyces cerevisiae*, mechanisms that control exit from mitosis have been well characterized. Two signaling networks, the mitotic exit network (MEN) and the Cdc14 early anaphase release (FEAR) network, regulate this transition and act to ensure that chromosome segregation between the mother and daughter cell is completed prior to exit from mitosis. The thesis presented here describes two methods that have identified novel regulators of mitosis in budding yeast. Characterization of one of these genes, *KIN4*, has shed light into how exit from mitosis is controlled by the position of the mitotic spindle.
Cell Cycle Overview

Cyclin dependent kinases control the cell cycle

The cell cycle is the sequence of events that leads to the production of two daughter cells from a single parent cell. Cyclin dependent kinases (CDKs) control progression through the cell cycle in all eukaryotes (reviewed in Morgan, 1997; Nasmyth, 1996). In budding yeast a single CDK (Cdc28) forms a complex with various regulatory subunits termed cyclins, which determines the specificity of the CDK (Figure 1). As their names imply, cyclin protein levels vary throughout the cell cycle, which provides a specific cycle of CDK activity. G1 cyclin (Cln1, Cln2, and Cln3 in budding yeast) CDK complexes promote entry into the cell cycle, which provides a specific cycle of CDK activity. S phase cyclins (Clb5 and Clb6) begin to accumulate at the end of G1 and are inactivated during G2 and mitosis. Active S phase cyclin CDK complexes induce DNA synthesis. Mitotic cyclin (Clb1, Clb2, Clb3, and Clb4) CDK complexes become active at the onset of mitosis and promote many processes including chromosome condensation and the formation of the mitotic spindle. In order for cells to exit mitosis, complete cytokinesis, and enter G1 of the next cell cycle, mitotic cyclins must be degraded (Koepp et al., 1999; Morgan, 1997).

Overview of Mitosis

Mitosis is the stage of the cell cycle when the chromosomes that were duplicated during S phase to form a pair of sister chromatids are segregated. To ensure that chromosomes are faithfully segregated, the two sister chromatids are held together until the metaphase to anaphase transition during mitosis (Figure 1). Concomitant with DNA synthesis a protein complex termed cohesin is formed around the two sister chromatids to hold them together (reviewed in Hirano, 2000).

Mitosis can be divided into four stages: prophase, metaphase, anaphase, and telophase. During prophase chromosomes condense and the mitotic spindle is formed. Sister chromatids attach to the mitotic spindle and are aligned in the middle of the spindle during metaphase. To ensure proper segregation, only when all pairs of sister chromatids make bipolar attachments to the mitotic spindle is cohesion between the sister chromatids lost during the metaphase to anaphase transition. This transition is triggered by the
Figure 1: CDKs regulate the cell cycle.

In *Saccharomyces cerevisiae* the cyclin dependent kinase Cdc28 associates with different cyclins to regulate the cell cycle. During G1, the levels of G1 cyclins (Cln1, Cln2, and Cln3) rise and the activity of G1 CDKs promotes entry into the cell cycle. S phase cyclins begin to increase near the end of G1 and are inactivated during G2 and mitosis. In budding yeast, Clb5 and Clb6-associated kinases are important for promoting DNA synthesis, however in their absence, the mitotic CDKs (Clb1, Clb2, Clb3, and Clb4) can substitute to initiate S phase. The mitotic CDKs are activated at the onset of mitosis and promote chromosome condensation and formation of the mitotic spindle. These functions can be performed by CDKs associated with Clb1, Clb2, Clb3, Clb4, as well as by Clb5 and Clb6. The inactivation of mitotic CDKs after completion of chromosome segregation allows cells to exit from mitosis, undergo cytokinesis, and enter G1 of the next cell cycle.
cleavage of one subunit of the cohesin complex (Scc1 in budding yeast) by the activity of the protease separase (Esp1 in budding yeast) (reviewed in Nasmyth, 2001). Esp1 is held inactive throughout most of the cell cycle by its inhibitor securin (Pds1 in budding yeast). As soon as all sister chromatids make bipolar attachments in metaphase, an ubiquitin ligase known as the Anaphase Promoting Complex or Cyclosome (APC/C) along with a specificity factor Cdc20 targets Pds1 for degradation. Once Pds1 is degraded, Esp1 becomes activated, thus allowing sister chromatids to separate and anaphase to proceed. During anaphase the sister chromatids are pulled to opposite poles of the mitotic spindle. Finally, in telophase the sister chromatids reach opposite poles, the chromosomes decondense, the spindle disassembles and then the cell divides to form two daughter cells.

**Mitotic exit in *Saccharomyces cerevisiae***

**Downregulation of Clb-CDK**

Once chromosomes have segregated, cells exit from mitosis and cytokinesis ensues. In order for cells to exit mitosis, mitotic CDK activity must be downregulated. If mitotic CDK activity remains, cells arrest in anaphase/telophase with an elongated spindle and segregated chromosomes (Surana et al., 1993). Mitotic CDK inactivation occurs through two mechanisms, degradation of the mitotic cyclins and the accumulation of CDK inhibitors.

Initiation of Clb cyclin degradation occurs at the metaphase to anaphase transition through the ubiquitination by the APC/C<sup>Cdc20</sup> (reviewed in Castro et al., 2005). In most eukaryotes the majority of Clb cylin degradation occurs at this transition, however in budding yeast a significant amount of Clb-CDK activity persists beyond this point (Surana et al., 1993). This pool of CDK activity is required to maintain spindle integrity (Grandin and Reed, 1993). During mitotic exit the remaining Clb cyclin is targeted for degradation by the action of APC/C complexed with the specificity factor Cdc20 or Cdh1 (reviewed in Morgan, 1999; Schwab et al., 1997; Visintin et al., 1997). Also at this time, the CDK inhibitor Sic1 accumulates to ensure that Clb-CDK inactivation is complete (Donovan et al., 1994; Schwob et al., 1994).
**Cdc14 promotes Clb-CDK inactivation**

The key regulator of mitotic exit in budding yeast is the protein phosphatase Cdc14. Cdc14 is required to promote the inactivation of the Clb-CDK pool that remains after anaphase onset. Cells lacking Cdc14 activity arrest with separated DNA masses, an elongated spindle and high Clb-CDK activity (Visintin et al., 1998). Conversely, CDK activity is inappropriately inhibited in cells that overexpress *CDC14* (Visintin et al., 1998). Cdc14 induces mitotic exit by reversing CDK phosphorylation (Figure 2). To date three main substrates, Cdh1, Sic1, and Swi5, have been identified that promote mitotic CDK inactivation. Dephosphorylation of Cdh1 promotes its association with the APC/C and therefore the degradation of Clb cyclins (Jaspersen et al., 1999; Visintin et al., 1998; Zachariae et al., 1998). Throughout most of the cell cycle the CDK inhibitor Sic1 is degraded in a phosphorylation dependent manner, however during mitotic exit stabilization of Sic1 is achieved through dephosphorylation by Cdc14 (Jaspersen et al., 1999; Visintin et al., 1998). *SIC1* transcription is also induced by Cdc14 dephosphorylating its transcription factor Swi5. Desphosphorylation of Swi5 allows it to translocate into the nucleus and perform its function (Knapp et al., 1996; Moll et al., 1991; Toyn et al., 1997; Visintin et al., 1998). Although these are the main substrates of Cdc14 to promote mitotic exit, it is believed that Cdc14 reverses most if not all of Clb-CDK phosphorylations thus resetting the cell to a G1 state.
Figure 2: Cdc14 controls mitotic CDK activity.

Cdc14 induces mitotic exit by reversing mitotic CDK dependent phosphorylation. Several Cdc14 substrates are important for downregulating mitotic CDKs, such as the CDK inhibitor Sic1, the transcription factor Swi5, and the APC/C specificity factor Cdh1.
Regulation of Cdc14

The fact that Cdc14 can ectopically induce Clb-CDK activation indicates that Cdc14 activity must be tightly regulated for correct cell cycle progression. Throughout most of the cell cycle Cdc14 is bound to its inhibitor Cfi1/Net1 in the nucleolus (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Cfi1/Net1 is thought to act as a competitive inhibitor by binding to the active site (Traverso et al., 2001). Upon entry into anaphase, Cdc14 is released from Cfi1/Net1 and now localizes to the nucleus and cytoplasm where it can dephosphorylate its substrates (Shou et al., 1999; Visintin et al., 1999).

Two signaling networks have been identified that promote the release of Cdc14 from its inhibitor Cfi1/Net1 (reviewed in Stegmeier and Amon, 2004). At the onset of anaphase the Cdc fourteen early anaphase release (FEAR) network becomes active to promote the release of Cdc14 from the nucleolus. At this point Cdc14 is seen throughout the nucleus and associates with the spindle pole bodies (SPBs, yeast homologue of the mammalian centrosome) and the mitotic spindle (Pereira et al., 2002; Pereira and Schiebel, 2003; Stegmeier et al., 2002; Yoshida et al., 2002). During later stages of anaphase, the mitotic exit network (MEN) becomes activated. The MEN promotes the further release of Cdc14 into the nucleus and cytoplasm and also maintains this release (Shou et al., 1999; Visintin et al., 1999).

The fact that the FEAR network and the MEN promote the same outcome, the release of Cdc14 from its inhibitor, why does the cell employ both mechanisms to regulate Cdc14? Analyzing the precise functions that Cdc14 performs when activated by only one of these networks provides insight into this phenomenon. The MEN is essential for cells to exit mitosis, evidenced by the fact that cells containing mutations in a MEN component arrest in late anaphase with segregated DNA, an elongated spindle, and high Clb-CDK activity (Jaspersen et al., 1998). On the other hand, FEAR network mutants do exit mitosis, albeit after a delay in anaphase (Pereira et al., 2002; Stegmeier et al., 2002). These findings indicate that the release of Cdc14 promoted by the MEN is sufficient to promote complete Clb-CDK inactivation, however release of Cdc14 promoted by the FEAR
network is not. This is likely due to the fact that the FEAR network is only active for a short time at the beginning of anaphase and that the FEAR network does not promote release of Cdc14 into the cytoplasm where many Cdc14 substrates are located (Jaquenoud et al., 2002; Moll et al., 1991; Nasmyth et al., 1990; Pereira et al., 2002; Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999; Yoshida et al., 2002). Close examination of FEAR network mutants has presented insight into the function of Cdc14 released early in anaphase. Cdc14 released by the FEAR network aids in chromosome segregation and regulates the localization of chromosomal passenger proteins (D'Amours et al., 2004; Pereira and Schiebel, 2003; Sullivan et al., 2004). Cdc14 released by the FEAR network is thought to promote MEN activation by dephosphorylating Cdc15, which enhances Cdc15 activity (Jaspersen and Morgan, 2000; Stegmeier et al., 2002). Employing Cdc14 to regulate a number of distinct processes acts to coordinate the regulation of these processes and thus ensures the correct sequence of events in the cell cycle.

The Mitotic Exit Network
The mitotic exit network was the first signaling cascade discovered to regulate Cdc14. Mutants in MEN components arrest in telophase with separated DNA masses, intact mitotic spindle, high Clb-CDK activity, and Cdc14 sequestered in the nucleolus (Shou et al., 1999; Visintin et al., 1999). The MEN resembles a Ras-like GTPase signaling cascade (Figure 3, Table 1; reviewed in Bardin and Amon, 2001; McCollum and Gould, 2001; Seshan and Amon, 2004; Simanis, 2003). Components of the MEN include the GTPase Tem1; the two component GTPase activating protein (GAP) Bub2-Bfa1/Byr4; the putative guanine nucleotide exchange factor (GEF) Lte1; the protein kinases Cdc15, Dbf2, Cdc5; the Dbf2 associated factor Mob1; and the scaffold protein Nud1.
Figure 3: The FEAR network and the MEN regulate Cdc14 activity in budding yeast.

During early anaphase the release of Cdc14 from its inhibitor Cfi1/Net1 is initiated by the Cdc14 Early Anaphase Release (FEAR) network. Components of the FEAR network include the kinetochore protein Slk19, separase (Esp1), polo kinase (Cdc5), the small nuclear protein Spo12 and its homologue Bns1, and the replication fork block protein Fob1. During later stages of anaphase, the Mitotic Exit Network (MEN) promotes and maintains Cdc14 in the released state. The GTPase Tem1, the two component GTPase activating complex Bub2-Bfa1, the protein kinases Cdc15 and Dbf1, and the Dbf2-associated factor Mob1 are all anchored at the spindle pole body (SPB) by the scaffold protein Nud1. Dashed lines indicate speculative interactions.
Both genetic and biochemical data have led to the model of the MEN depicted in Figure 3. The GTPase Tem1 functions at the top of the signaling cascade (Jaspersen et al., 1998; Shirayama et al., 1994b; Visintin and Amon, 2001). It is believed that the GTP bound form of Tem1 is the active form due to the comparison with the homologue Spg1 in fission yeast (Sohrmann et al., 1998), however this has not been proven for budding yeast. Tem1 is positively regulated by Lte1 and negatively regulated by the GAP complex Bub2-Bfa1/Byr4 (Alexandru et al., 1999; Geymonat et al., 2002; Hoyt et al., 1991; Krishnan et al., 2000; Li, 1999; Shirayama et al., 1994a). Upon formation of the mitotic spindle, Tem1 becomes localized to one spindle pole body (SPB) and specifically the SPB that is destined to migrate into the bud (Bardin et al., 2000; Pereira et al., 2000). The inhibitors of Tem1, Bub2-Bfa1, also localize to the daughter bound SPB and actually the localization of Tem1 to the SPB depends on Bub2 and Bfa1 (Fraschini et al., 1999; Lee et al., 1999b; Li, 1999; Pereira et al., 2000). All MEN components except for Lte1 bind to the SPB component Nud1 on the cytoplasmic face of the SPB, thus indicating that Nud1 acts as a scaffold for this signaling cascade (Adams and Kilmartin, 2000; Bardin et al., 2000; Fraschini et al., 1999; Gruneberg et al., 2000; Pereira et al., 2000; Visintin and Amon, 2001). Activated Tem1 propagates the signal to the protein kinase Cdc15, which also localizes to the SPB (Asakawa et al., 2001; Bardin et al., 2003; Lee et al., 2001a; Menssen et al., 2001; Visintin and Amon, 2001). Cdc15 then activates the protein kinase Dbf2, which must be bound to its accessory factor Mob1 (Mah et al., 2001).

The precise role of Cdc5 in promoting mitotic exit has been difficult to discern. Indeed cells lacking Cdc5 have a similar phenotype to other mutants in MEN components, that is arresting in late anaphase with separated DNA masses, elongated spindles and Cdc14 in the nucleolus (Jaspersen et al., 1998; Kitada et al., 1993; Lee et al., 2001a; Shou et al., 1999; Visintin et al., 1999). Genetic analysis has not been able to place Cdc5 within the MEN signaling cascade and recent evidence further supports the idea that Cdc5 is not a core component of the signaling cascade but acts to regulate MEN activity through a number of mechanisms. Cdc5 appears to regulate components at the top of the MEN by inactivating the GAP complex Bub2-Bfa1 to promote Tem1 activation (Hu and Elledge, 2002; Hu et al., 2001; Lee et al., 2001b). Cdc5 also promotes Dbf2 kinase activity in a
Bub2-independent manner, through Cdc5’s role in the FEAR network (see below; Jaspersen and Morgan, 2000; Lee et al., 1999b; Stegmeier et al., 2002; Visintin et al., 2003).

Activation of the MEN

Activation of the MEN is controlled through a number of processes. As mentioned above, Cdc5 induces activation of the MEN through at least two mechanisms, inactivation of the GAP complex Bub2-Bfa1 and activation of Cdc14 through its role in the FEAR network, which will be discussed in more detail in the following section. Cdc15 activity is also thought to be regulated through an autoinhibitory C-terminal domain, however the precise mechanism is not understood (Bardin et al., 2003). The subcellular localization of MEN components also impacts coordination of MEN activation. Tem1, Bub2, and Bfa1 preferentially localize to the SPB destined to migrate into the bud, whereas Cdc15 and Dbf2 localize to both SPBs (Bardin et al., 2003; Bardin et al., 2000; Cenamor et al., 1999; Menssen et al., 2001; Molk et al., 2004; Pereira et al., 2000; Visintin and Amon, 2001; Xu et al., 2000). Therefore, the SPB appears to be a signaling center for the MEN given that all MEN components except Lte1 localize to the cytoplasmic face of the SPB at some point in the cell cycle (Bardin et al., 2003; Bardin et al., 2000; Cenamor et al., 1999; Gruneberg et al., 2000; Pereira et al., 2000; Visintin and Amon, 2001; Xu et al., 2000; Yoshida and Toh-e, 2001). Once a bud is formed, Lte1 localizes to the bud cortex; however Tem1 is localized to the SPB destined to migrate into the bud (Bardin et al., 2000; Pereira et al., 2000). Thus Tem1 only comes into contact with its activator, Lte1, when the daughter bound SPB migrates into the bud during spindle elongation in anaphase (Bardin et al., 2000; Pereira et al., 2000). This appears to be one mechanism by which the cell monitors the position of the mitotic spindle and ensures that mitotic exit only occurs when the spindle has elongated into the bud.

Mitotic exit and cytokinesis are regulated by MEN

Cdc14 activated by the MEN is responsible for downregulating mitotic CDK activity and thus promotes mitotic exit. However, Cdc14 and the MEN appear to play a role in regulating cytokinesis independently of their function in regulating mitotic exit. MEN
components Cdc15, Dbf2, Mob1, and Cdc5 localize to the bud neck, the site of cytokinesis, during late anaphase (Frenz et al., 2000; Luca and Winey, 1998; Song et al., 2000; Song and Lee, 2001; Xu et al., 2000; Yoshida et al., 2002). The localization of Dbf2 and Mob1 to the bud neck depends on Cdc14 (Frenz et al., 2000; Yoshida et al., 2002). Furthermore, when MEN function is bypassed for mitotic exit, severe defects in cytokinesis are seen (Jimenez et al., 1998; Lippincott et al., 2001; Luca et al., 2001; Shou et al., 1999; Song and Lee, 2001). Therefore mitotic exit and cytokinesis are coordinated with one another because the same proteins are involved in both processes.

The FEAR Network

Upon careful examination of the localization of Cdc14, it became apparent that Cdc14 is released from its inhibitor during early anaphase before the MEN becomes active (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). A number of genes have been identified that regulate this early anaphase activation of Cdc14 and were termed the FEAR network (Figure 3). This network consists of the polo-like kinase Cdc5, the separase Esp1, the kinetochore protein Slk19, the small nuclear protein Spo12 and its homologue Bns1, and the replication fork block protein Fob1 (Pereira et al., 2002; Stegmeier et al., 2004; Stegmeier et al., 2002; Visintin et al., 2003; Yoshida et al., 2002). Through genetic epistasis analyses the FEAR network can be divided into at least two branches. ESP1 and SLK19 function in parallel to SPO12/BNS1 and FOB1. CDC5 is likely downstream of both these branches, however this has not been proven due to the fact that Cdc5 also functions in the activation of the MEN.

Functions of Cdc14 released by the FEAR network

Although the FEAR network is not essential for completion of the cell cycle, cells do lose viability when the FEAR network is inactive (D'Amours et al., 2004). Several functions of Cdc14 released by the FEAR network have recently been discovered. Cdc14 released by the FEAR network but not the MEN promotes the segregation of telomeres and the rDNA (Buonomo et al., 2003; D'Amours et al., 2004; Granot and Snyder, 1991; Sullivan et al., 2004; Torres-Rosell et al., 2004). It has been proposed that Cdc14 regulates some aspects of chromosome condensation given that it is required for rDNA condensation and
for targeting condensins to the rDNA during anaphase (D'Amours et al., 2004; Guacci et al., 1994; Sullivan et al., 2004).

Cdc14 released by the FEAR network also regulates spindle stability during anaphase by promoting the downregulation of microtubule dynamics (Higuchi and Uhlmann, 2005). If sister chromatid separation is activated in the absence of Cdc14, chromosome movement towards the spindle poles as well as spindle elongation is hampered (Higuchi and Uhlmann, 2005). Two known substrates of Cdc14 involved in this process have been identified, Sli15 and Ask1. Dephosphorylation of Sli15 by Cdc14 in early anaphase induces the translocation of Sli15 and Ipl1 to the spindle and spindle midzone during anaphase (Pereira et al., 2001). Sli15 and Ipl1 are part of a family of chromosomal passenger proteins that localize to kinetochores during metaphase, but translocate to the spindle and spindle midzone in anaphase, which is thought to aid in the stabilization of the mitotic spindle (reviewed in Carmena and Earnshaw, 2003). Ask1 is part of the kinetochore and is also dephosphorylated in early anaphase by Cdc14 (Li and Elledge, 2003; Sullivan et al., 2004). Cells expressing Ask1 mutated at two CDK phosphorylation sites exhibit reduced microtubule dynamics at the kinetochore and improved chromosomal movement towards the spindle poles in a Cdc14 mutant (Higuchi and Uhlmann, 2005). There are likely other substrates of Cdc14 that regulate microtubule dynamics during anaphase that await identification.

Finally, Cdc14 released by the FEAR network promotes MEN activation and thus promotes its own activation in later stages of anaphase. Cdc14 dephosphorylates Cdc15 to enhance its activity. How this dephosphorylation promotes Cdc15 activity is now known (Jaspersen and Morgan, 2000; Stegmeier et al., 2002). Furthermore, Cdc14 is thought to regulate the Bub2-Bfa1 GAP complex. Cdc14 binds to Bub2 at the SPB upon release from the nucleolus and has been proposed to inactivate the GAP in a phosphatase independent manner at this time (Pereira et al., 2002; Yoshida et al., 2002). However, at the end of mitosis, Cdc14 dephosphorylates Bfa1, which is thought to reactivate the GAP complex (Geymonat et al., 2003; Pereira et al., 2002). Future studies to elucidate Cdc14’s
precise regulation of the GAP complex will be important to better understand how Cdc14 controls its own activity and how the GAP complex is regulated.

**Inactivation of Cdc14**

Inactivation of a signal can be just as important as the activation of that signal. Cells overexpressing Cdc14 are inviable, indicating that inactivation of Cdc14 after mitotic exit is important for resetting the cell to the G1 state and thus for cell cycle progression (Visintin et al., 1998; Visintin et al., 1999). It is therefore imperative that FEAR network and MEN activity are inhibited after mitotic exit.

FEAR network activity is restricted to a short window of time during early anaphase, however the precise mechanisms that control this are unknown (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). Aspects of the regulation of individual components are known, but how these data fit together to inactivate the FEAR network is still an open question. The APC/C^{Cdh1} at the end of mitosis targets both Cdc5 and Spo12 for degradation, however this does not explain why FEAR is only active during early anaphase (Charles et al., 1998; Cheng et al., 1998; Shah et al., 2001). Spo12 phosphorylation is required for its FEAR network function and it is speculated that Cdc14 may dephosphorylate Spo12 to downregulate the FEAR network (Shah et al., 2001; Stegmeier et al., 2004).

Inactivation of the MEN is better understood. Cdc14 itself induces the downregulation of the MEN and therefore leads to its own inactivation. Cdc14 dephosphorylates Bfa1, which is thought to restore the inhibition of Tem1 by Bub2-Bfa1 (Geymonat et al., 2003; Hu et al., 2001; Pereira et al., 2002). Depophosphorylation of Lte1 by Cdc14 causes the release of Lte1 from the bud cortex, which presumably reduces Tem1 activation (Bardin et al., 2000; Jensen et al., 2002; Seshan et al., 2002). As mentioned above, the MEN activator Cdc5 is targeted for degradation by the APC/C^{Cdh1}, which is activated at the end of mitosis by Cdc14 (Charles et al., 1998; Cheng et al., 1998; Shah et al., 2001). These data indicate an elegant regulation of Cdc14 activity. Cdc14 released by the FEAR network promotes its sustained release by dephosphorylating Cdc15 and thus activating
the MEN; however, Cdc14 also promotes its own deactivation inducing the
downregulation of the MEN. Therefore, the activation and inhibition of Cdc14 at the end
of mitosis are connected to one another, thus confining Cdc14 activity to a small window
of time during exit from mitosis.

Signals controlling mitotic exit
In order to maintain genomic integrity it is imperative that cells do not exit mitosis before
chromosomes have faithfully segregated given the fact that cytokinesis is an irreversible
process. A number of mechanisms have been identified that couple the activation of
Cdc14 and thus mitotic exit in budding yeast to chromosome segregation. One theme has
evolved in studying how the cell coordinates events, that the cell uses the same protein to
perform several functions, thus ensuring that multiple processes are simultaneously
regulated.

Coupling chromosome segregation and mitotic exit
Mitotic exit and chromosome segregation are coupled to another due to the fact that the
separase Esp1 and the polo kinase Cdc5 are involved in both processes (Stegmeier et al.,
2002). As mentioned above, Cdc14 activated by the FEAR network but not the MEN
plays a role in promoting the segregation of telomeres and the rDNA regions. Therefore
activation of Cdc14 in early anaphase ensures that completion of chromosomal and
activation of the MEN occur concurrently. In addition, Cdc14 released by the FEAR
network promotes the change in subcellular localization of chromosomal passenger
proteins (Pereira and Schiebel, 2003). Chromosomal passenger proteins are localized to
the kinetochores during metaphase and translocate to the spindle midzone during
anaphase. This translocation to the spindle midzone is thought to help stabilize the
mitotic spindle and therefore ensure proper segregation.

Mitotic spindle integrity and DNA damage
If the mitotic spindle is defective or if DNA damage has occurred, it is imperative that the
cell does not exit mitosis and divide before these problems have been resolved. The
spindle assembly checkpoint monitors attachment of microtubules to kinetochores and
the DNA damage checkpoint monitors damage to the DNA (reviewed in Lew and Burke, 2003; Nyberg et al., 2002). Both of these checkpoints inhibit mitotic exit by preventing the degradation of the securin Pds1 and thus cells arrest in metaphase. Pds1 stabilization prevents sister chromatid separation and activation of the FEAR network. The spindle assembly checkpoint components Bub1, Bub3, Mad1, Mad2, and Mad3 prevent Pds1 degradation by inhibiting the APC/C<sub>Cdc20</sub>, whereas the Bub2-Bfa1 GAP complex is needed to inhibit MEN activation (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Lee et al., 2001b; Li, 1999; Wang et al., 2000). Why would the MEN need to be held inactive if the APC/C<sub>Cdc20</sub> is inhibited? The current idea is that the Bub2-Bfa1 complex acts during every cell cycle but is essential to inhibit the MEN during all delays or arrests in mitosis. It has been proposed that the GAP activity is enhanced when there is damage, however direct evidence has not been provided to date (Fesquet et al., 1999; Hu and Elledge, 2002). Examination of Bfa1 modifications during activation of these checkpoints correlates with this hypothesis. Cdc5 dependent phosphorylation of Bfa1 is inhibited in response to spindle damage (Hu et al., 2001). In contrast, Bfa1 is modified in a Rad53 and Dun1 dependent manner in response to DNA damage. Further studies into how these modifications affect Bub2-Bfa1 activity are needed.

**Spindle position**

To ensure that each daughter cell receives a full complement of DNA, it is imperative that the site of cytokinesis bisects the mitotic spindle. In budding yeast, the site of cytokinesis (the bud neck) is determined long before the mitotic spindle is formed. One nucleus must be pulled into the bud to ensure proper segregation. A surveillance mechanism termed the spindle position checkpoint has been identified that ensures that these two events are coupled to one another. The first evidence for a spindle position checkpoint came from analyzing cells lacking the microtubule motor dynein (Yeh et al., 1995). When the spindle resided entirely within the mother cell, mitotic exit was delayed indicating that the cell was monitoring the position of the spindle (Yeh et al., 1995). Two partially redundant mechanisms to position the spindle along the mother-bud axis have been identified in budding yeast (reviewed in Heil-Chapdelaine et al., 1999; Pearson and Bloom, 2004). Astral microtubules generated from the SPB are very dynamic and are
continuously scanning the mother cell and bud cortex. One pathway to position the spindle involves microtubule motors, dynein, Kar3, Kip2 and Kip3 to slide astral microtubules along the cortex. The second pathway involves capture of microtubule ends at the bud cortex by Kar9 along with Bni1, Bud6, myosin, and actin (Beach et al., 2000; Miller et al., 2000; Yin et al., 2000).

The Bub2-Bfa1 complex is required to inhibit mitotic exit in response to misaligned spindles (Adames et al., 2001; Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Pereira et al., 2000; Wang et al., 2000). Deleting either BUB2 or BFA1 allows cells with spindles contained entirely within the mother cell to exit mitosis, thereby generating multinucleate and anucleate cells. Bub2 and Bfa1 along with Tem1 are localized to the SPB destined to migrate into the bud and this localization of Tem1 is dependent on Bub2 and Bfa1 (Bardin et al., 2000; Fraschini et al., 1999; Lee et al., 1999a; Li, 1999; Pereira et al., 2000). In cells with misaligned spindles, these proteins are now at both SPBs indicating that the Bub2-Bfa1 complex is influenced by spindle position (Pereira et al., 2001). The specific mechanism that relates spindle position to Bub2-Bfa1 localization and activity is not known, but one suggestion is that interaction of astral microtubules with the bud neck may play a role (Adames et al., 2001; Pereira et al., 2001).

Another mechanism that acts to inhibit mitotic exit when the spindle is mispositioned depends on the spatial segregation of the MEN components Tem1 and Lte1 (Figure 4). Tem1 is localized to the SPB destined to migrate into the bud, whereas its activator Lte1 is sequestered to the bud cortex once a bud is formed (Bardin et al., 2000; Pereira et al., 2000; Seshan and Amon, 2004). Therefore only when the Tem1 bearing SPB migrates into the bud during spindle elongation in anaphase will Tem1 become in contact with its activator Lte1 (Bardin et al., 2000; Pereira et al., 2000). Two lines of evidence have confirmed that this spatial segregation is important to inhibit mitotic exit when the spindle is misaligned. Overexpression of Lte1 causes the protein to be localized to both the mother and the bud, which allows cells with misaligned spindles to inappropriately
Figure 4: Mechanisms sensing spindle position in budding yeast.

(A) In the absence of the microtubule motor dynein (\textit{dyn1}\Delta), approximately 10\% have misaligned spindles such that spindle elongation occurs entirely within the mother cell. These cells do not exit mitosis until the spindle becomes properly oriented along the mother-bud axis due to the activity of the spindle position checkpoint.

(B) The spatial segregation of the MEN components Tem1 and Lte1 until the migration of the Tem1-bearing SPB into the bud is important for preventing inappropriate mitotic exit in cells containing misaligned spindles. Overexpression of Lte1 (GAL-LTE1) causes defects in Lte1 localization, which allows cells with misoriented spindles to exit from mitosis and results in the accumulation of multinucleate and anucleate cells.

(C) Proteins in the mother cell also involved in sensing the position of the mitotic spindle. Deletion of the protein kinase Kin4, which localizes to the mother cell cortex and the mother SPB, allows cells with misaligned spindles to exit from mitosis inappropriately. Kin4 likely regulates Tem1 in a Lte1-independent manner.
exit mitosis generating multinucleate and anucleate cells (Bardin et al., 2000). In addition, if the septin ring is disrupted, Lte1 is no longer maintained in the bud and cells with misaligned spindles exit mitosis (Castillon et al., 2003). However, this cannot be the only mechanism that monitors spindle position because certain mutants with misaligned spindles have been shown to exit mitosis even in the absence of Lte1 and also given the fact that Lte1 is not essential under most conditions (Adames et al., 2001; Castillon et al., 2003).

Recent evidence has indicated that signals from the mother cell also act to inhibit mitotic exit (D'Aquino et al., 2005; Pereira and Schiebel, 2005). The protein kinase Kin4 is required to inhibit mitotic exit in cells with misaligned spindles. Biochemical and genetic data indicate that Kin4 regulates the Bub2-Bfa1 complex by preventing its phosphorylation, thus preventing its inactivation and MEN activation. Localization of Kin4 to the mother cell cortex and to the mother SPB implies that Kin4 acts to create a zone of MEN inhibition within the mother cell (Figure 4). Therefore only when the SPB bearing Tem1 moves out of the inhibitory domain created by Kin4 in the mother cell into the bud containing the activator Lte1, will mitotic exit be induced. Whether Kin4 is constitutively active and/or plays a role in monitoring spindle position is not known. However it is likely that astral microtubules play a role due to the fact that Kin4 is present at the SPB and the cell cortex.

Monitoring spindle position does not appear to be unique to budding yeast. A centrally placed cleavage site ensures that each daughter cell receives a similar number of organelles and molecular components. In contrast, asymmetric cell divisions can aid in determining cell fate during embryonic development (Strome, 1993). In fission yeast it is believed that the position of the nucleus determines the site of cytokinesis (Chang et al., 1996). Tubulin mutants frequently have mispositioned spindles, however the site of cytokinesis always corresponds to the position of the nucleus (Chang et al., 1996). Mutants defective in astral microtubule function arrest in metaphase until the spindle is correctly aligned along the long axis of the cell (Oliiferenko and Balasubramanian, 2002). In mammalian rat epithelial cells with mispositioned spindles produced by micro-
manipulation, anaphase onset is delayed once again until the spindle is correctly positioned along the long axis of the cell (O'Connell and Wang, 2000). Spindle positioning in these mammalian cells is dependent on the microtubule motor dynein and astral microtubules (O'Connell and Wang, 2000). In contrast to budding yeast, dynein is required for the anaphase delay triggered by mispositioned spindles (O'Connell and Wang, 2000). Nonetheless, this data suggests that the same machinery, microtubule motors and astral microtubules, are involved in positioning the spindle within the cell in budding and fission yeast and mammalian cells. How the position is monitored in these different organisms needs to be investigated further to determine if there are similarities between them.

**Homologues in other eukaryotes**

All eukaryotes need to couple mitotic events with each other and with cytokinesis. The septation initiation network (SIN) is a homologous pathway to the MEN in *Schizosaccharomyces pombe* and has been well characterized. Homologues to MEN components in mammalian cells have been identified but their functions are less well understood. (Table 1)

**The septation initiation network**

The fission yeast *S. pombe* is a rod shaped organism that grows at both tips. Cytokinesis usually occurs at the center of the cell at the end of mitosis mediated by the contraction of an actomyosin ring and the placement of a division septum. SIN activity is necessary for ring contraction and for synthesis of the septum (reviewed in Krapp et al., 2004; McCollum and Gould, 2001; Simanis, 2003). Cells that do not activate the SIN are elongated, have multiple nuclei, cannot form a septum and eventually lyse. However, hyperactivation of the SIN induces the formation of multiple septa without complete cell division. The SIN resembles a GTPase signaling cascade similar to the MEN. Components of the SIN include the GTPase spg1p; the two component GAP cdc16p-byr4p; the protein kinase cdc7p; the protein kinase sid1p with its associated factor cdc14p; the protein kinase sid2p with its associated factor mob1p; the polo kinase plo1p; and the scaffolding proteins sid4p and cdc11p.
## TABLE 1 MEN and SIN components and mammalian homologs

<table>
<thead>
<tr>
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<th><em>S. cerevisiae</em> (MEN)</th>
<th><em>S. pombe</em> (SIN)</th>
<th>Mammals putative homolog</th>
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<td><strong>GTPase</strong></td>
<td>Tem1</td>
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<td>Lte1</td>
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<td>Byr4</td>
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<td>Dbf2</td>
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<td>WARTS/LATS</td>
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<td>Mob1</td>
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<td>Cdc11</td>
<td>Centriolin</td>
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<td>?</td>
<td>Sid4</td>
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<td>Clp1/Flp1</td>
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Figure 5: Comparison of the MEN and the SIN signaling pathways.

(A) The MEN regulates exit from mitosis in *S. cerevisiae*. Many components are localized to the SPB at some point during the cell cycle through interactions with the scaffold protein Nud1.

(B) The main function of the SIN in *S. pombe* is to regulate cytokinesis. Many SIN components also localize to the SPB with sid4 and cdc11 acting as anchors on the SPB. Dashed lines indicate speculative interactions.
The order of function and subcellular localization of SIN components are very similar to the MEN in *S. cerevisiae* (Figure 5). At the top of the pathway is the GTPase spg1p. The two component GAP cdc16p-byr4p negatively regulates spg1p, however no GEF for spg1p has been identified to date (Furge et al., 1999). Through localization studies the protein kinases cdc7p, sid1p and sid2p are thought to act in a linear order downstream of spg1p, yet there is no biochemical data to date to support this model (Guertin et al., 2000). All the SIN components have been shown to localize to the SPB at some point in the cell cycle, which is mediated by the scaffolding proteins cdc11p and sid4p (reviewed in Simanis, 2003). sid4p acts as a link to the SPB and binds to the polo kinase polo1p. cdc11p binds to sid4p and to the remaining SIN components (Krapp et al., 2001; Tomlin et al., 2002). The GTPase localizes to SPBs throughout the cell cycle, however it is not activated until metaphase. Using an antiserum that preferentially recognizes GDP-spg1p, it has been shown that the GTP bound form of spg1p is the active form (Sohrmann et al., 1998). The GAP complex byr4p-cdc16p binds to both SPBs keeping spg1p inactive until metaphase when it dissociates from both SPBs (Cerutti and Simanis, 1999; Li et al., 2000). Activation of spg1p at both SPBs by an unknown mechanisms recruits cdc7p to both SPBs (Sohrmann et al., 1998). Upon entry into anaphase and inactivation of mitotic CDK activity, byr4p-cdc16p now localize to only one SPB. This results in the inactivation of spg1p and the loss of cdc7p specifically at this SPB, which has been determined to be the old SPB (Grallert et al., 2004; Sohrmann et al., 1998). The SPB bearing spg1p and cdc7p recruits the sid1p-cdc14p complex, which is dependent on the inactivation of mitotic CDKs, leading to the activation of sid2p-mob1p (Chang et al., 2001; Guertin et al., 2000). Therefore high mitotic CDK activity antagonizes both the inactivation of spg1p at one SPB and the recruitment of the sid1p-cdc14p complex to the SPB (Chang et al., 2001; Guertin et al., 2000). sid2p is present at the SPB throughout the cell cycle while mob1p is seen on the SPB throughout mitosis (Hou et al., 2000; Salimova et al., 2000). Once the sid2p-mob1p complex becomes activated the proteins localize to medial ring and flank the developing septum (Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). Since sid2p and mob1p are the only SIN components seen at the division site, it is believed that these proteins provide the signal to make a division septum.
It is interesting that asymmetry in the localization of SIN components, similar to the MEN in budding yeast, is generated even though the cell divides symmetrically. A simple explanation for this phenomenon is that this asymmetry just reflects that the two SPBs are different, one old and one new. This was first investigated in budding yeast and was found not to be true. Normally the old SPB faces the bud and it is this SPB that many MEN components localize to (Menssen et al., 2001; Pereira et al., 2001). If cells are treated transiently with nocodazole, this bias is abolished and either the new or the old SPB enters the bud. Despite this random SPB inheritance, MEN components still localize asymmetrically to the SPB destined to migrate into the bud (Pereira et al., 2001). This same experiment conducted in fission yeast yielded the opposite result. After transient nocodazole treatment, the active SIN remained on the new SPB similar to untreated cells (Grallert et al., 2004). This indicates that the generation of asymmetry is different between budding yeast and fission. Why asymmetry is generated and the purpose of this asymmetry still remain to be determined.

**Clp1/Flp1 controls mitotic CDK inactivation**

The homologue of Cdc14, clp1p/flp1p, does regulate mitotic CDK activity in *S. pombe* but in contrast to Cdc14 is not essential for mitotic exit. Cells lacking clp1p/flp1p enter mitosis prematurely, have cytokinesis defects, and have defects in chromosome segregation while overexpression of clp1p/flp1 induces a G2 arrest (Cueille et al., 2001; Trautmann et al., 2001). Clp1p/flp1p prevents the dephosphorylation of a conserved tyrosine residue in cdc2p, the only CDK in *S. pombe*, by down regulating the phosphatase cdc25p (Esteban et al., 2004; Wolfe and Gould, 2004). Although clp1p/flp1p appears to regulate the G2 to M transition, there is mounting evidence that it also influences mitotic CDK activity during exit from mitosis, which corresponds to the role Cdc14 plays in budding yeast (Esteban et al., 2004; Wolfe and Gould, 2004). The role of clp1p/flp1p at the end of mitosis may be to maintain mitotic CDK inhibition to allow the SIN to remain active and promote the timely completion of cytokinesis (Wolfe and Gould, 2004). Indeed sid1p and cdc14p do not localize to the SPB until mitotic CDK activity drops below a certain threshold (Guertin et al., 2000). Furthermore, increased SIN activation can
lead to ectopic septum formation but only during stages of the cell cycle when mitotic CDK activity is low (Ohkura et al., 1995; Schmidt et al., 1997).

Similar to Cdc14 in budding yeast, the localization of clp1p/flplp is highly dynamic throughout the cell cycle. During G1 and S phase, clp1p/flplp is present in the nucleolus and at the SPB (Cueille et al., 2001; Trautmann et al., 2001). Clp1p/flplp is released from the nucleolus and localizes to the mitotic spindle, the SPBs, and the medial ring upon entry into mitosis (Cueille et al., 2001; Trautmann et al., 2001). It is thought that changes in clp1p/flplp localization affect its activity, however this is yet to be proven. The SIN is not required to release clp1p/flplp from the nucleolus, however SIN inactivation is needed for clp1p/flplp to be resequestered into the nucleolus at the end of mitosis. Furthermore, SIN is required to maintain clp1p/flplp in the released state when the cytokinesis checkpoint is activated, which is analogous to the maintenance function of the MEN in budding yeast (Cueille et al., 2001; Trautmann et al., 2001). The mechanisms that hold clp1p/flplp in the nucleolus and how the SIN regulates clp1p/flplp localization are not known.

**Mammalian homologues**

It appears that higher eukaryotes have homologues of many MEN/SIN components but in most cases little is known about their precise roles in the cell cycle (Table 1). It also remains to be seen whether a signaling pathway similar to MEN/SIN exists. Cdc5/polo1p are part of the polo kinase family. In higher eukaryotes polo kinases are required for bipolar spindle formation, exit from mitosis, and cytokinesis (Donaldson et al., 2001; Glover et al., 1998)(reviewed in Donaldson et al., 2001 and Glover et al., 1998). WARTS in Drosophila and LATS in mammalian cells show similarity to the kinase Dbf2/sid2p. Both these proteins associate with the centrosome, the spindle, and the midbody (Nishiyama et al., 1999). The Dbf2/sid2p associated factor Mob1/mob1p has homologues in all eukaryotes, however little is known about their function. Proteins that share similarity to Bub2/cdc16p are also found in mammalian cells and appear to localize to the centrosome (Cuif et al., 1999). Centriolin, a homologue of Nud1/cdc11p and a centrosomal protein, plays a role in cytokinesis and G1 progression (Gromley et al.,
2003). It will be interesting to see if the centromere acts as a signaling center similar to the SPB in budding and fission yeasts. Finally two homologues of Cdc14/clp1p/flp1p, hCdc14a and hCdc14B, exist in human cells. hCdc14a localizes to centrosomes and is thought to play a role in regulating centrosome function (Bembenek and Yu, 2001; Kaiser et al., 2002; Mailand et al., 2002). hCdc14b localizes to the nucleolus during interphase but not mitosis, however little is known about its function (Bembenek and Yu, 2001; Kaiser et al., 2002). It will be interesting to see if other homologues can be identified and whether a regulatory network similar to the MEN and SIN exist in mammalian cells.
Conclusions and Perspectives

Over the past several years a lot of progress has been made in understanding the mechanisms controlling mitotic exit in budding yeast. The protein phosphatase Cdc14 performs a critical role in promoting mitotic exit by reversing mitotic CDK phosphorylation. However recently Cdc14 has been shown to play other roles in mitosis, such as regulating chromosome segregation, influencing the mitotic spindle, and promoting cytokinesis, indicating that it acts to couple various events in mitosis to ensure genomic stability. The FEAR network and the MEN respond to various signals to regulate the activation of Cdc14. Understanding all the signals that control the FEAR network and the MEN will give insight into the precise regulation of mitotic exit.

Two screens were conducted to identify novel regulators of mitotic exit in budding yeast, one to identify negative regulators and one to identify positive regulators. The gene \textit{KIN4} was identified as a novel negative regulator of mitotic exit. Data presented in Chapter 2 will show that Kin4 acts in the spindle position checkpoint. Characterization of \textit{KIN4} has shed light on how position of the mitotic spindle influences mitotic exit. A screen for positive regulators of mitotic exit is presented in Chapter 3. Four uncloned genes have been identified and await further analysis.
Literature Cited


Chapter II:

The protein kinase Kin4 inhibits exit from mitosis in response to spindle position defects.

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Dr. Fernando Monje-Casas contributed to Figure 5B, Figure 6E, Figure 7, and Figure 8 and Dr. Vladimir Reiser contributed to Figure 11A.
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 mis-positioned nuclei by inhibiting the Mitotic Exit Network, a GTPase signaling cascade that promotes exit from mitosis. Kin4 is active in cells with mis-positioned nuclei and predominantly localizes to mother cells, where it is ideally situated to inhibit MEN signaling at spindle pole bodies 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 Network (FEAR network) and the Mitotic Exit Network (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 prevent MEN signaling in cells with a mis-positioned 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 while Tem1 and other MEN components localize to the daughter-bound spindle pole body (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 \textit{LTE1} or deleting the septin ring component \textit{SHS1}, which causes Lte1 accumulation in the mother cell, allows cells with mis-oriented 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 \textit{BUB2} or \textit{BFA1} also allows cells with mis-positioned 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 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 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 Mitotic Exit Network 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, 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 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-l 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 cells (Figure 1B). Upon release from a pheromone-induced G1 arrest, cells lacking KIN4 progressed through the cell cycle with similar kinetics as wild-type 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.
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, A12342), lte1Δ esp1-1 -/+ kin4Δ (A3722, A12356), and lte1Δ slk19Δ -/+ kin4Δ (A4394, 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 (A1411) and kin4Δ (A8453) cells carrying a CDC14-HA fusion were arrested in G1 using 5μg/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.
High levels of Kin4 delay Cdc14 release from the nucleolus and exit from mitosis by antagonizing MEN activity.

To investigate how \textit{Kin4} inhibits mitotic exit we analyzed the consequences of overproducing Kin4. Cells overexpressing \textit{Kin4} from the galactose inducible \textit{GALI-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 \textit{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 \textit{CDC14} function failed to localize Sli15 to anaphase spindles but the protein did localize to anaphase spindles in cells mutated for the MEN component \textit{CDC15} (Pereiera and Schiebel 2003; Figure 2C; Figure 3). Overexpression of \textit{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 assessed the activity of the protein kinase Dbf2, which constitutes the most downstream component of the MEN. In wild-type 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 \textit{Kin4} (Figure 2B). Furthermore, the transient release of Cdc14 from the nucleolus observed in \textit{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.
Figure 2

A

Wild Type

Percent Cells

Time (min)

metaphase
anaphase
Cdc14 release

0 45 60 75 90 105 120 135 150 165 180

- Clb2

- Kar2

B

Wild Type

Percent Cells

Time (min)

metaphase
anaphase

0 45 60 75 90 105 120 135 150 165 180

Dbf2 kinase

Dbf2

Kar2

C

Percent anaphase cells with Shi15 on the spindle

WT cdc15 cdc14 GAL-KIN4

D

wild type
bub2Δ
GAL-CDC15[1-750]
TAB6-1
GAL-KIN4
GAL-KIN4, bub2Δ
GAL-KIN4, GAL-CDC15[1-750]
GAL-KIN4, TAB6-1

Glucose
Galactose
Figure 2: Overexpression of KIN4 delays mitotic exit.

(A) Wild type (A1411) and GAL-KIN4 (A9249) cells carrying a CDC14-HA fusion were arrested in G1 in YEP Raffinose (YEPR) using 5µg/ml α factor. GAL-KIN4 transcription was induced for one hour 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) Wild type (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 was determined.

(C) Wild type (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 minutes after release.

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

Figure 3: Sli15 localization to the mitotic spindle depends on the FEAR network.
Examples of Sli15 localization in cdc14-3 and cdc15-2 mutants. In wild-type cells and MEN mutants but not cdc14-3 and FEAR network mutants, Sli15 localizes to the mitotic spindle during anaphase.
**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 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 allowing 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 hyper-phosphorylation and to ensure that wild-type and *GAL-KIN4* cells were in the same cell cycle stage during the analysis, we conducted this analysis in a MEN mutant, in which, as 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 the drug to cells carrying the *cdc15-as1* allele caused a first cycle arrest in anaphase (**Figure 4A**) 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, wild type cells are unaffected (Bishop et al., 2000). Both Bfa1 and Bub2 accumulated in their hyperphosphorylated forms in *cdc15-as1* cells (**Figure 4B**) as expected of cells that arrest in anaphase. Furthermore, Bfa1 localization resembled that of wild-type cells, with the majority of cells exhibiting Bfa1 localization on the SPB that is present in the bud (**Figure 4A**). In *KIN4* overexpressing cells, the slowest migrating forms of Bfa1 and Bub2 observed in *cdc15-as1* cells did not accumulate (**Figure 4B**). The migration pattern of Bfa1 in *GAL-KIN4* cells differed from that observed in a *cdc5-2* mutant, which is partially impaired in Bfa1
Figure 4

A

\[ \text{Percent Cells} \]

\[ \begin{align*}
\text{Time (min)} & \\
0 & 30 & 60 & 90 & 120 & 150 & 180
\end{align*} \]

- metaphase
- anaphase
- Bfa1 on 1 SPB
- Bfa1 on 2 SPBs

B

\[ \text{Time (min)} \]

\[ \begin{align*}
0 & 30 & 45 & 60 & 75 & 90 & 105 & 120 & 135 & 150 & 165 & 180
\end{align*} \]

- Bfa1
- Bub2
- Pgk1

C

\[ \text{cdc15-as1} \]

D

\[ \text{cdc15-as1 GAL-KIN4} \]

\[ \text{cdc5-2 kin4A} \]

\[ \text{Bfa1} \]

\[ \text{WT} \]

\[ \text{GAL-KIN4} \]
Figure 4: *KIN4* overexpression interferes with the regulation of Tem1, Bub2 and Bfa1.

(A, B, 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 legend to Figure 2. Cells were released into YEPRG containing 10μM 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 minutes after release, *cdc15-as1 GAL-KIN4* mutants 180 minutes after release, and *cdc5-2* (A13313) and *cdc5-2 kin4Δ* (A13312) cells grown at 37°C for two hours. * indicates a cross reacting band that serves as a loading control.

(D) Wild type (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 spindle pole body was determined.
phosphorylation (Hu et al., 2001; Figure 4C). 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 4A), 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 4D). 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 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
Figure 5: KIN4 is not required for the spindle assembly checkpoint.

(A) Wild type (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 5μg/ml α factor and released into medium containing 15μg/ml nocodazole. The percentage of cells with Cdc14 released from the nucleolus (left graph) and the percent of rebudded cells (right graph) was 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 8mM methionine to shut off MET-CDC20 transcription and the percentage of rebudded cells was determined.
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 5A; 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 5A). Next we considered the possibility that an intact microtubule cytoskeleton was needed in kin4A 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 5B), 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 mis-positioned we examined the effects of deleting KIN4 in cells lacking cytoplasmic dynein (DYNI). 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 6A). At 16°C, the temperature at which the percentage of dyn1Δ cells with mispositioned spindles is around 10% (Figure 6A), 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 6A). It was nevertheless obvious that deletion of KIN4 allowed dyn1Δ cells with mis-positioned 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 6A, Figure 7; data not shown). The extent to which multinucleate and anucleate cells accumulated was similar in dyn1Δkin4Δ, dyn1Δbub2Δ and dyn1Δbfa1Δ cells and
Figure 6

A. Anaphase cells with correctly positioned spindles

B. Anaphase cells with mis-positioned spindles

C. Metaphase and Anaphase

D. Bar chart showing percentage of cells with 1 SPB or 2 SPBs

E. Anaphase cells with correctly and mis-positioned spindles
Figure 6: Kin4 functions in the spindle position checkpoint.

(A) kin4Δ (A8453), kar9Δ act5" (A2025) and kar9Δ act5" kin4Δ (A12004) cells were grown for 3 hours 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), dyn1Δ bub2Δ bfa1Δ kin4Δ (A11987) cells were grown for 24 hours at 16°C. The percentage of cells with mis-positioned anaphase nuclei (white bars) and the percentage of cells with more than one bud, multiple nuclei or no nucleus (gray bars) was determined. Error bars indicate standard deviation.

(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 wild type (A4378, A12597) and kin4Δ (A11772, 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 hours and the percentage of cells with Bfa1 on no, one, or two SPB(s) in cells with misaligned anaphase spindles was determined.

(E) Wild type (A2266), kin4Δ (A12121), dyn1Δ (A12123), and dyn1Δ kin4Δ (A12122) cells containing a TEM1-13MYC allele were arrested in a factor at 25°C and released into medium at 16°C. Samples were collected at 5, 5.5, 6 and 6.5 hours after release to analyze Tem1 localization at SPBs.
Figure 7: Cell morphology of dyn1Δ cells with a misaligned nucleus or a defective spindle position checkpoint.

Examples of dyn1Δ cell types when the spindle position checkpoint is intact (top panel) or when escape from the checkpoint occurred (2nd, 3rd and 4th panel).
triple and quadruple mutant combinations (Figure 6A) indicating that BUB2, BFA1 and KIN4 function in the same pathway.

kar9Δ act5ts cells also accumulate cells with mis-positioned anaphase nuclei due to a defect in anchoring cytoplasmic microtubules at the bud cortex (reviewed in Pearson and Bloom, 2004; Figure 6A). Deletion of KIN4 also affected the ability of kar9Δ act5ts cells with mis-positioned spindles to arrest in anaphase, though 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 mis-positioned anaphase spindles.

To begin to address how KIN4 inhibits MEN signaling in cells with mis-positioned 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 6B) 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 6C) or Bfa1 localization in cells with misaligned anaphase spindles (Figure 6D). 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 6E). 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 mis-positioned anaphase spindles (Figure 6E, Figure 8). 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 mis-positioned spindles (Figure 6E, Figure 8). 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 mis-positioned spindles.

**Kin4 is active in metaphase-arrested cells and anaphase cells with mis-positioned 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 9A), 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 9A). Immunoprecipitation of Kin4 led to a dramatic loss of the slower migrating species of Kin4 in the precipitate (Figure 10) 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 the slower migrating forms of Kin4 indicating that they were due to phosphorylation (Figure 10). Our results suggest that phosphorylation of Kin4 changes during the cell cycle.

Although Kin4 protein is readily detectable, Kin4 kinase activity was barely detectable, if at all in exponentially growing cells when measured using several artificial substrates in vitro (Figure 9B; data not shown). Even when the protein was overproduced from the galactose inducible GAL1-10 promoter, we detected little kinase activity in cells progressing through the cell cycle in a synchronous manner (Figure 9C). However, using Myelin Basic Protein (MBP) as a substrate we did detect kinase activity associated with
Figure 8: Kin4 affects Tem1 localization to the SPB

Tem1-13MYC localization and spindle morphology in wild type, dyn1Δ, and dyn1Δ kin4Δ cells grown as described in Figure 6E.
Figure 9: Characterization of Kin4 during the cell cycle.

(A) Wild type 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) Wild-type 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 act5a (A2025) and kar9Δ act5a KIN4-HA (A12003) cells were grown at 37°C for two hours. Kin4-associated kinase activity (top panel; Kin4 kinase), the amount of immunoprecipitated Kin4-HA (middle panel; IPed Kin4) and the total amount of Kin4-HA in cell extracts (bottom panel; total Kin4-HA) was determined.

(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 panel) or containing 15µg/ml nocodazole (circles, bottom panel). 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 threonine 209 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 (McCarteney and Schmidt 2001). * indicates a cross reacting band that serves as a loading control.
Figure 10: Kin4 appears to be phosphorylated.

Kin4-HA was immunoprecipitated from exponentially growing A9233 cells. The precipitates were then either incubated with buffer or buffer and calf intestinal alkaline phosphatase (CIP) for one hour at 37°C. Phosphorylated forms of Kin4 appear to be unstable during the immunoprecipitation and/or cannot be immunoprecipitated leading to only a subtle migration difference between phosphatase treated and untreated Kin4-HA.
endogenous (Figure 9B) and overproduced Kin4 (Figure 9C) 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 9D). 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 arbitrary units, which is similar to that of exponentially growing cells (1.44 arbitrary units; Figure 9B). The specific activity was increased to 4.57 arbitrary units in kar9A act5ts cultures, in which 20% of cells contained misaligned anaphase spindles (Figure 9B). This difference in Kin4 activity between cdc15-2 and kar9A act5ts mutants was neither due to less Kin4 protein being present in cdc15-2 mutants than in kar9A act5ts mutants nor a decreased ability to immunoprecipitate Kin4 from cdc15-2 mutant extracts (Figure 9B). Our results suggest that Kin4 kinase activity is low in cells with correctly positioned anaphase nuclei and slightly increased in cells with mis-positioned 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 11A). 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 11A). Kin4-GFP was also detected transiently on the mother
cell SPB during anaphase (E. Schiebel and G. Pereira, personal communications). Similar results were obtained when Kin4-GFP was overproduced (Figure 12).

To determine when exactly during mitosis Kin4 diminished from the mother cell cortex and associated with the mother – bud neck we marked SPBs 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 using a GFP filter set. This analysis revealed that in budded cells with an SPB distance between 0 – 2 μm, which represents cells in S phase and metaphase, Kin4-GFP localized exclusively to mother cells (Figure 11A, S phase; n=12). In 60 percent 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 11A, early anaphase; n=22). In the remaining 40 percent 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 11A, 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 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 mis-positioned (Figure 11B; 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 wild-type cells that are in early anaphase but comparable to that observed in late anaphase wild-type cells. However, in contrast to wild-type late anaphase cells some Kin4-GFP accumulated in the vacuole. Our results indicate that in cells with mis-positioned 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.
Figure 11: 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 a 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 mis-positioned spindles were examined.
**Figure 12: Kin4 is concentrated at the mother cell cortex when overexpressed.**

The localization pattern of Kin4-GFP overproduced from the *GAL1-10* promoter was examined after growing cells for 2 hours in the presence of galactose in strain A11997. The left panel shows a cell in G1, the middle panel a cell in early S phase, and the right panel a cell in mitosis.
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 mis-positioned spindles by inhibiting the MEN. The observation that Kin4 kinase activity is detectable in cells with mis-positioned anaphase spindles and the fact that deletion of \textit{KIN4} does not cause defects during an unperturbed cell cycle indicates that the protein kinase’s main function is in the spindle position checkpoint. The 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.

\textbf{A genetic selection identifies \textit{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 \textit{KIN4} functions to inhibit MEN activity rather than FEAR network function. Although \textit{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 \textit{KIN4} suppresses the synthetic lethality of \textit{lte1Δ spo12Δ, lte1Δ slk19Δ} cells, and \textit{lte1Δ esp1-1} double mutants suggests so. However during an unperturbed cell cycle deleting \textit{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 \textit{BUB2} and \textit{BFA1} are readily detectable (Stegmeier et al., 2002; Yoshida et al., 2002; \textbf{Figure 5A}) didn’t \textit{KIN4} reveal itself as a negative regulator of MEN activity. It is possible that a role for \textit{KIN4} in an unperturbed cell cycle is masked by a redundant factor present in the yeast genome. However inactivation of the closest homolog of \textit{KIN4}, \textit{YPL141c} (the two protein kinases are 43% identical and 56% similar) in a \textit{kin4Δ} background did not expose a defect in cell cycle progression (K. D. unpublished observations, see Appendix I). Thus, we conclude that \textit{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 shed 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 4C) would be consistent with this idea. However, deletion or overexpression of KIN4 affected neither Cdc5 localization nor its kinase activity (K. D. unpublished observations, see Appendix I) 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 mis-positioned 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, that is in the absence of KIN4 the GAP complex is still to some extent active. 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 mis-positioned 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 percent 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 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, (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 hypo-phosphorylated. 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 trans-membrane 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 mis-positioned spindles compared to anaphase cells with correctly positioned spindles even though only 20 percent of kar9Δ ac5ts cells have mis-positioned 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 mis-position.

A model for how exit from mitosis is inhibited in cells with mis-positioned 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 Supplemental Table1. 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 threonine209 to alanine using the Quik Change XL Site Directed Mutagenesis Kit. cdc15-asl (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 using transposon mutagenesis (Burns et al., 1994). Linkage analysis revealed 4 insertions in 66 000 mutagenized lte1Δ slk19Δ colonies and 6 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 minutes in 5% trichlor acetic acid. Cells were broken in 100μl lysis buffer (50mM TRIS (pH7.5), 1mM EDTA, 50mM DTT, complete EDTA-free protease inhibitor cocktail [Roche]) with glass beads for 40 minutes 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 200M inverted microscope. Slidebook Software version 4 was used for deconvolution processing of the images.

Kin4 kinase assays
Cell extracts were made using IP buffer (1% NP40, 150 mM NaCl. 50 mM Tris [pH 7.5], 60 mM β-glycerophosphate, 0.1 mM NaVO₃, 15 mM para-Nitro-phenyl phosphate, complete EDTA-free protease inhibitor cocktail [Roche], 1mM DTT). Kin4-HA was immunoprecipitated 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 MgCl₂, 50 μCi γP³²-ATP and 2 μg Myelin Basic Protein were added to each sample and allowed to incubate for 15 minutes at room temperature.
Literature Cited


Chapter III:

Positive regulators of mitotic exit are identified in a screen for mutants dependent on the overexpression of a mitotic exit network component.
Summary

Mitosis is the process by which a cell segregates its chromosomes. To accurately transmit its genetic material, it is important for the cell that cytokinesis does not begin before all chromosomes have been faithfully segregated. After chromosome segregation, cells exit from mitosis by inactivating mitotic cyclin dependent kinase (CDK) activity. The phosphatase Cdc14 is responsible for this inactivation in *Saccharomyces cerevisiae*. A Ras-like signal transduction cascade termed the Mitotic Exit Network (MEN) and the Cdc14 early anaphase release (FEAR) network control Cdc14 activity. Many components of the MEN and the FEAR network have been identified, however gaps remain in our understanding of how the signal to exit is sensed, transmitted and regulated. To identify novel regulators or components of the MEN and/or FEAR, a screen was conducted that searched for mutants that rely on high levels of MEN components for survival.
Introduction

Proper segregation of chromosomes is essential for cell survival in all organisms. Aneuploidy, an abnormal number of chromosomes, can lead to cell death or abnormal cell division. Uninhibited cell multiplication could form a tumor and increase the probability of cancer in a multicellular organism. Therefore it is imperative that during mitosis each cell receives a complete set of chromosomes and exit from mitosis does not occur until all chromosomes have been correctly segregated.

In the budding yeast *Saccharomyces cerevisiae*, the protein phosphatase Cdc14 regulates the inhibition of mitotic cyclin dependent kinase (CDK) activity, which is required for cells to exit mitosis and enter G1 of the next cell cycle (Jaspersen et al., 1998). During most of the cell cycle Cdc14 is held inactive in the nucleolus by its inhibitor Cfi1/Net1 (Visintin et al., 1999). During anaphase, Cdc14 is released from its inhibitor and now localizes to the nucleus and cytoplasm (Visintin et al., 1999). Cdc14 is then able to reach its targets and induce the inhibition of mitotic CDK activity by two different mechanisms. Cdc14 promotes mitotic cyclin degradation by dephosphorylating Cdh1, a specificity factor for an ubiquitin ligase known as the anaphase promoting complex or cyclosome (APC/C) (Jaspersen et al., 1999; Schwab et al., 1997; Visintin et al., 1998; Visintin et al., 1997). Cdc14 also promotes the accumulation of the CDK inhibitor Sic1 through two mechanisms. It stabilizes the Sic1 protein by dephosphorylating it and increases Sic1 transcription by dephosphorylating its transcription factor Swi5 (Jaspersen et al., 1999; Toyn et al., 1997; Visintin et al., 1998). There are many other Cdc14 substrates, as it is thought that the role of Cdc14 is to reverse the phosphorylation events of CDK.

Two signaling networks have been discovered that regulate the dissociation of Cdc14 from its inhibitor (reviewed in Stegmeier and Amon, 2004), the Cdc14 early anaphase release (FEAR) network and the mitotic exit network (MEN). The FEAR network promotes the release of Cdc14 into the nucleus during early anaphase (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). This network consists of the polo-like kinase Cdc5, the separase Esp1, the small nuclear protein Spo12 and its homologue Bns1, and the replication fork block protein Fob1 (Pereira et al., 2002; Stegmeier et al., 2004;
Through genetic epistasis analysis the FEAR network can be divided into two branches. *ESPI* and *SLK19* function in parallel to *SPO12/BNS1* and *FOBI*. *CDC5* is likely downstream of both these branches, however this has not been proven. During later stages of anaphase, the mitotic exit network (MEN) becomes activated. The MEN promotes the further release of Cdc14 into the nucleus and cytoplasm and also maintains this release (Shou et al., 1999; Visintin et al., 1999). Components of this pathway include a Ras-like GTPase Tem1, the two-component GTPase activating protein (GAP) Bub2/Bfa1, and the putative guanine nucleotide exchange factor (GEF) Lte1 (Shirayama et al., 1994a; Shirayama et al., 1994b). Downstream of Tem1 are the serine/threonine kinases Cdc15 and Dbf2 (Lee et al., 2001). Cdc15 functions upstream of Dbf2 and has been shown to activate Dbf2 (Lee et al., 2001; Mah et al., 2001). Dbf2 activity *in vitro* also requires that it be bound to the protein Mob1 (Komarnitsky et al., 1998; Luca and Winey, 1998; Mah et al., 2001). Nud1 is a spindle pole body (SPB) component and seems to serve as an anchor for Tem1, Dbf2, and Cdc15. Correct pathway function does not occur in a *nud1-44* mutant, in which Tem1, Cdc15, and Dbf2 are not appropriately localized, suggesting that SPB localization of these components is critical for pathway function (Bardin et al., 2000; Gruneberg et al., 2000; Pereira et al., 2000; Visintin and Amon, 2001). The polo-like kinase, Cdc5, has also been shown to be involved in the MEN (Kitada et al., 1993). However, attempts to place Cdc5 at a specific point within the pathway have failed, suggesting that it may act at multiple levels (Lee et al., 2001).

A pathway similar to the MEN also exists in *Schizosaccharomyces pombe* to control septation (reviewed in Bardin and Amon, 2001; McCollum and Gould, 2001). The septation initiation network (SIN) is comprised of the GTPase spg1p; protein kinases cdc7p, sid1p, sid2p and plo1p; the sid1p-associated protein cdc14p; the sid2p-associated protein mob1p; the two component GAP cdc16p-byr4p; and two components of the SPB, sid4p and cdc11p. The Cdc14 homolog, clp1p/flp1p promotes CDK inactivation (Cueille et al., 2001; Trautmann et al., 2001). Comparing the SIN and the MEN, the SIN contains two additional components (sid1p and cdc14p). This raises the possibility that the homologs of these components in *S. cerevisiae* have not yet been identified.
While much detail has been learned about the MEN over the last ten years, many questions still remain. Are there other factors involved in propagating the signal? There is no biochemical data to show that Tem1 directly activates Cdc15. Can other proteins be identified that are responsible for activating this kinase? Another question to be addressed is how Dbf2 activity leads to the release of Cdc14 from its inhibitor Cfi1. Dbf2 has not been shown to localize to the nucleolus or to interact with Cdc14 directly. In addition, what is the role of Cdc5 in MEN activation? At how many levels is Cdc5 involved? Finally, can novel regulatory mechanisms be identified that control mitotic exit?

Here we describe a screening method designed to identify novel components or regulators of the MEN or pathways parallel to it by focusing on the components Cdc5, Cdc15, and Dbf2. This approach may also provide clues as to the regulation of Cdc5 or its precise roles within mitotic exit.

**Results**

**Isolation of mutants dependent on high levels of a MEN component**

To identify new components or regulators of the MEN, a high dosage-dependent screen was conducted based on an approach described by Cullen et al. (2000). The objective of this method is to identify partial loss of function mutants that can be rescued by the overexpression of the gene of interest. This approach is likely to identify regulators or effectors of the gene being overexpressed, or gene products that act in a parallel pathway. If a gene has more than one essential function, this screen is more appropriate than a suppressor screen. A suppressor screen is used to identify genes that restore the wild type phenotype to a mutant; however, unless alleles that are known to only affect one specific function are used, it may be difficult to obtain suppressors. This screening method was successfully used by Cullen et al. to identify mutants that were dependent on the overexpression of *plo1*<sup>+</sup>, the homolog of *CDC5* in *S. pombe*. They identified five known components of SIN as well as other genes not yet implicated in this pathway. This
screening method allowed them to identify many new components, indicating that this method is a powerful tool to identify genes that function in a related process.

An adaptation of the screen described by Cullen et al. (2000) was implemented in *S. cerevisiae* to identify new components or regulators of the MEN and also to further elucidate the mechanisms that regulate Cdc5 or Cdc5's role in mitotic exit. The screen was conducted using three different components of the MEN, *CDC5, CDC15*, and *DBF2*, to maximize the number of genes that can be identified. Each gene was integrated into the genome under the control of the inducible *GAL1-10* promoter at a different locus than the wild type gene. Therefore, overexpression is achieved by growing cells in the presence of galactose and a normal level of expression from the endogenous copy is obtained in the presence of glucose. By southern blotting the copy number of the integration was verified to be one. It was determined that overexpression of each of these genes in these strains is not lethal to the cell (unpublished results). To induce point mutations, each strain was exposed to ethyl methanesulfonate (EMS) and allowed to grow on galactose. Colonies were then replica plated to glucose at various temperatures to identify mutants that only grow in the presence of galactose. In the primary screening, the plates contained the dye Phloxin B to allow for easier detection of colonies that are inviable. A total of 725,960 colonies from the three different strains were screened resulting in 77 mutants (Table 1).

### Table 1: Screen Summary

<table>
<thead>
<tr>
<th>Total Colonies Screened</th>
<th>Number of Mutants</th>
<th>Sugar Utilization</th>
<th>DBF2 alleles</th>
<th>CDC5 alleles</th>
<th>TEM1 alleles</th>
<th>Unknown Mutants</th>
</tr>
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<tr>
<td><strong>GAL-DBF2</strong></td>
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<td>12</td>
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<td>4</td>
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<tr>
<td><strong>GAL-CDC5</strong></td>
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<td>2</td>
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<tr>
<td><strong>GAL-CDC15</strong></td>
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<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>725960</td>
<td>77</td>
<td>29</td>
<td>6</td>
<td>19</td>
<td>4</td>
</tr>
</tbody>
</table>
Elimination of false positives

It is possible that mutations in genes involved in sugar utilization will give false positives due to growth on two different carbon sources as a screening method. Two methods were used to eliminate these mutants. The primary method was to analyze the growth on plates containing both glucose and galactose compared to growth on each sugar alone. Glucose metabolism is the default pathway in the cell; therefore when glucose is present, galactose metabolism and thus GAL responsive genes are repressed. This phenomenon is termed catabolite repression or glucose repression (reviewed in Gancedo, 1998). If there is a mutation in the glucose repression pathway such that glucose was no longer repressing, then galactose metabolism is active and therefore growth on both sugars appears the same as growth on galactose alone. However, if the mutant is not involved in glucose repression, then growth on both sugars appears the same as growth on glucose.

The second testing method involved tetrad analysis. Mutants were backcrossed to wild type and the resulting tetrads were analyzed for growth on the two different carbon sources. Genes involved in glucose utilization and/or repression when mutated should show growth on galactose and not on glucose whether the overexpression construct is present or not. If there is a mutation affecting glucose utilization/repression, then the differential growth on glucose versus galactose should not depend on the presence of the overexpression construct. If the mutation is not involved in glucose utilization/repression, then growth on galactose should be the same as on glucose if the overexpression construct is absent and growth on galactose should be better than on glucose if the overexpression construct is present. The above two methods identified 29 mutants with a defect in glucose utilization/repression. The remaining mutants were called doe for dependent on overexpression.

Identification of known regulators of mitotic exit

This screening method is likely to identify genes already known to be involved in the MEN or the FEAR network. Conducting complementation tests with mutants of the various MEN components led to the identification of mutations of known MEN components. Identifying known components provided evidence that the screening method
is working. The isolated mutants were crossed to temperature sensitive alleles of the MEN components \textit{TEM1}, \textit{CDC15}, \textit{DBF2}, \textit{MOB1}, \textit{NUD1}, \textit{CDC5}, and \textit{CDC14}. Complementation testing was also conducted with the FEAR network component \textit{ESP1}. The resulting diploids were incubated at the restrictive conditions and viability was monitored. If the diploid was viable, then the two mutations are likely in different genes; however, if the diploid was unable to grow, then this suggests that the mutations are in the same gene. Complementation was also conducted by spore matings using deletions of the essential genes \textit{DBF2}, \textit{TEM1}, \textit{NUD1}, and \textit{CDC15}. Because the complementation studies with temperature sensitive alleles of \textit{CDC5} and \textit{ESP1} did not give clear results for some of the mutants, linkage analysis was used to determine whether doe\textsubscript{1}, doe\textsubscript{2}, doe\textsubscript{3}, or doe\textsubscript{4} is allelic to \textit{CDC5} and whether doe\textsubscript{2} is allelic to \textit{ESP1}. These studies with known components of the MEN yielded seven \textit{DBF2} alleles, four from the \textit{GAL-DBF2} screen, two from the \textit{GAL-CDC5} screen, and one from the \textit{GAL-CDC15} screen; nineteen \textit{CDC5} alleles, all from the \textit{GAL-CDC5} screen; and four \textit{TEMI} alleles, one from the \textit{GAL-CDC5} screen and three from the \textit{GAL-CDC15} screen (Table 1, Table 2). It is interesting to note that seven alleles, three \textit{DBF2} alleles and all four \textit{TEM1} alleles, were identified that complemented temperature sensitive alleles but did not complement deletions. Also two cold sensitive \textit{CDC5} alleles were identified. All of these alleles are useful, in particular the mutants just mentioned, but the goal is to first identify novel genes.

**Basic characterization of dependent on overexpression (doe) mutants**

After the above secondary screening was conducted, nineteen mutants remained. These mutants were tested for dominance by crossing to wild type and incubating the diploid at the restrictive condition. There was one dominant mutation (see discussion below), one mutant with a mating defect, and the remaining 17 mutations were recessive. These 17 mutants were retested for growth at different temperatures on galactose or glucose containing medium. Four mutants with the strongest growth differential between galactose and glucose were chosen for further analysis. All four were found to be temperature sensitive. The temperature sensitivity should allow for relatively easy cloning of the wild type genes. Through backcrossing to a wild type strain, the temperature sensitive mutations were isolated from the \textit{GAL-DBF2}, \textit{GAL-CDC5}, or
Table 2: *DBF2*, *TEM1*, and *CDC5* alleles are identified.

<table>
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<th>25°C</th>
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<th>37°C</th>
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<td>Gal</td>
<td>Glu</td>
<td>Gal</td>
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<td><strong>DBF2 Alleles</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>+++</td>
</tr>
<tr>
<td>K16</td>
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<td>++</td>
</tr>
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- indicates no growth
+ indicates level of growth
Shaded areas indicate strains of interest
GAL-CDC15 constructs. These mutants had a lower restrictive temperature on galactose without the overexpression construct, confirming the fact that the mutant depended on the overexpression of the MEN component for viability.

**Cell cycle analysis of dependent on overexpression (doe) mutants**
The next step in characterizing doe mutants was to determine if they have an effect on the cell cycle and in particular mitotic exit. Temperature sensitive mutants of the mitotic exit network components, TEM1, CDC15, DBF2, CDC5, and CDC14, all arrest as dumbbells with separated chromosomes, anaphase spindles, and Cdc14 sequestered in the nucleolus (Hartwell et al., 1973; Johnston and Thomas, 1982; Kitada et al., 1993; Shirayama et al., 1994a; Shirayama et al., 1994b). If the doe mutants positively regulated mitotic exit upstream of Cdc14 release, then their terminal phenotypes are likely be similar to that described above. Cultures of the doe mutants were shifted to the non-permissive temperature and the morphology of the cells, unbudded, small buds, or large buds, was examined three and five hours later (Figure 1). The phenotypes observed were similar at the two time points. Mutant doe2 was the most homogeneous with 88% of the cells appearing as dumbbells at five hours. doe1, doe3, and doe4 were more heterogeneous. doe1 had more unbudded cells than any other mutant (46%). The most abundant population in both doe3 and doe4 cultures was cells with large buds at 44% and 49% respectively. It is interesting to note that doe4 had a small percentage of cells (7%) with more than one bud.

To analyze the phenotype of these doe mutants in more detail, cell cycle progression was monitored in synchronous cultures. Cells were released from a pheromone-induced G1 block at the non-permissive temperature and spindle morphology was monitored to determine cell cycle progression. Progression through the cell cycle was impaired in the doe1 mutant (Figure 2A). A maximum of ten percent of cells were in metaphase or anaphase after 120 or 140 minutes, indicating that either these cells could not progress from the G1 block or were highly asynchronous. In contrast, doe2 mutants appeared to have a metaphase and/or anaphase delay (Figure 2B). Finally, doe4 mutants were delayed in entering metaphase (Figure 2C). It is unclear whether there was also a delay
Figure 1: Phenotype of *doe1*, *doe2*, *doe3*, and *doe4* at the restrictive temperature.

*doe1* (A12755), *doe2* (A12756), *doe3* (A12757), and *doe4* (A12758) cells were grown at 37°C for 3 hours (left graph) and 5 hours (right graph). The morphology of the cell was examined using a light microscope and the percentage of cells that were unbudded, had a small bud, had a large bud, or had more than one bud was determined.
Figure 2

A

Wild Type

GAL-DBF2

Gal-DBF2 doe1

B

Wild Type

GAL-CDC5

GAL-CDC5 doe2
doe2

C

Wild Type

GAL-CDC15

GAL-CDC15 doe4
doe4
Figure 2: *doe1*, *doe2*, and *doe4* do not progress through the cell cycle like wild type.

(A) Wild type (A2587), *GAL-DBF2* (A2546), and *GAL-DBF2 doe1* (K32) cells were arrested in G1 in YEP Raffinose and Galactose (YEPRG) using 5μg/ml α factor. *GAL-DBF2* transcription was inhibited for one hour by addition of glucose to the medium. Cells were released into YEP Glucose lacking pheromone. The percentage of cells with metaphase spindles (squares) and anaphase spindles (triangles) was determined.

(B) Wild type (A2587), *GAL-CDC5* (A4083), *GAL-CDC5 doe2* (K99), and *doe2* (K100) were grown as described in (A). The percentage of cells with metaphase spindles (squares) and anaphase spindles (triangles) was determined.

(C) Wild type (A2587), *GAL-CDC15* (A5211), *GAL-CDC15 doe4* (K193), and *doe4* (K194) were grown as described in (A). The percentage of cells with metaphase spindles (squares) and anaphase spindles (triangles) was determined.
in anaphase because the time course was not carried out further. Analysis of doe3 has not been conducted.

Temperature sensitive mutants of the mitotic exit network components CDC15, DBF2, and TEM1 are bypassed by high osmolarity (see Appendix II, Grandin et al., 1998). To further test the similarity between DOE1,2,3,4 and MEN components, the mutants were grown on media containing 1.5M sorbitol. doe3 and doe4 were suppressed by sorbitol, however doe1 and doe2 were not suppressed (Figure 3).

**Basic characterization of the dominant mutant DOE5**
As mentioned above, one dominant mutant was identified in the GAL-CDC5 screen. This mutant was crossed to the parent strain containing GAL-CDC5 and to a wild type strain. Both diploids displayed the same phenotype as the haploid mutant; however, we couldn’t determine if this phenotype was a result of a single mutation because both diploids failed to sporulate. We speculated that a mutation in Cfl that made the protein bind more tightly to Cdc14 could result in a dominant mutation. To test this hypothesis, we deleted CFII in the DOE5 mutant strain as well as the GAL-CDC5 strain used for the screen. If the dominant mutation was in the CFII gene, then deleting CFII should restore the viability of the mutant strain at the restrictive condition. However, deleting CFII had no effect on the growth phenotype, indicating that DOE5 is not an allele of CFII.
Figure 3: A hypertonic environment rescues the temperature sensitive growth defect of doe3 and doe4.

doe1 (A12755), doe2 (A12756), doe3 (A12757), and doe4 (A12758) strains were spotted on solid media supplemented with 1.5M sorbitol and incubated at 25°C, 34°C, and 37°C.
Discussion

Described here is a method that has identified mutants dependent on the overexpression of mitotic exit network components in *S. cerevisiae*. A similar screen was conducted in *S. pombe* (Cullen et al., 2000). Each screen has identified novel regulators of the cell cycle, indicating that this screening method may be applicable to other systems to identify new components or regulators of signaling networks.

**Screening methodology identifies known and novel genes involved in mitosis**

From our primary screen of 725,960 colonies, we acquired 75 mutants. Out of this mutant pool we identified 6 *DBF2* alleles, 19 *CDC5* alleles, and 4 *TEMI* alleles. The fact that we obtained mutants in components of the MEN validated the assumptions we made about the screening method. The identification of known MEN components also corroborated the results obtained by Cullen et al. (2000) in their overexpression screen of *plo1* in *S. pombe*. They were able to identify five known components of the SIN. These alleles of known MEN components could be useful in gaining insight into the regulation of these proteins. Of particular interest are the three *DBF2* alleles that could complement the temperature sensitive mutant *dbf2-2* and the four *TEMI* alleles that could complement the temperature sensitive mutant *teml-3* (Table 2). Overexpression of *TEMI* through the introduction of a 2μ plasmid in the four different strains carrying the above *TEMI* alleles, did not rescue the temperature sensitive defect of any strain. Therefore, the intragenic suppression is likely not the result of the reduction in the amount of Tem1 protein in the diploid cell carrying two temperature sensitive alleles of *TEMI*. Identification of intragenic suppressors suggests that these proteins function as multimers or in a complex or that they contain multiple independent domains. Future studies of these alleles should provide greater insight into the regulation and activity of these proteins. All 19 *CDC5* alleles came from the *GAL-CDC5* screen and most appear to be null alleles given that they are not able to grow at any temperature in the absence of overexpression of *CDC5*. However, there are a few alleles that appear to have interesting phenotypes and should be further studied (Table 2). K65 and K72 are temperature sensitive. K74 appears to grow at 30°C but not at room temperature or 37°C. Finally, K75 and K77 grow at 37°C but not at 30°C or room temperature indicating that they are cold sensitive. These interesting
alleles of DBF2, TEM1, and CDC5 should be sequenced to determine the location and nature of the mutation. Also, cell cycle analysis and genetic interactions with other MEN components should be conducted.

Although we did identify known MEN components, we did not obtain alleles of CDC15, MOB1, or NUD1. High expression of CDC5 has been shown to suppress mutations in CDC15 and TEM1 (Jaspersen et al., 1998; Kitada et al., 1993). An allele of TEM1 was identified in the GAL-CDC5 screen, however CDC15 was not. A number of possibilities could explain these results. One explanation could be that the screen was not saturating. Alleles of CDC15 should have been identified in the GAL-CDC15 screen given the fact that alleles of DBF2 were identified in the GAL-DBF2 screen and alleles of CDC5 were identified in the GAL-CDC5 screen. However, the fact that multiple alleles of DBF2, TEM1, and CDC5 were obtained would argue against this. The nature of EMS mutations could be a second explanation. EMS mostly causes GC to AT transversions. It is possible that this type of mutation in CDC15, MOB1, or NUD1 would not result in an allele that would depend on the overexpression of another MEN component. A strong possibility could be that these genes are required for signaling to occur. Nud1 is the scaffold at the spindle pole body where Tem1, Cdc15, and Dbf2 bind (Gruneberg et al., 2000; Visintin and Amon, 2001). nud1-66 arrest in anaphase presumably because these proteins do not get localized to the SPB and the MEN is not activated.

The identification of known MEN components was important in validating the screen, however the goal was to discover novel regulators of mitotic exit. Four mutants that had a strong phenotype in our screen and were not known MEN components were identified. Preliminary analysis of these dependent on overexpression (doe) mutants has been conducted.

**Future cell cycle analysis**

The initial results from cell cycle analysis of the recessive doe mutants indicate that each mutant has a unique profile. The terminal phenotype of doe1 appears the most heterogeneous, thus indicating that this gene may play a role in number of processes
throughout the cell cycle. DOE2 looks particularly interesting given the fact that the mutant arrests as dumbbells and that in a synchronous time course the mutant is delayed in anaphase. Like most MEN mutants, doe3 and doe4 are suppressed by sorbitol. Also, both these mutants exhibit a metaphase and/or anaphase delay. These data indicate that at least DOE2, DOE3, and DOE4 play a role in mitosis.

There are a number of future studies that need to be conducted to understand what precise roles these genes are playing in mitosis. All MEN mutants have Cdc14 sequestered in the nucleolus. To determine if these DOE genes are involved in controlling mitotic exit, it is imperative to determine the localization of Cdc14 in these mutants. If Cdc14 remains sequestered, then localization of other MEN components should be studied to determine where within the MEN pathway these genes are acting. However, if Cdc14 gets released from the nucleolus, then the DOE gene could function downstream of or parallel to CDC14. Further analysis of events downstream of CDC14 would need to be conducted to determine the roles the DOE genes play in mitosis.

**Identification of DOE genes**

To gain better insight into the function of the DOE genes it is imperative to determine where these mutations lie within the genome. Tetrad analysis showed that the doe1, doe2, doe3, and doe4 mutations lie within a single locus, therefore cloning by complementation with a yeast genomic library should reveal what gene is mutated. Screening for complementation can be conducted at 37°C due to the fact that these doe mutants are temperature sensitive. However, first attempts at cloning have not been successful, possibly due to a high revertant rate at the restrictive temperature. To try to reduce the number of revertants, homozygous diploids were made of each of these four doe mutants. Now these diploids can be used for the complementation cloning. Once the genes have been identified, there are number of initial experiments to be conducted. First, a full deletion of the genes should be made and the phenotype analyzed to determine whether the phenotype resembles the temperature sensitive phenotype. Second, the protein should be tagged so analysis on the protein levels and localization throughout the cell cycle can
be conducted. These initial experiments should give greater insight into the function of the $DOE$ genes and thus regulation of mitosis.

To determine what gene is mutated in the dominant $DOE5$ mutant a similar strategy can be used. A genomic library from $DOE5$ mutant should be made and transformed into the $GAL$-$CDC5$ strain used for the screen. The growth of the transformants will be analyzed in a way similar to the original screen. Therefore only colonies that grow in the presence of galactose, but are inviable in the presence of glucose should have the $DOE5$ allele. Once the gene has been identified, the $DOE5$ allele should be sequenced, which could provide a clue to the nature of the mutation.

**Advantages to screening methodology**
This screening method has a number of advantages. First, multifunctional genes can be studied. If a gene is required for more than one essential pathway, then suppression of one pathway is not able to suppress the lethality of the mutation. Therefore suppressor screening would not yield results unless a mutant allele that affected only one function is used. In this screen, a mutant allele of the gene being studied is not needed. Secondly, nonessential genes could be studied if they are involved in an essential process. Finally, the alleles identified in this screen are ideal for a multicopy suppressor screen. The allele should be suppressed by at least one gene, the used to obtain it. A multicopy suppressor screen using an allele of $cdc7$ obtained in the high dose dependent screen in $S. pombe$ was very successful (Cullen et al., 2000). Four genes known to be involved in the SIN as well as two previously uncharacterized genes were identified. A multicopy suppressor screen of the alleles identified in this study should be conducted in the future.
Experimental Procedures

Yeast strains
All strains are derivative of W303 (K699). To generate a GAL-DBF2, GAL-CDC5 or GAL-CDC15 fusion, the DBF2, CDC5 or CDC15 open reading frame was ligated under the control of the GAL1-10 promoter fragment. These constructs were integrated into the genome and transformants with a single integrant were determined by Southern blotting.

Mutagenesis procedure
GAL-DBF2 (A2546), GAL-CDC5 (A4083), or GAL-CDC15 (A5211) cells in stationary phase were treated with ethyl methanesulfonate (EMS) for 90 minutes and sodium thiosulfate was added to stop the mutagenesis. Survival rate was 50% under these conditions. 1000 cells/plate were spread on galactose. Cells were then replica plated onto glucose + Phloxin B (10mg/L) and raffinose + galactose + Phloxin B and incubated at 30°C. Colonies that did not grow on glucose (red), but grew on raffinose + galactose (white/pink) were picked and analyzed further.

Cell synchronization
Cells were arrested in G1 in YPRaffinose+Galactose medium containing α-factor (5 μg/ml) at room temperature. One hour prior to release, glucose was added to the medium to turn off the galactose promoter. Cells were then released into α-factor-free YPD at 37°C.
Literature Cited


Chapter IV:

Discussion and Future Directions
Conclusions

Over the past fifteen years, our understanding of how exit from mitosis is regulated in Saccharomyces cerevisiae has expanded. Mitotic CDK activity must be downregulated for cells to exit mitosis and enter G1 of the next cell cycle (Surana et al., 1993). The protein phosphatase Cdc14 has taken center stage in regulating mitotic CDK activity given the fact that CDC14 has been shown to be both necessary and sufficient to downregulate mitotic CDK activity (Jaspersen et al., 1998; Visintin et al., 1998; Wan et al., 1992).

The first step in understanding mitotic exit has been to focus on the regulation of Cdc14 itself. In 1998, a group of proteins comprised of Tem1, Cdc15, Dbf2, Cdc5, and Cdc14 were found to act together to control mitotic CDK activity and were termed the mitotic exit network or MEN (Jaspersen et al., 1998). The fact that Cdc14 activity is necessary and sufficient to promote mitotic exit suggested that it was the most downstream component and the effector of this signaling pathway (Visintin et al., 1998). Indeed it was shown that the MEN controlled the activity of Cdc14 by regulating the association of Cdc14 with its inhibitor Cfl1/Net1 (Shou et al., 1999; Visintin et al., 1999). Throughout most of the cell cycle Cdc14 is bound to Cfl1/Net1 and tightly sequestered in the nucleolus (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Activation of the MEN induces the disassociation of Cdc14 from Cfl1/Net1, which allows Cdc14 to localize to the nucleus and cytoplasm where it can dephosphorylate its substrates (Shou et al., 1999; Visintin et al., 1999).

In 2002, a second set of proteins were identified that control Cdc14 activity, Spo12, Bns1, Esp1, Slk19, and Cdc5 (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). This signaling network has been termed the Cdc fourteen early anaphase release (FEAR) network. As the name indicates, the FEAR network is active for a short period of time in early anaphase to regulate Cdc14. Activation of Cdc14 by the FEAR network has been shown to be important for promoting the segregation of telomeres and the rDNA, for regulating the localization of chromosomal passenger proteins, and for promoting the

Genetic and biochemical evidence has expanded our understanding of the interactions between components of the MEN and the FEAR network signaling cascades and how these two networks act to control Cdc14 activity. Now that the basic signaling cascades involved in controlling mitotic exit have been identified, it is imperative to study how these networks are regulated. What signals activate or inhibit the FEAR network and the MEN? How do other cellular events or environmental conditions influence exit from mitosis? To start to address these questions I have conducted two screens, one to identify negative regulators of exit from mitosis and one screen to identify positive regulators of exit from mitosis. The data presented here describes the identification of the negative regulator \( KIN4 \) and the identification of positive regulators, dependent on overexpression (DOE). Characterization of Kin4 as a component of the spindle position checkpoint has led to a more detailed model of how mitotic exit is inhibited when the spindle is mispositioned.

**Kin4 identified as negative regulator of mitotic exit**

Three negative regulators of mitotic exit have been identified to date. The GAP complex Bub2-Bfa1 acts at the top of the MEN to negatively regulate Tem1 (Geymonat et al., 2002; Geymonat et al., 2003; Hoyt et al., 1991; Krishnan et al., 2000; Li, 1999). Fob1 is a negative regulator of the FEAR network and has been proposed to prevent the dissociation of Cdc14 from its inhibitor Cfi1/Net1 (Stegmeier et al., 2004). To identify novel negative regulators of mitotic exit, a genetic selection for suppressors of the \( spo12\Delta\text{tel1}\Delta \) or \( slk19\Delta\text{tel1}\Delta \) synthetic lethality was conducted. Only three genes were identified, the two known negative regulators, \( BUB2 \) and \( BFA1 \), and the novel negative regulator \( KIN4 \).

In an unperturbed cell cycle, deletion of \( KIN4 \) had little if any effect on progression through the cell cycle. However, when Kin4 is overexpressed mitotic spindle disassembly and degradation of the mitotic cyclin Clb2 is severely delayed. This defect in exit from
mitosis is due to the fact that Cdc14 remains in the nucleolus. To investigate whether Kin4 inhibited the FEAR network, the MEN, or both, the consequences of overexpressing *KIN4* was examined. The relocalization of the chromosomal passenger protein Sli15 from the kinetochore to the spindle midzone during anaphase is dependent on the FEAR network (Pereira and Schiebel, 2003). Cells overexpressing Kin4 relocalized Sli15 similar to wild type and a MEN mutant (*cdc15-2*). To examine MEN function, Dbf2 kinase activity was monitored. Wild type cells accumulated high Dbf2 kinase activity during anaphase, in contrast cells overexpressing Kin4 showed no accumulation of Dbf2 kinase activity. These results indicated that Kin4 inhibits the MEN but not the FEAR network. Using epistasis analysis, *KIN4* was found to function at or near the top of the MEN.

The observation that deletion of *KIN4* had little effect on progression through the cell cycle but elevated levels of the protein inhibited MEN activity, led to the hypothesis that the primary role of *KIN4* is to act in a surveillance mechanism that inhibits the MEN. Two known surveillance mechanisms, the spindle assembly checkpoint and the spindle position checkpoint, have been shown to inhibit mitotic exit in budding yeast (reviewed in Lew and Burke, 2003). The negative regulators *BUB2* and *BFA1* are essential for both of these checkpoints. In contrast, *KIN4* was found to function in the spindle position checkpoint but not the spindle assembly checkpoint.

How does Kin4 inhibit activation of the MEN? To address this question, I analyzed the regulation of components at the top of the MEN: Bub2, Bfa1, and Tem1. Bub2 and Bfa1 were not fully phosphorylated in cells overexpressing *KIN4*, suggesting that Kin4 regulates the association of the GAP complex with Tem1. Cdc5 phosphorylates both Bfa1 and Bub2 during anaphase to promote the inactivation of GAP activity (Hu and Elledge, 2002; Hu et al., 2001). However, Kin4 likely acts in parallel to Cdc5 given the fact that deleting *KIN4* or overexpressing *KIN4* had no effect on Cdc5 localization or activity. Overexpressing *KIN4* not only affected Bub2-Bfa1 activity but also affected Bfa1 localization with fifty percent of cells showing no Bfa1 on the SPBs. It is not yet clear whether this is an additional mode of regulation or an artifact of high levels of Kin4.
However, Kin4 clearly influences the localization of Tem1 to the SPB. In cells overexpressing Kin4, Tem1 is not detected on the SPB in either metaphase or anaphase. On the other hand, deletion of KIN4 allowed Tem1 to associate with the SPB in cells with misaligned spindles, when the protein is normally not found there. The next question to address is how Kin4 regulates Tem1 localization. Is there a direct interaction between Kin4 and Tem1 or does Kin4 regulate Tem1 through a different mechanism?

To gain better insight into the role of Kin4 in mitotic exit, regulation of the protein itself was analyzed. Kin4 encodes a protein with a kinase domain in the amino terminus and a carboxy terminus that shows no homology to known proteins or protein motifs. Phosphorylation status and localization within the cell appear to be two modes of regulation of Kin4. A correlation between the phosphorylation state and Kin4’s localization was seen. During S phase and early mitosis, Kin4 is localized to the mother cell cortex and is hypophosphorylated. During late stages of mitosis, Kin4 is enriched at the bud neck and the protein becomes phosphorylated. Kin4 has also been shown to localize to the SPB and specifically the mother SPB (Pereira and Schiebel, 2005). In contrast to bud neck localization, SPB localization does not correlate with phosphorylation status. Kin4 is associated with the mother SPB in midanaphase, which is before Kin4 becomes phosphorylated, and remains at the SPB until cells exit from mitosis. These data indicate that a number of mechanisms likely regulate the localization of Kin4. The next step in understanding the regulation and the functions of Kin4 is to elucidate how its localization is controlled.

Identification and characterization of KIN4 has expanded the model for how exit from mitosis is inhibited in cells with misaligned spindles (Figure 1). Previously it was shown that the spatial segregation of the MEN activator Lte1 into the bud from the GTPase Tem1 at the SPB is important for inhibiting mitotic exit in response to spindle misalignment (Bardin et al., 2000; Pereira et al., 2000). Now we can expand on this model in which an activator, Lte1, and an inhibitor, Kin4, define the spindle position checkpoint. Lte1 establishes a domain of MEN activation within the bud, whereas Kin4 generates a domain of MEN inhibition within the mother cell. Thus, MEN activation and
Figure 1: Model for how Kin4 functions in the spindle position checkpoint to inhibit MEN signaling.

A zone of activation and a zone of inhibition of mitotic exit are created by the localization of Lte1 and Kin4 respectively. If the spindle is mispositioned such that it is solely within the mother cell, exit from mitosis is prevented by the inhibition of the GTPase Tem1. Tem1 is held inactive by the action of Kin4 and Bub2-Bfa1, the two component GAP. Tem1 activation is also inhibited because it is not in contact with its activator Lte1.
consequently mitotic exit does not occur unless the daughter bound SPB, which functions as the center for MEN signaling, moves from the Kin4 inhibition domain into the Lte1 activation domain within the bud. How the position of the spindle is monitored and how the signal is propagated remains to be determined.

**Dependent on overexpression (doe) mutants identified**

To identify novel positive regulators of mitotic exit, a screen for mutants dependent on the overexpression of the MEN component *DBF2*, *CDC5*, or *CDC15* was conducted. This screening methodology was adapted from a screen conducted in *S. pombe* where mutants that were dependent on the overexpression of *plo1* (S. pombe homolog of *CDC5*) were identified (Cullen et al., 2000). The objective of this screening method is to isolate partial loss of function mutants that can be rescued by the overexpression of the gene of interest. Regulators or effectors of the gene being overexpressed or genes that act in a parallel pathway could be identified using this approach. Using this screening method is more appropriate than a suppressor screen for genes that have more than one essential function because an allele that only affects one function is not needed. The analogous screens described in Chapter III and in *S. pombe* by Cullen et al. (2000) have identified known and novel regulators of the cell cycle, indicating that this screening method is a valid approach. These two screens also indicate that this method could be adapted to other systems to identify components or regulators of signaling networks.

From screening 725,960 colonies, four dependent on overexpression *(doe)* alleles as well as six *DBF2* alleles, nineteen *CDC5* alleles, and four *TEMI* alleles were identified. Obtaining known positive regulators of mitotic exit reassured us that the screening method was valid. Moreover, these alleles could potentially be useful in studying the regulation of these individual genes. Of particular interest are three *DBF2* alleles and all four *TEMI* alleles that showed intragenic complementation, as well as two cold sensitive *CDC5* alleles. Identification of alleles that show intragenic complementation suggests that the protein forms a multimer, has multiple functional domains or that the protein functions in a complex. Obtaining these alleles also indicate that this screening approach can be used to study genes with multiple functions.
To determine whether the DOE genes play a role in mitosis, basic characterization of the doe mutants was conducted. Analyzing the terminal phenotype has provided some clues as to the point in the cell cycle that these genes act. The terminal phenotype of doe1 is very heterogeneous indicating that it may play a role in multiple processes throughout the cell cycle. Analyzing the cell cycle profile of doe2, doe3, and doe4 mutants indicates that these genes may play a role in mitosis. The most interesting mutant is doe2 given the fact that it arrests as dumbbells and is delayed in anaphase. Data supporting the idea that DOE3 and DOE4 play a role in mitosis are that the mutants are suppressed by sorbitol similar to most MEN mutants and that both these mutants exhibit a metaphase and/or anaphase delay. This initial characterization of the doe mutants has validated our approach of identifying novel regulators of mitosis.
Unanswered Questions and Future Directions

As our knowledge of the signaling networks that control exit from mitosis expands, it has become clear that a variety of signals regulate this transition. Cell cycle cues and other cellular events and environmental conditions all appear to influence exit from mitosis. Regulation of the GAP complex Bub2-Bfa1 has taken center stage given the fact that these proteins are required to respond to defects in the assembly and position of the mitotic spindle and to DNA damage. However, we are only beginning to understand how these signals are coordinated to inhibit mitotic exit. The data described in this thesis has shed light onto one mechanism that regulates exit from mitosis in budding yeast, the spindle position checkpoint. Although it is known that misaligned spindles inhibit mitotic exit, it is still unclear what monitors the position of the spindle and how the signal is propagated. In the next sections I will discuss what is known about the function and regulation of Kin4 and what should be done to further our understanding of this protein. Data from Chapter II has shown, that the protein kinase Kin4 is required to inhibit mitotic exit when the spindle becomes misaligned, however the precise role Kin4 plays is not known. Next I will explain what is known about the regulation of the negative regulators Bub2 and Bfa1 and our understanding of the regulation of Tem1. Finally I will briefly describe how the HOG pathway has been shown to regulate mitotic exit.
Regulation and function of Kin4

The recent identification of Kin4 as a negative regulator of exit from mitosis has expanded the model of how the position of the mitotic spindle influences mitotic exit. However our knowledge of how Kin4 is regulated is very limited. Understanding how Kin4 is regulated and the proteins that it interacts with should provide insight into how the position of the spindle influences the cell cycle.

How is localization regulated?
A number of FEAR network and MEN components have dynamic localization patterns, which serves as a mode of regulation. The localization pattern of Kin4 also appears to be dynamic and therefore could be a mode of regulation for this protein. Analyzing how Kin4 is localized may provide evidence for this hypothesis. Kin4 is found at the mother cell cortex throughout most of the cell cycle and then detected at the bud neck and the mother SPB during later stages of mitosis (D'Aquino et al., 2005; Pereira and Schiebel, 2005). To date there are no known proteins reported that share the same localization pattern as Kin4. Only a few proteins have been shown to localize specifically to the mother cell cortex, Stt4, Sfk1, and Num1 (Audhya and Emr, 2002; Heil-Chapdelaine et al., 2000). Stt4 encodes 1-phosphatidylinositol 4-kinase and Sfk1 a trans-membrane protein that anchors Stt4 at the plasma membrane. Both proteins localize to the mother cell cortex in cells with small buds, and to the mother and bud cortex in large budded cells; however they are not detected at the bud neck (Audhya and Emr, 2002). Num1 is required for dynein-dependent sliding of microtubules along the mother cell cortex into the bud (Heil-Chapdelaine et al., 2000). Num1 localizes to the mother cortex in budded cells, but localizes to the bud tip in large budded cells rather than the bud neck (Heil-Chapdelaine et al., 2000). If the localization of Kin4 to the mother cell cortex depends on any of these three proteins, this might provide a clue to how Kin4 is regulated. Given the fact that Num1 and dynein function together to slide microtubules along the cell cortex, which is required for nuclear position, a dependence of Kin4 regulation on Num1 would be appealing.
Kin4 is also seen at the SPB, similar to most MEN components, in a Nudl dependent manner (Bardin et al., 2000; Gruneberg et al., 2000; Pereira and Schiebel, 2005; Visintin and Amon, 2001). In contrast to Tem1, Bub2, and Bfa1 that bind predominantly to the SPB destined to migrate into the bud and to Cdc15 and Dbf2 that localize to both SPBs, Kin4 is detected only at the mother SPB in an unperturbed cell cycle (Bardin et al., 2003; Bardin et al., 2000; Cenamor et al., 1999; Menssen et al., 2001; Molk et al., 2004; Pereira et al., 2000; Pereira and Schiebel, 2005; Visintin and Amon, 2001; Xu et al., 2000). Kin4 is the first reported protein that we know of that selectively localizes to the mother SPB. However, if the spindle is misaligned or microtubules are depolymerized, Kin4 can now be detected on both SPBs, similar to Bub2 and Bfa1 (Pereira and Schiebel, 2005). Binding of Kin4 to the SPB has been shown to be dependent on Cdc5 but independent of Bub2, Bfa1, and Tem1 (Pereira and Schiebel, 2005). It will be important to determine whether there is a direct interaction between Kin4 and Cdc5. Conducting an in vitro kinase assay using Kin4 as a substrate and Cdc5 as the kinase could provide evidence that Kin4 is a substrate of Cdc5 and therefore there is a direct interaction between the two proteins. It will also be imperative to understand why Kin4 is only detected on the mother SPB. Answering this question should provide a clue into how spindle alignment is monitored.

One way to address this question is to find what in Kin4 is responsible for its localization through domain analysis. The N-terminus bears the kinase domain while the C-terminus shows no homology to known protein motifs. Determining the minimal domain required for proper localization could be accomplished by constructing truncated versions of Kin4 and analyzing their localization patterns. If the kinase domain is required, studying the localization of the kinase dead mutant should establish whether Kin4 kinase activity is required for proper localization. Once the minimal motif is determined, it could be used to find interactors.

Another question to address is whether proper localization is required for Kin4 function. When Kin4 is overexpressed it localizes to the mother cell cortex and bud neck, however in contrast to cells with normal levels of expression, it can now be detected in the bud
albeit at lower levels than in the mother (D'Aquino et al., 2005). This suggests that overproduced Kin4 performs the same functions as Kin4 expressed at endogenous levels, however the protein found in the bud may exert ectopic functions. One way to address this would be analyze the consequence of mislocalizing Kin4. If Kin4 is localized to both the mother and bud cortex, is mitotic exit prevented? Do cells with Kin4 localized to only the bud cortex, or to the daughter SPB exit mitosis? Do cells with aberrant Kin4 localization have a functional spindle position checkpoint? Performing these studies should determine how localization affects Kin4 function and may provide insight into the mechanisms that monitor spindle position.

*What is the function of Kin4?*

Biochemical and genetic data indicate that Kin4 regulates Bub2 and Bfa1. Cdc5 phosphorylates Bfa1 during anaphase, which causes the GAP complex to dissociate from Tem1 and thus allowing for MEN activation (Hu et al., 2001). Bub2 phosphorylation, which is partially dependent on **CDC5**, also correlates with inactivation of GAP activity during anaphase (Hu and Elledge, 2002). Overexpression of Kin4 prevents hyperphosphorylation of both Bub2 and Bfa1 (D'Aquino et al., 2005). One speculation is that Kin4 regulates Cdc5. However, cells lacking **KIN4** or overexpressing **KIN4** exhibit wild type levels of Cdc5 kinase activity and Cdc5 localizes normally (see Appendix I). Therefore, Kin4 likely does not regulate Cdc5 directly and as a result may regulate a phosphatase that controls the phosphorylation of Bfa1 and/or Bub2. Nevertheless, we cannot rule out the possibility that Kin4 regulates Cdc5 activity only at the SPB, which may not be detectable by our assays.

*Is kinase activity required for function?*

Data from Chapter II has shown that Kin4 associated kinase activity can be detected. Attempts to detect Kin4 kinase activity above background levels in a wild type cell have failed, indicating that Kin4 kinase activity might not get induced during normal progression through the cell cycle. This finding is consistent with the fact that cells lacking **KIN4** show no cell cycle defects. However, our kinase assays are not very sensitive presumably because we use the artificial substrate myelin basic substrate due to
the fact that we do not know the *in vivo* substrates of Kin4. Kinase activity over background levels can be detected in *kar9Δ act5ts* cells when approximately 20% of cells have mispositioned spindles, providing evidence that the kinase is active in cells with misaligned spindles. To show that the kinase activity of Kin4 is essential for its function, I made a point mutant in the kinase domain and assayed the phenotype of this mutant in cells with misaligned spindles. Cells containing a kinase dead allele of Kin4 can bypass the spindle position defect of *dynlΔ* cells similar to *kin4Δ* cells, demonstrating that the kinase activity is crucial for the function of Kin4 in the spindle position checkpoint (see Appendix I).

Curiously, cells treated with nocodazole exhibited the most robust Kin4 kinase activity of all the conditions tested. This result is surprising given the fact that, unlike Bub2 and Bfa1, Kin4 is not required for the spindle assembly checkpoint that is triggered by depolymerizing microtubules (D'Aquino et al., 2005; Pereira and Schiebel, 2005). Two models to explain this phenomenon have been proposed. One, Kin4 only partially activates the Bub2-Bfa1 GAP complex when there is spindle damage. Phosphorylation of Bfa1 by Cdc5 has been shown to only partially inhibit GAP activity *in vitro* (Geymonat et al., 2003). Deletion of either *BUB2* or *BFA1* would presumably eliminate all GAP activity, while deletion of *KIN4* would only partially reduce GAP activity. This remaining GAP activity in *kin4Δ* would be sufficient to maintain the arrest. One argument against this hypothesis is the fact that deletion of *BUB2* or *BFA1* does not enhance the phenotype of *dynlΔ kin4Δ* double mutants (D'Aquino et al., 2005). However, developing an assay to measure Bub2-Bfa1 GAP activity *in vivo* could provide supporting data for this hypothesis. A second hypothesis is that there are additional signals in response to the activation of the spindle assembly checkpoint versus the activation of the spindle position checkpoint. In *dynlΔ* cells with mispositioned spindles only the spindle position checkpoint inhibits mitotic exit, whereas mitotic exit is inhibited by both the spindle position checkpoint and the spindle assembly checkpoint in nocodazole treated cells. Therefore a Kin4 independent signal along with Kin4 acts to maintain Bub2-Bfa1 GAP activity in nocodazole treated cells. Isolating mutants that only
arrest in nocodazole when combined with $kin4\Delta$ would provide evidence for this hypothesis.

**What proteins are phosphorylated by Kin4 and which proteins interact with Kin4?**

A key to understanding the mechanisms in which Kin4 inhibits mitotic exit is to identify substrates and protein interactors. A first approach could be to purify candidate proteins to be used as substrates for the Kin4 kinase assay. Co-immunoprecipitation assays could also be performed between epitope tagged Kin4 and candidate proteins. Tem1 is a good candidate given the fact that cells overexpressing $KIN4$ fail to localize Tem1 to the SPB and cells lacking $KIN4$ inappropriately allow Tem1 to associate with SPBs when the spindle is misaligned. To our dismay, purified Tem1 was not phosphorylated using the Kin4 kinase assay (Leon Chan personal communication). However, this negative result does not eliminate the possibility that Tem1 is a substrate. Accessory factors or a priming modification on Tem1 that are not present in the kinase reaction could be required for phosphorylation. Co-immunoprecipitations of Tem1 and Kin4 have not been conducted as of yet. In addition, Cdc5 and Nud1 could be interactors and/or substrates given the fact that Kin4 localization depends on Cdc5 and Nud1.

There are a number of approaches to identify substrates and interactors of Kin4 that could be employed and I will mention just a few here. Performing a yeast two-hybrid assay using Kin4 could identify protein interactors. This approach may not detect substrates given the fact that a kinase substrate interaction is typically short-lived. An attempt at using a full length Kin4 protein as bait in the two-hybrid system has not yielded any results, however this could be due to technical issues (K.D., L.C. personal communication). Using truncated versions of Kin4 in this assay might also provide clues into the function of the C terminus, which shares no homology to known protein motifs. A second approach to identifying interactors would be to immunoprecipitate an epitope tagged version of Kin4 and perform mass spectrometry on the associated proteins. Finally conducting a suppressor screen of $GAL-KIN4$ could identify substrates and/or interactors. Screening for both high dosage suppressors and loss of function suppressors should likely identify genetic interactors of $KIN4$. 


Recently a kinase substrate map was constructed for budding yeast (Ptacek et al., 2005). Eighty-two kinases out of the known 122 yeast protein kinases were each purified and incubated separately with a yeast proteome array in the presence of radioactive ATP (Ptacek et al., 2005; Zhu et al., 2000). Proteins were identified as substrates if they were reproducibly phosphorylated when an active kinase was present versus a control. Kin4 was tested in this assay resulting in 19 positive hits. A number of these hits appear interesting and should be studied further. Two proteins that localize to the plasma membrane were identified, Ist2 and Akr1. Ist2 is particularly appealing given the fact that it localizes only to the mother in small budded cell, however it does localizes to the bud in medium and large budded cells. Dependence of Kin4 localization to the cell cortex on Ist2 should be investigated. The only MEN component identified was Cdc15. This is intriguing given the fact that Kin4 appears to regulate events upstream of Cdc15. Determining if Cdc15 is a substrate in our kinase activity assay as well as testing for a protein-protein interaction between Kin4 and Cdc15 should solve this discrepancy. Unfortunately many of the FEAR network and MEN components were not on the protein array, including Bub2, Bfa1, Lte1, Cdc14, Esp1, Spo12, and Fob1. Therefore it will be worthwhile to identify substrates through other approaches.

**Bub2-Bfa1 Regulation**

Cells lacking \textit{BUB2} or \textit{BFA1} are viable when the cell cycle in unperturbed; however, if damage occurs, such as spindle damage or DNA damage, \textit{BUB2} and \textit{BFA1} become essential (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999; Wang et al., 2000). Overexpression of \textit{BUB2} or \textit{BFA1} has also been shown to induce a mitotic arrest (Lee et al., 1999; Ro et al., 2002). A current view is that \textit{BUB2} and \textit{BFA1} are required to inhibit mitotic exit in response to most, if not all, mitotic arrests (Alexandru et al., 1999; Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Fesquet et al., 1999; Fraschini et al., 1999; Hoyt et al., 1991; Krishnan et al., 2000; Lee et al., 2001; Li, 1999; Pereira et al., 2000; Wang et al., 2000). Therefore once checkpoint signals are removed, control mechanisms must exist to turn off Bub2-Bfa1 activity.
Phosphorylation of Bfa1 and Bub2 has been reported to regulate the GAP activity (Hu and Elledge, 2002; Hu et al., 2001; Lee et al., 2001). Cdc5 has been shown to phosphorylate both Bfa1 and Bub2 (Hu and Elledge, 2002; Hu et al., 2001). The phosphorylated form is believed to be inactive given the fact that phosphorylated Bfa1 has weaker affinity for Tem1 (Hu et al., 2001). A Bfa1 mutant that has 11 potential phosphorylation sites mutated (BFA1-11A) has been constructed (Hu et al., 2001). Expressing this allele in wild type cells does not induce a cell cycle arrest, which would be expected for a hyperactive allele of BFA1. This indicates that there may be other mechanisms to regulate Bfa1-Bub2 in response to spindle position. The protein kinase Kin4 has been shown to function upstream of Bub2 and Bfa1 and likely in parallel to Cdc5, however it is not known how Kin4 regulates the GAP complex (described above). Future studies on the functions of Kin4 in controlling mitotic exit should provide greater insight into the regulation of Bub2 and Bfa1.

**Regulation of Tem1**

The GTPase Tem1 acts at the top of the MEN and is essential for mitotic exit. Bub2 and Bfa1 negatively regulate Tem1, whereas Lte1 positively regulates Tem1. Bub2 and Bfa1 are also required for Tem1 localization to the SPB in S phase and early mitosis (Pereira et al., 2001). Deletion or overexpression of KIN4 affects Tem1 localization, suggesting that Kin4 may also regulate Tem1. One hypothesis is that Kin4 regulates Tem1 through its affects on Bub2 and Bfa1. However, deletion of KIN4 did not affect Bfa1 localization and overexpression of KIN4 only resulted in the loss of Bfa1 from the SPB in 50 percent of the cells. Consequently the hypothesis that Kin4 influences Tem1 localization independently from its regulation of Bub2 and Bfa1 seems more likely. Therefore, the next critical question to answer is how Kin4 affects Tem1 localization and activity. Is there a direct interaction between Kin4 and Tem1? Determining if Tem1 and Kin4 can co-immunoprecipitate should address this question. Tem1 is a phosphoprotein and Kin4 is a kinase, does Kin4 phosphorylate Tem1? We tested if Tem1 is a substrate of Kin4 and found that purified Tem1 was not phosphorylated in our Kin4 kinase assay (Leon Chan, personal communication). However, we cannot rule out the possibility that other factors
are needed for Tem1 phosphorylation that are not present in our reaction. Analyzing the localization of Tem1 in cells carrying the kinase dead allele of KIN4 should determine if Kin4 kinase activity is required for proper Tem1 localization. These studies on Tem1 localization may provide clues as to how asymmetry at the SPB is achieved.

**How is asymmetry generated at the SPB?**

Generation of asymmetry appears to be a theme in the regulation of late stages of mitosis in budding yeast. How is this asymmetry established? SPBs duplicate through a conservative mechanism, generating an “old” and “new” SPB (Adams and Kilmartin, 2000). In budding yeast, it has been shown that the “old” SPB is always the one to migrate into the bud (Pereira et al., 2001). The old SPB maintains astral microtubule interactions with the bud, whereas the new SPB is only capable of forming interactions with the mother cell (Carminati and Stearns, 1997; Segal et al., 2000a; Segal et al., 2000b; Shaw et al., 1997). If microtubules are transiently depolymerized, then the asymmetric pattern of SPB inheritance is disrupted. Now either the old or the new SPB migrates into the bud; however, the MEN components Tem1, Bub2, and Bfa1 are still detected at the SPB migrating into the bud (Pereira et al., 2001). This data indicates that asymmetry does not just reflect a difference between the old and new SPBs but that there is a mechanism in place to establish asymmetry. The nature of this mechanism is not known, but one hypothesis is that forces exerted on the SPBs by the astral microtubules generates asymmetry whereas a second hypothesis is that the microtubule interactions with the mother or bud cortex of the two different SPBs creates asymmetry. In budding yeast, one nucleus must be pulled into the bud. As a result, a greater pulling force is exerted on the SPB migrating into the bud than the SPB remaining in the mother cell. In the temperature sensitive esp1-I mutants, the nucleus gets pulled into the bud (Jansen et al., 1996). If these cells are treated transiently with nocodazole to disrupt astral microtubule interactions with the cortex and then released from the restrictive temperature, anaphase now occurs in the opposite direction with one nucleus being pulled into the mother cell. In these cells asymmetry is generated, however now MEN components are localized to the SPB that is found in the mother cell (Fernando Monje-
Casas, personal communication). This data shows that astral microtubule interactions with the bud cortex are not necessary for generating asymmetry and supports the hypothesis that forces exerted on the SPB are important for asymmetry. Somehow this force differential would be translated into a signal for the localization of MEN components.

Curiously, components of the SIN pathway, homologs of the MEN in *S. pombe*, also localize in an asymmetric manner at the SPB. In a recent study on SPB inheritance, based on experiments in budding yeast, it was found that active SIN complexes associate with the new SPB and remain at the new SPB even after transient microtubule depolymerization (Grallert et al., 2004). Thus in *S. pombe* the SPBs seem to be intrinsically different, which dictates where the activated SIN module is localized. This might be explained by the fact that two proteins, Cdc11 and Sid4, act as scaffolds to anchor SIN components to the SPB, whereas only Nud1 acts as a scaffold for the MEN (Gruneberg et al., 2000; Krapp et al., 2001; Tomlin et al., 2002).

**How is position of the spindle monitored?**

Much is known about how the spindle is properly positioned within the cell (reviewed in Morris, 2003; Pearson and Bloom, 2004). It is clear that dynamic microtubule interactions with the cell cortex play a key role in establishing nuclear position. Astral microtubules generated from the SPBs scan the mother cortex, bud cortex and bud neck. Interactions with these various sites orient the mitotic spindle along the mother-bud axis and help pull one nucleus into the bud. Dynein and the kinesins Kar3, Kip2, and Kip3 are microtubule dependent motors required for orienting the spindle. A microtubule capture apparatus is found at the bud tip. Microtubules are stabilized once they are captured. Then shrinkage of the microtubules acts to pull one nucleus into the bud. Despite all the knowledge gained about positioning the spindle, how the cell monitors the position of the spindle and how the signal that the spindle is misaligned is propagated are not known.
One proposed mechanism involves the interaction of astral microtubules with the bud neck (Adames et al., 2001). Several cells inappropriately exited mitosis with misaligned spindles when a loss of the interaction between cytoplasmic microtubules and the bud neck was detected. However, this evidence is only correlative and does not establish a direct cause and effect relationship. If the interaction between cytoplasmic microtubules and the bud neck could somehow be controlled, then looking at the consequence of disrupting this interaction may provide direct evidence for this hypothesis.

Another mechanism involves the delivery of specific components into the bud by the SPB. Therefore the cell ensures mitotic exit does not occur until the bud has received one nucleus. Tem1, the GTPase at the top of the MEN, is localized to the daughter SPB whereas its activator Lte1 is sequestered in the bud (Bardin et al., 2000; Pereira et al., 2000). Only when the Tem1 bearing SPB migrates into the bud will Tem1 become activated and trigger mitotic exit. However, this cannot be the only mechanism by which the MEN is activated given the fact that Lte1 is only essential at low temperatures and that certain mutants with misaligned spindles have been shown to exit mitosis even in the absence of Lte1 (Adames et al., 2001; Castillon et al., 2003).

A third mechanism suggests that forces generated at the SPB as the mitotic spindle is elongated are monitored. In cells with misaligned spindles, Tem1, Bub2, and Bfa1 are detected on both SPBs. Presumably the forces exerted on the SPBs are equal in these cells with mispositioned spindles. Once the spindle repositions along the mother-bud axis, Tem1 accumulates at the daughter bound SPB, which now should experience greater forces than the SPB remaining in the mother cell. These three mechanisms mentioned are not mutually exclusive and therefore it is possible that the cell utilizes all of them.

**Role for the high osmolarity and glycerol (HOG) pathway in mitotic exit**

Mounting evidence indicates a role in regulating mitotic exit for the HOG pathway, the MAP kinase signaling cascade responsible for mediating the cell’s response to high
osmolarity (HOG pathway reviewed in Hohmann, 2002). Data described in Appendix II begins to address how a hypertonic environment can promote exit from mitosis. High osmolarity (1.2M sorbitol), which activates the HOG pathway, can bypass the proliferation defects of the temperature sensitive MEN mutants *cdc15-2, dbf2-2,* and *tem1-3* (Grandin et al., 1998; Appendix II). The HOG pathway MAP kinase kinase *PBS2* is required for this bypass. In contrast to other MEN mutants, temperature sensitive mutants *cdc14-1* and *cdc14-3* were not viable in hypertonic conditions, indicating that the MEN effector *CDC14* is required for the HOG pathway to regulate mitotic exit.

An unanswered question is how the HOG pathway regulates mitotic exit. In a *cdc15-2* mutant, Cdc14 is released from the nucleolus when exposed 1.2M sorbitol while in a *cdc15-2 pbs2Δ* Cdc14 release is impaired. The HOG pathway could enhance signaling through the defective MEN or through a parallel pathway like the FEAR network. To look at MEN activation, Dbf2 kinase activity was monitored. As expected Dbf2 kinase activity was low in *cdc15-2* mutants (Appendix II; Visintin and Amon, 2001) and this was not stimulated upon addition of sorbitol (Appendix II). This data suggests that the HOG pathway does not restore MEN signaling in temperature sensitive MEN mutants. Next we tested if the FEAR network is required for the HOG pathway induced Cdc14 release in MEN mutants. Temperature sensitive *cdc15-2* mutants lacking the FEAR network components *SPO12* or *SLK19* were no longer viable in the presence of sorbitol. In addition, high osmolarity no longer induced Cdc14 release in *cdc15-2 spo12Δ* mutants. These data support the hypothesis that the HOG pathway functions upstream of the FEAR network; however these observations do not rule out the possibility that the HOG pathway regulates a parallel pathway. The HOG pathway may regulate Cdc5 function, however more thorough data is needed to support this hypothesis.

Overexpression of *STE20*, a conserved PAK kinase involved in several signaling cascades including the HOG pathway, can bypass the telophase arrest of *lte1Δ* at 14°C as well as alleviate the growth defect of *cdc15-2* mutants (Hofken and Schiebel, 2002; Appendix II). *STE20* appears to play a role in addition to the HOG pathway in regulating mitotic exit given the fact that *lte1Δ ste20Δ* mutants have a more severe growth defect.
than \textit{lte1Delta pbs2Delta} or \textit{lte1Delta hog1Delta} mutants. It has been proposed that Ste20 activates Cdc5 given the fact that PAK kinase can activate polo kinase in mammalian cells (Ellinger-Ziegelbauer et al., 2000). Elucidating all the roles that Ste20 plays in mitotic exit will expand our knowledge of how mitotic exit is regulated.

**Concluding remarks**

As our knowledge expands on how mitotic exit is controlled in budding yeast, it has become clear that this is a highly regulated transition. It is imperative that the cell coordinates segregation of its genetic material with exit from mitosis and the generation of two daughter cells. It is clear that the cell integrates cell cycle cues, cellular events, and environmental conditions to regulate this transition. As homologues in higher eukaryotes are beginning to be studied, it will be interesting to determine what aspects are conserved and what aspects are unique to budding yeast.
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Appendix I:

An analysis of *KIN4* genetic interactions
Background

To ensure accurate chromosome segregation it is imperative that the site of cytokinesis is coordinated with the position of the mitotic spindle. Surveillance mechanisms are in place to guarantee that this coordination occurs. In the budding yeast *Saccharomyces cerevisiae*, a surveillance mechanism known as the spindle position checkpoint ensures that exit from mitosis only occurs when the mitotic spindle is positioned along the mother-bud axis (reviewed in Lew and Burke, 2003). This checkpoint was first identified in cells that were defective in guiding or capturing cytoplasmic microtubules at the cell cortex. Cells bearing mutations in factors regulating cytoplasmic microtubules often exhibit misaligned spindles (Yeh et al., 1995). These mutants complete chromosome segregation solely within the mother cell, however exit from mitosis does not occur.

The spindle position checkpoint inhibits mitotic exit by preventing the activation of the protein phosphatase Cdc14. Cdc14 is the key regulator of exit from mitosis in budding yeast (reviewed in Stegmeier and Amon, 2004). Throughout most of the cell cycle Cdc14 is held inactive in the nucleolus by its inhibitor Cfi1/Net1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Upon transition into anaphase, release of Cdc14 from Cfi1/Net1 is promoted and mitotic exit is triggered. This dissociation in anaphase is regulated by two signaling networks, the Cdc fourteen early anaphase release (FEAR) network and the mitotic exit network (MEN). The MEN resembles a GTPase signaling cascade and is essential for mitotic exit. The GTPase Tem1 is at the top of the pathway and is thought to be positively regulated by Lte1 and is negatively regulated by the GAP complex Bub2-Bfa1 (Alexandru et al., 1999; Geymonat et al., 2002; Hoyt et al., 1991; Jaspersen et al., 1998; Krishnan et al., 2000; Li, 1999; Shirayama et al., 1994a; Shirayama et al., 1994b; Visintin and Amon, 2001). Activated Tem1 propagates the signal to the protein kinase Cdc15, which then activates the protein kinase Dbf2 and its associated factor Mob1 (Asakawa et al., 2001; Bardin et al., 2003; Lee et al., 2001a; Mah et al., 2001; Menssen et al., 2001; Visintin and Amon, 2001).

Genetic evidence indicates that the spindle position checkpoint inhibits MEN activation through a number of mechanisms. The first mechanism identified depends on the spatial...
segregation of MEN components until one nucleus has moved into the bud. The activator Lte1 becomes localized to the bud cortex upon bud formation while Tem1 localizes to the daughter bound spindle pole body (SPB; Bardin et al., 2000; Pereira et al., 2000; reviewed in Seshan and Amon, 2004). As a result, Tem1 and Lte1 only come into contact with one another when the daughter SPB moves into the bud during spindle elongation in anaphase (Bardin et al., 2000; Pereira et al., 2000). Accumulation of Lte1 in the mother cell allows with misaligned spindles to inappropriately exit mitosis, indicating that this spatial segregation is important (Bardin et al., 2000; Castillon et al., 2003).

Another mechanism to ensure that cells do not exit mitosis when the spindle is misaligned involves the negative regulators Bub2 and Bfa1. Deletion of either \textit{BUB2} or \textit{BFA1} bypasses the spindle position checkpoint, resulting in the formation of multinucleate and anucleate cells (Adames et al., 2001; Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Pereira et al., 2000; Wang et al., 2000). However, the mechanisms by which Bub2 and Bfa1 are activated in response to mispositioned spindles are not known. Recent evidence has identified the protein kinase Kin4 as a component of the spindle position checkpoint (D'Aquino et al., 2005; Pereira and Schiebel, 2005). Biochemical and genetic data indicate that Kin4 maintains the Bub2-Bfa1 complex in an active state, likely by preventing Bub2 and Bfa1 hyperphosphorylation. Whether Kin4 is constitutively active and/or plays a role in monitoring spindle position is not known. To gain further insight into the function of Kin4, I have analyzed genetic interactions between MEN components and \textit{KIN4}. Data indicates that Kin4 only inhibits mitotic exit through the Bub2-Bfa1 complex and likely functions in parallel to Cdc5. In addition, redundant functions between \textit{KIN4} and a gene with high similarity, \textit{YPL141c}, were examined. The experimental results suggest that, in contrast to Kin4, Ypl141c does not act as a negative regulator of mitotic exit.

**Results and Discussion**

**Inhibition of mitotic exit through overexpression of \textit{KIN4} is dependent on \textit{BUB2}.

Analyzing the phenotype of cells with elevated protein levels of Kin4 reveals the inhibitory function of Kin4. Cells overexpressing \textit{KIN4} exhibit a delay in anaphase.
spindle disassembly, a defect in release of Cdc14 from the nucleolus, and persistent Clb2 protein levels (Figure 1B; D'Aquino et al., 2005). Kin4 is thought to inhibit MEN activation by preventing the hyperphosphorylation of the GAP complex components Bub2 and Bfa1. To determine whether Kin4 is capable of regulating other components of the MEN, the phenotype of overexpressing KIN4 in cells lacking BUB2 was examined (Figure 1). Upon release from a pheromone induced G1 arrest, cells lacking BUB2 progressed through the cell cycle normally (Figure 1A), whereas cells overexpressing KIN4 exhibited a defect in mitotic exit as reported previously (Figure 1B). Overexpression of KIN4 in bub2A cells appeared almost identical to bub2A when comparing spindle morphology, release of Cdc14 from the nucleolus, and Clb2 protein levels (Figure 1A, C). These data indicates that Kin4 regulates mitotic exit through the GAP complex and that this is its only essential function for inhibition of mitotic exit.

**Kin4 does not regulate Cdc5 kinase activity or localization.**

Phosphorylation of Bub2 and Bfa1 is believed to regulate their GAP activity (Hu and Elledge, 2002; Hu et al., 2001; Lee et al., 2001b). The polo kinase Cdc5 is capable of phosphorylating both Bub2 and Bfa1 (Hu and Elledge, 2002; Hu et al., 2001). To investigate whether Kin4 controls Bub2-Bfa1 phosphorylation by regulating Cdc5, we examined Cdc5 activity and localization in Kin4 mutants (Figure 2). Deletion of KIN4 did not affect Cdc5 kinase activity or Cdc5 localization in comparison to wild type cells (Figure 2A, C). Cells overexpressing Kin4 had normal Cdc5 localization; however, Cdc5 kinase activity accumulated in comparison to wild type cells (Figure 2B, D). This accumulation of Cdc5 kinase activity is likely the result of the anaphase delay in cells with abundant Kin4 and does not represent a mode of regulation. These results suggest that Kin4 functions in parallel to Cdc5 to regulate the Bub2-Bfa1 complex, though the precise mechanism by which Kin4 regulates the GAP complex is not known.
Figure 1: Deletion of BUB2 suppresses the anaphase delay induced by overexpression of KIN4 and Kin4 kinase activity is required for its function.

(A, B, C) bub2Δ (A1901), GAL-KIN4 (A9249), and bub2Δ GAL-KIN4 (A9252) cells carrying a CDC14-HA fusion were arrested in G1 in YEP Raffinose (YEPR) using 5μg/ml α factor. GAL-KIN4 transcription was induced for one hour 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.

(D) dynlΔ (A2077), kin4Δ (A8453), dynlΔ kin4Δ (A9290), and dynlΔ KIN4-T209A (A14393) cells were grown for 24 hours 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) was determined.
Figure 2: Deletion or overexpression of KIN4 has no effect on Cdc5 kinase activity and localization.

(A) Wild type (A2399) and kin4Δ (A12800) cells carrying a CDC5-HA fusion were arrested in G1 using 5μg/ml α factor followed by a release into medium lacking pheromone. The amount of Cdc5 associated kinase activity throughout the cell cycle was determined.

(B) Wild type (A2399) and GAL-KIN4 (A12801) cells carrying a CDC5-HA fusion were arrested in G1 in YEP Raffinose (YEPR) using 5μg/ml α factor. GAL-KIN4 transcription was induced for one hour by the addition of galactose (YEPRG) while in α factor. Cells were released into YEPRG lacking pheromone. The amount of Cdc5 associated kinase activity was determined. Unfortunately, the 90 minute wild type sample was lost.

(C) Wild type (A2976) and kin4Δ (A13106) cells carrying a CDC5-MYC fusion were grown as described in (A). The number of metaphase and anaphase cells with Cdc5 localized to the nucleus, one SPB, or both SPBs was determined.

(D) Wild type (A2976) and GAL-KIN4 (A12802) cells carrying a CDC5-MYC fusion were grown as described in (B). Cdc5 localization to the nucleus, one SPB, or both SPBs in metaphase and anaphase cells was analyzed.
Kin4 kinase activity is required for the spindle position checkpoint.

The protein kinase Kin4 has recently been identified as a component of the spindle position checkpoint in budding yeast, yet the role it plays is unknown (D'Aquino et al., 2005; Pereira and Schiebel, 2005). Clearly KIN4 is essential for this checkpoint given the fact that deletion of KIN4 allows cells with misaligned spindles to exit mitosis, thus generating multinucleate and anucleate cells. To examine the role of Kin4 in this checkpoint we first analyzed Kin4 kinase activity. In kar9Δ act5ts cycling cultures, which contain 20% of cells with misaligned spindles, Kin4 kinase activity is increased approximately two fold in comparison to wild type cells (D'Aquino et al., 2005). This result suggested that Kin4 kinase activity is induced in cells with misaligned spindles. To determine if kinase activity is required for a functional checkpoint, a kinase dead allele was constructed (D'Aquino et al., 2005). Cultures of cells lacking the microtubule motor dynein (DYNJ) accumulate cells with misaligned spindles at low temperatures, whereas cultures of dyn1Δ kin4Δ and dyn1Δ KIN4-T209A accumulate multinucleate and anucleate cells (Figure 1D). Thus this result indicates that the kinase activity of Kin4 is essential for its function in the spindle position checkpoint. To better understand Kin4’s function in this checkpoint it will be imperative to identify the substrates of Kin4.

KIN4 interacts genetically with MEN components TEM1, CDC5, and NUD1.

If KIN4 is a negative regulator of the MEN during a normal cell cycle, then deletion of KIN4 could result in a hyperactive MEN. However, deletion of KIN4 in wild type cells has no effect on progression through the cell cycle (Figure 4A; D'Aquino et al., 2005; Pereira and Schiebel, 2005). To determine if KIN4 exhibited any genetic interactions with the MEN, KIN4 was deleted in strains carrying temperature sensitive mutants of the MEN components CDC14, DBF2, CDC5, TEM1, NUD1, and CDC15. Deletion of KIN4 by itself exhibited no growth defects in comparison to wild type at all temperatures tested (Figure 3). In contrast to what was expected for a negative regulator, deletion of KIN4 actually lowered the restrictive temperature of cdc5-1, tem1-3, and nud1-44 and very slightly affected the growth of dbf2-2 (Figure 3). It appears that KIN4 interacts with components at the top of the MEN, TEM1 and CDC5, and the SPB scaffold NUD1, which coincides with the epistasis analysis (D'Aquino et al., 2005). Why does the
Figure 3: Deletion of KIN4 lowers the restrictive temperature of some MEN temperature sensitive mutants.

Serial dilutions of cultures of wild type (A2587 and A8453), cdc14-3 (A795 and A9234), dbf2-2 (A852 and A9236), cdc5-1 (A860 and A9238), teml-3 (A1782 and A9240), nud1-44 (A1965 and A9242), and cdc15-2 (A2597 and A9244) -/+ kin4Δ cells were spotted on plates containing glucose and incubated at the indicated temperatures.
deletion of \textit{KIN4} lower rather than raise the restrictive temperatures? The answer to this question is not known, but one speculation could be that Kin4 promotes the localization of a MEN component while also inhibiting it. Therefore, in the absence of Kin4, inhibition is relieved but proper localization is impaired resulting in lower level of MEN signaling. Kin4 has been shown to regulate the localization of the MEN activator Tem1 in cells with misaligned spindles (D'Aquino et al., 2005). However these data seem to contradict one another. In cells with misaligned spindles deletion of \textit{KIN4} restores Tem1 localization to the SPB, whereas the genetic interaction data would suggest that deletion of \textit{KIN4} impairs localization of Tem1. Future studies on the regulation of Tem1 localization are needed to solve this discrepancy.

\textit{YPL141c}, a kinase with similarity to \textit{KIN4}, is not an inhibitor of mitotic exit.

Examination of the yeast genome revealed that the open reading frame \textit{YPL141c} is 43% identical and 56% similar to \textit{KIN4}. The fact that deletion of \textit{KIN4} has no effect on a normal cell cycle could be due to the action of a redundant factor. To determine if these genes have redundant functions, cell cycle progression was analyzed in single and double mutants. Deletion of \textit{KIN4} or deletion of \textit{YPL141c} had no effect on progression through an unperturbed cell cycle (Figure 4A). The double mutant may have a 15 minute delay in entry into metaphase and anaphase given that the number of metaphase cells peaks at 75 minutes and the number anaphase cells peaks at 90 minutes in wild type and single mutants, whereas metaphase peaks at 90 minutes and anaphase peaks at 105 minutes in the double mutant (Figure 4A). To validate this delay, this experiment should be repeated several times. Even so, overall progression through the cell cycle appeared similar in all strains indicating that \textit{KIN4} and \textit{YPL141c} are not required for normal cell cycle progression.

Cells lacking \textit{KIN4} have an intact spindle assembly checkpoint (D'Aquino et al., 2005; Pereira and Schiebel, 2005). The spindle assembly checkpoint is activated in response to unattached kinetochores and induces a metaphase arrest through inhibition of the APC/C\textsuperscript{Cdc20} and inhibition of the MEN (reviewed in Lew and Burke, 2003). To test if \textit{KIN4} and \textit{YPL141c} have redundant functions in the spindle assembly checkpoint, we
Figure 4: **YPL141c** is not essential for mitotic exit.

(A) Wild type (A1411), *kin4Δ* (A8453), *ypl141cΔ* (A9286), and *kin4Δ ypl141cΔ* (A9288) cells carrying a *CDC14-HA* fusion were arrested in G1 using 5μg/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.

(B) Wild type (A1411, squares), *kin4Δ* (A8453, triangles), *ypl141cΔ* (A9286, diamonds), and *kin4Δ ypl141cΔ* (A9288, circles) cells carrying a *CDC14-HA* fusion were arrested with 5μg/ml α factor and released into medium containing 15μg/ml nocodazole. The percentage of cells with Cdc14 released from the nucleolus (left graph) and the percent of rebudded cells (right graph) was determined. Rebudding signifies that cells exited from the nocodazole block and began a new cell cycle.
analyzed the phenotype of single and double mutants when treated with nocodazole, which causes microtubules to depolymerize and thus activates the spindle assembly checkpoint. Wild type cells treated with nocodazole did not exit mitosis and enter into a new cell cycle as evidenced by the failure to form a new bud (re budding) and the maintenance of Cdc14 in nucleolus (Figure 4C). In contrast to wild type cells, kin4Δ, ypl141cΔ, and kin4Δ ypl141cΔ strains all exhibited a transient release of Cdc14 from the nucleolus (Figure 4B). However, this release of Cdc14 was not sufficient to bypass the spindle assembly checkpoint given the fact that these cells did not rebud when treated with nocodazole (Figure 4B). Analyzing the release of Cdc14 in these mutants does indicate that they may share some overlapping function because the release is greater when both proteins are absent than when cells only lack one of the proteins (Figure 4B). This experiment should be repeated to ensure that this result is reproducible.

*KIN4* was identified as an inhibitor of mitotic exit in a screen for suppressors of the synthetic lethality between deletion of the MEN activator LTE1 and the deletion of the FEAR network component SPO12 or SLK19 (D'Aquino et al., 2005; Stegmeier et al., 2002). The only genes identified in this screen were *KIN4* and the two known negative regulators *BUB2* and *BFA1*. In spite of this, suppression of ite1Δ spo12Δ by deleting *YPL141c* was tested to determine if *YPL141c* can act a negative regulator of mitotic exit, In contrast to cells lacking *KIN4*, deletion of *YPL141c* was not able to suppress the *ite1Δ spo12Δ* synthetic lethality. This data along with the experiments described above indicate that *YPL141c* likely does not function as a negative regulator of mitotic exit. To provide additional evidence for this conclusion, the phenotype of a *dyn1Δ ypl141cΔ* mutant should be examined to determine if *YPL141c* plays a role in the spindle position checkpoint similar to *KIN4*. Given the fact that *ite1Δ spo12Δ* is not suppressed by deleting *YPL141c*, it would be surprising to find *YPL141c* functioning in this checkpoint.
Experimental Procedures

All strains are derivatives of W303 (A2587). The *ypI41c::HIS3* strain was constructed by a PCR-based method Longtine et al. (1998). Immunoblot analysis of the total amount of Clb2 and Kar2 was performed as described in Cohen-Fix et al. (1996). Cdc5 kinase activity was assayed as described in Charles et al. (1998). Indirect immunofluorescence methods and antibody concentrations were as described in Visintin et al. (1999). Growth conditions for individual experiments are described in the figure legends.
Literature Cited


Appendix II:

The stress-activated MAP kinase signaling cascade promotes exit from mitosis.

A version of this manuscript has been submitted for publication by Vladimír Reiser, Katharine E. D’Aquino, Ly-Sha Ee and Angelika Amon.

K.D. contributed to Figure 3C and Figure 7C.
Summary

In budding yeast, a signaling network known as the Mitotic Exit Network (MEN) triggers exit from mitosis. We find that hypertonic stress allows MEN mutants to exit from mitosis in a manner dependent on the HOG MAP kinase cascade. The HOG pathway drives exit from mitosis by promoting the activation of the MEN effector, the protein phosphatase Cdc14. Activation of Cdc14 depends on the FEAR network, a group of proteins that function in parallel to the MEN to promote Cdc14 function. Notably, exit from mitosis is promoted by the signaling branch defined by the Sho1 osmosensing system, but not by the Sln1 osmosensor of the HOG pathway. Our results suggest that the stress MAP kinase pathway mobilizes programs to ensure completion of the cell cycle and entry into G1 under unfavorable conditions and defines the first extracellular signal regulating Cdc14 and exit from mitosis.
Results and Discussion

Exposure of cells to stress leads to an elaborate response that includes stress adaptation and induction of repair mechanisms. These processes are typically associated with a halting or slowing down of cell proliferation (Hohmann, 2002). We observed that in budding yeast under certain circumstances, hypertonic stress promotes rather than delayed cell cycle progression. Temperature-sensitive mutants in the Mitotic Exit Network (MEN) components \textit{CDC15}, \textit{TEM1} and \textit{DBF2} (Stegmeier and Amon, 2004; a scheme of the MEN is shown in Figure 1) arrest in anaphase due to a failure to inactivate Clb-cyclin dependent kinases (Clb-CDKs) and as a consequence cells do not exit from mitosis. However, when grown in hypertonic medium (such as YPD supplemented with sorbitol or sodium chloride) temperature sensitive MEN mutants were able to proliferate (Figure 2A). In contrast, the proliferation defect of other temperature sensitive cell cycle mutants was not suppressed by a hypertonic environment (Table 1), indicating that a hypertonic environment does not promote proliferation of any temperature sensitive cell cycle mutant.

To investigate whether the rescue of the temperature sensitive growth defect of MEN mutants by high osmolarity was due to an increase in intracellular glycerol levels that occurs in response to a high osmotic environment (reviewed in Hohmann, 2002) we examined the consequences of deleting \textit{GPD1}. \textit{GPD1} encodes the major NAD-dependent glycerol-3-phosphate dehydrogenase that is responsible for glycerol accumulation in response to an increase in the osmotic environment. Interference with glycerol accumulation in response to osmotic stress did not affect the ability of MEN mutants to grow at 37°C more than wild-type cells. Deletion of \textit{GPD1} reduced the ability of MEN mutants to grow on medium containing sorbitol to the same extent as that of wild-type cells (data not shown). Several other observations further argue against the idea that an increase in intracellular glycerol is responsible for the suppression of the temperature sensitive lethality of MEN mutants by high osmolarity. First, overexpression of \textit{GPD1} did not promote proliferation of MEN mutants that were defective in the HOG pathway, the MAP kinase signaling cascade responsible for mediating the cell’s response to high
Figure 1: A schematic representation of the FEAR network and the MEN.

The release of Cdc14 from its nucleolar inhibitor is regulated by the FEAR network in early anaphase and then enhanced and maintained in late anaphase by the MEN pathway (for review see Stegmeier and Amon, 2004).
Figure 2

A

![Image of yeast strains with different conditions and temperatures.]

<table>
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<th>Strain</th>
<th>Condition 1</th>
<th>Condition 2</th>
<th>Condition 3</th>
<th>Condition 4</th>
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<td>YPD + 0.5M Sorb</td>
<td>YPD + 1.0M Sorb</td>
</tr>
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<td>YPD + 1.0M Sorb</td>
</tr>
</tbody>
</table>

25°C  37°C

B

![Graph showing cell progression through mitosis.]

Progression through mitosis (min)

D

![Images of yeast cell division under different conditions.]

Sorb  cdc15-2  cdc15-2 pbs2Δ

0 min  60 min  120 min

37°C
Figure 2: A hypertonic environment rescues the mitotic exit defect of MEN mutants.

(A) Strains carrying temperature sensitive alleles of genes encoding components of the MEN were spotted on solid media supplemented with sorbitol or NaCl. Strains used (from top) are: A1411, A1740, A1674, A851, A5321 and A859. Note that the cdc5-1 strain was sensitive to 0.5 M NaCl.

(B) A hypertonic environment does not accelerate exit from mitosis: wild-type cells were arrested with nocodazole (15 mg/ml) followed by release from the block in the presence or absence of 1.5 M sorbitol. The percentage of cells with metaphase and anaphase spindles was determined at the indicated times.

(C) Budding pattern as well as spindle pole body (SPB) number and distribution were analyzed in cdc15-2 (A9943) cells arrested in anaphase at restrictive temperature before (- Sorb) and 60 minutes after (+ Sorb) addition of 1.5M sorbitol. SPB number and distribution was monitored using CFP (green) fused to the SPB protein Spc42 (localization indicated by white arrows).

(D) cdc15-2 (A1674) and cdc15-2 pbs2A (A10710) cells were arrested in anaphase at 37°C (0 minute time point), treated with sorbitol (1.5 M) and then examined at the indicated time points by light microscopy using a DIC filter.

Table 1

<table>
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<tr>
<td>cdc23-1</td>
<td>+++</td>
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155
osmolarity (Hohmann, 2002). MEN mutants lacking the HOG pathway MAP kinase kinase PBS2 fail to proliferate at 37°C in the presence of sorbitol (Figure 2D). Overexpression of GPD1 did not ameliorate this defect (data not shown). Second, overexpression of the HOG pathway component STE20 rescued the temperature sensitive growth defect of cdc15-2 mutants in a GPD1 independent manner (Figure 3C). Finally, inactivation of the osmosensor SHO1 prevents cdc15-2 mutants to grow at 37°C in the presence of sorbitol (Figure 3B), yet GPD1 induction and thus presumably glycerol production is not affected by deleting SHO1 (O'Rourke and Herskowitz, 2004). Together these results indicate that the rescue of MEN mutants by high osmolarity is not due to an increase in intracellular glycerol levels.

In contrast to MEN mutants, strains carrying temperature sensitive mutations in the MEN effector CDC14 (Shou et al., 1999; Visintin et al., 1999) were not able to grow under hypertonic culture conditions. Cells bearing either the cdc14-1 or cdc14-3 allele failed to proliferate in sorbitol-containing medium at 37°C (Table 1). SIC1, which encodes an inhibitor of Clb-CDKs and CDH1, which functions as an activator of Clb cyclin degradation are critical targets of CDC14 in promoting exit from mitosis (reviewed in Stegmeier and Amon, 2004). Deletion of either gene prevented cdc15-2 mutants to proliferate at 37°C in the presence of sorbitol (Table 1) indicating that CDK inactivation is required for high osmolarity to promote proliferation in MEN mutants. Our results suggest that a hypertonic environment either restores signaling through the MEN or promotes Cdc14 activity and Clb-CDK inactivation by a parallel mechanism.

To examine whether a hypertonic environment also affects the kinetics of exit from mitosis during an unperturbed cell cycle we analyzed the effects of 1.5 M sorbitol on wild-type cells exiting mitosis upon release from a nocodazole-induced metaphase arrest. Cells treated with sorbitol progressed through mitosis less efficiently than untreated cells but the kinetics of exit from mitosis was similar under both culture conditions (Figure 2B). Furthermore, exit from mitosis upon release from a MEN mutant block was also not accelerated by addition of sorbitol to the medium (data not shown). These results indicate that a hypertonic environment cannot accelerate exit from mitosis when this cell
Figure 3

A

Hyperosmotic stress

! Turgor pressure

? ?

Sln1 Snk1 Snk2 Ypd1 Pbs2 Cdc42

MAPKK

MAPK

Adaptation

B

wild type

ssk1Δ

ste20Δ

cle4Δ

ste11Δ

sho1Δ

pbs2Δ

hog1Δ

rpd3Δ

sin3Δ

YPD YPD YPD YPD

YPD YPD +Sorb YPD YPD +Sorb

25°C 37°C

D

Sorb (min) 0 10 30 60 120 0 10 30 60 120

wild type

cdc15-2

cdc15-2 ste11Δ

cdc15-2 ssk1Δ

α-phospho Hog1 α-Hog1

cdc15-2

cdc15-2 spo12Δ

cdc15-2 hog1Δ

cdc15-2 pbs2Δ

cdc15-2 rpd3Δ

cdc15-2 sin3Δ

cdc15-2 gpd1Δ

(°C) 25 34

CDC15 overexpress
Figure 3: Suppression of the temperature sensitive growth defect of MEN mutants by high osmolarity depends on the Sho1 signaling branch of the HOG pathway.

(A) A schematic representation of the HOG pathway.

(B) The Sho1 branch, but not the Sln1 branch of the HOG pathway, is necessary for the high osmolarity-induced suppression of MEN mutants. Strains were spotted onto media with or without 1.5 M sorbitol.

(C) Cells carrying a multicopy plasmid containing STE20 were grown on YPD plates at the indicated temperatures. Strains used (from the top): A1674, A12227, A12228, A12229, A12230, A12231, A12232, A12233, A12227 And A14535.

(D) Activation of Hog1 in cdc15-2 strains in response to osmotic stress. cdc15-2 cells were arrested at 37°C and exposed to 1.5 M sorbitol. Samples were taken at the indicated times to determine Hog1 activity using an antibody that recognizes double phosphorylation of the TGY amino acid motif in Hog1. The total amount of Hog1 was determined using an anti-Hog1 antibody. Signals were quantified and normalized to protein levels of Hog1 (graph). Strains used (from the top) are: A1411, A1674, A11682 and A11681.

Figure 4: The HOG pathway drives exit from mitosis in MEN mutants in response to hypertonic stress.

(A) cdc15-2 (A1674) and cdc15-2 pbs2A (A10710) cells were synchronized in G1 using a-factor (5mg/ml) and released into the cell cycle at 37°C. Sorbitol was added 120 minutes after release from the G1 block (indicated by the orange arrow) and the percentage of cells with small buds was determined at the indicated times.

(B - J), Cells were arrested in G1 with a-factor and released from the block at 37°C. The percentage of cells with metaphase and anaphase spindles (B - F, I) or Cdc14 release from the nucleolus (G, H, J) was scored at the indicated times. Sorbitol (D, E, G - J) or sorbitol and cycloheximide (1mg/ml) (F) were added 120 minutes after release from the G1 arrest (indicated by the orange arrow) with exception of the control strains (B, C). Note that in contrast to anaphase spindles, metaphase spindles do not appear to be sensitive to osmotic stress. Spindle depolymerization upon sorbitol addition did not occur in cdc13-1 (A2598; not shown).
Figure 4

A

B

C

D

E

F

G

H

I

J
cycle transition occurs with wild-type kinetics. Thus, the exit from mitosis promoting function of a hypertonic environment is only revealed when cell cycle progression is halted or delayed in anaphase.

In budding yeast, hypertonic stress activates a specific MAPK signaling pathway, the HOG pathway that is essential for stress adaptation (Brewster et al., 1993; O'Rourke et al., 2002; a schematic of the HOG pathway is shown in Figure 3A). We found that suppression of the proliferation defect of MEN mutants by hypertonic environment depended on this MAP kinase signaling cascade. Transfer to a hypertonic environment allowed cdc15-2 cells to exit from mitosis efficiently, and enter the next cell cycle as judged by their ability to form new buds, duplicate spindle pole bodies and subsequently assemble a mitotic spindle (Figure 2C, D; 4A, C, D). In contrast, cdc15-2 mutants lacking the HOG pathway MAPKK encoding gene PBS2 failed to exit from mitosis and to form new buds (Figure 2D, 4A) yet cells retained high viability (Figure 5). Our results indicate that activation of the HOG pathway by hypertonic stress allows MEN mutants to exit from mitosis. However, the Hog1 MAPK is activated only transiently in response to hypertonic stress and its activity returns to pre-stress levels within 20 minutes following exposure to hypertonic conditions at 30 °C (Brewster et al., 1993; Maeda et al., 1995). How is it possible that this transient activation supports continuous proliferation of MEN mutants? We observed that conditions that rescued MEN mutants (1.5M sorbitol at 37 °C) significantly lengthened HOG pathway activation (Figure 3D). Our results indicate that sustained activation of the HOG pathway allows MEN mutants to proliferate at the restrictive temperature.

The MEN stimulates exit from mitosis by promoting the dissociation of the protein phosphatase Cdc14 from its inhibitor Cfi1/Net1 in the nucleolus (Shou et al., 1999; Visintin et al., 1999). In the absence of MEN function, cells arrest in anaphase with Cdc14 sequestered in the nucleolus. To determine whether exit from mitosis in MEN mutants brought about by HOG pathway activation was associated with the release of Cdc14 from the nucleolus, we analyzed cell cycle progression in cdc15-2 and cdc15-2 pbs2Δ mutants in detail. Cells were synchronized in G1 and released into the cell cycle.
Figure 5: *cdc15-2 pbs2Δ* cells retain high viability in the presence of sorbitol.

Strains described in Figure 2 were analyzed for their metabolic activity (red) and cell wall integrity (blue) before and 150 minutes after osmotic stress (1.5M sorbitol) at 37 °C (LIVE/DEAD yeast viability assay, Molecular Probes). The percentage of viable cells is shown for each strain and conditions.
at the restrictive temperature. When 80 percent of cdc15-2 mutants were arrested in anaphase, sorbitol (1.5 M) was added. Cdc14 appeared released from the nucleolus immediately following sorbitol addition in cdc15-2 cells (Figure 4G, H, J; Figure 6A, B) and anaphase spindle disassembly occurred (Figure 4C, D, I). Cells then entered the next cell cycle as judged by their ability to form new buds and metaphase spindles (Figure 4A, C, D, I). Cdc14 was also initially released from the nucleolus in 60% of cdc15-2 pbs2A cells but was then found re-sequestered in the nucleolus within 20 minutes (Figure 4H). Anaphase spindles also disassembled in 40% of cdc15-2 pbs2A cells (Figure 4E, I) but cells never exited mitosis and entered the next cell cycle as judged by their inability to form new buds and metaphase spindles (Figure 2D, 4A, 4E).

Curiously, after prolonged incubation periods (260 minute time point) anaphase spindles appeared to reform in cdc15-2 pbs2A cells (Figure 4E, I). Our results indicate that release of Cdc14 from the nucleolus is bi-phasic in cdc15-2 cells with no significant difference between the cdc15-2 and the cdc15-2 pbs2A strain during the initial phase of the Cdc14 release (Figure 4G, H, 140 min. time point). However, the second wave of Cdc14 release from the nucleolus depends on the HOG pathway. We believe that the first HOG pathway independent wave of Cdc14 loss from the nucleolus reflects high osmolarity induced disturbances of the nucleolar compartment (Nanduri and Tartakoff, 2001), rather than the dissociation of Cdc14 from its inhibitor. This idea is supported by two findings. First, cdc15-2 pbs2A cells do not exit from mitosis (Figure 4E). Second, in agreement with data reported previously (Nanduri and Tartakoff, 2001) we also observed that the nucleolar protein Nop1 (Schimmang et al., 1989) temporarily diffused (for up to 60 minutes) throughout the cell after sorbitol addition at 37 °C (data not shown). The disassembly of the mitotic spindle in 40 percent of cdc15-2 pbs2A cells also did not reflect exit from mitosis, as entry into a new cell cycle did not occur (Figure 4A, E). Instead, spindle disassembly is likely to be caused by a sensitivity of anaphase spindles to mechanical stress imposed by cell volume shrinkage (Reiser et al., 2003) during the initial exposure to hypertonic conditions. Consistent with this idea is the observation that a similar collapse of anaphase spindles occurred in the cdc14-3 mutant, whose proliferation defect was not rescued by a hypertonic environment (data not shown). Our results demonstrate two responses of MEN mutants to osmotic stress.
Figure 6: Hypertonic stress promotes the release of Cdc14 from nucleolus.

cdc15-2 and cdc15-2 pbs2Δ cells were analyzed as described for Figure 4g, h for release of Cdc14 in telophase cells (A, B) and in metaphase cells (C, D). Sorbitol (1.5 M) was added to cell cultures 120 minutes after release from the G1 block (indicated by the orange arrow).
A transient HOG pathway independent response that leads to a temporary disruption of nuclear and nucleolar structures and a sustained, HOG pathway dependent response that leads to the suppression of the mitotic exit defect of MEN mutants. Our results further indicate that the HOG pathway promotes exit from mitosis by inducing Cdc14 release from the nucleolus. This release likely requires the transcriptional response induced by the HOG pathway because it did not occur in the presence of the translation inhibitor cycloheximide (Figure 4F) and depended on the chromatin remodeling factors Rpd3 and Sin3, which are necessary for the transcriptional induction of HOG pathway responsive genes (De Nadal et al., 2004; Figure 3B).

How does activation of the HOG pathway promote Cdc14 release from the nucleolus and exit from mitosis in MEN mutants? We know of two pathways that promote the dissociation of Cdc14 from its inhibitor in the nucleolus during anaphase. The Cdc14 Early Anaphase Release Network (FEAR network) promotes Cdc14 release from the nucleolus during early anaphase whereas the MEN maintains Cdc14 in the released state during late anaphase (reviewed in Stegmeier and Amon, 2004; Figure 1). To test whether high osmolarity restores signaling to MEN mutants we first examined whether a hypertonic environment allowed cells deleted for MEN components to grow. A high osmolarity environment did not allow cells lacking MEN components to proliferate (data not shown), raising the possibility that induction of the HOG pathway restores signaling through a compromised MEN pathway. However when we examined the activity of Dbf2 kinase (DBF2 is the most downstream component of the MEN identified to date; see Figure 1) in cdc15-2 mutants treated with sorbitol we observed no stimulation of Dbf2 activity (Figure 7A). Dbf2 kinase activity was low in cdc15-2 mutants (Visintin and Amon, 2001; Figure 7A) and addition of sorbitol did not stimulate Dbf2 kinase activity (Figure 7A). This finding suggests that the HOG pathway does not restore MEN signaling to temperature sensitive MEN mutants at least not to an extent measurable by Dbf2 kinase assays.
Figure 7

A

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phospho-histone H1

Dbf2 input (α-myc)

wild type  cdc15-2 (37 °C)

B

1.5M Sorb - + - +

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YPD

C

cdc15-2 + Sorb

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Cdc14 release (% of cells)

Time (min)

D

Cycloheximide

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Casein phosphorylation

Input

Gdc5 kinase assay

cdc15-2, 37 °C

cdc15-2 + Sorb (60 min)

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% Cells

Time (min)

F

wild type

pbs2Δ

hog1Δ

ste20Δ

25°C 33°C

G

lte1Δ

lte1Δ ste20Δ

lte1Δ hog1Δ

lte1Δ pbs2Δ

YPD 5-FOA

30°C
Figure 7: A hypertonic environment stimulates the activity of the FEAR network component Cdc5.

(A) Hypertonic environment does not increase activity of the MEN kinase Dbf2. \textit{cdc15-2} (A2096) cells were arrested at non-permissive temperature (0 minutes), treated with sorbitol (1.5 M) with or without cycloheximide (1 mg/ml) and then assayed for Dbf2 kinase activity at indicated time points using histone H1 as a substrate. As a control Dbf2 kinase activity was measured in wild-type cells (A2747 arrested in G1 (G1) and in wild-type cells that were in anaphase (Ana). Anaphase cells were obtained by first synchronizing cells in G1 and then releasing cells from the G1 block. Cells were harvested when more than 90% of cells were in anaphase.

(B) The FEAR network is required for the suppression of MEN mutants by high osmolarity. Strains were spotted on plates with or without sorbitol. Strains used (from top) are: A1411, A1674, A4168 and A10010.

(C) \textit{cdc15-2} mutants (A1674) and \textit{cdc15-2 spo12D} mutants (A10408) were arrested in G1 with a-factor and released from the block at 37°C. Sorbitol was added 120 minutes after release from the G1 arrest and the localization of Cdc14 in anaphase cells was examined.

(D) Hyperosmotic stress stimulates Cdc5 activity in \textit{cdc15-2} mutant. \textit{cdc15-2} cells carrying a \textit{CDC5-HA} fusion (A6438) were arrested at 37°C and then exposed to 1.5M sorbitol in absence or presence of cycloheximide (1 mg/ml). Samples were taken at indicated time points to determine Cdc5 kinase activity using casein as substrate. The specific activity of Cdc5 (ratio between casein phosphorylation and Cdc5 protein[Input]) before (-Sorb) and after addition of sorbitol (+Sorb, 60 min) was determined in three independent experiments. The activity of Cdc5 before addition of sorbitol (-Sorb) was assigned a value of 1. Addition of sorbitol led to an approximately two fold (2.12 +/- 0.36) increase in Cdc5 activity after 60 minutes.

(E) Wild type (A1411) and \textit{pbs2A} (A9610) strains were released from a pheromone-induced G1 arrest and the percentage of cells with anaphase spindles (ana) and Cdc14 released from nucleolus (Cdc14) was determined at the indicated times.

(F) Mutations in the HOG pathway enhance the temperature sensitivity of \textit{cdc15-2} mutants. Strains were spotted on YPD plates and cultured at the indicated temperatures. Strains used (from the top, left \textit{CDC15}) are: A1674, A9610, A9608, A11812 (\textit{CDC15}) and (from the top right, \textit{cdc15-2}) A1674, A10710, A10707, A11813 (\textit{cdc15-2}).

(G) Mutations in the HOG pathway enhance the proliferation defect of \textit{lte1A} cells. Serial dilutions of strains with the indicated genotype and carrying an \textit{LTE1-URA3} plasmid were spotted on YPD and on plates containing 5'-fluoroorotic acid (5-FOA). Strains used (from the top) are: A4101, A6307, A12253 and A12254.
Next we tested whether the HOG pathway promotes Cdc14 release from the nucleolus and exit from mitosis in MEN mutants in a FEAR network-dependent manner. The FEAR network is not essential for exit from mitosis, but components of the FEAR network such as Spo12 or Cdc5 can, when overproduced, suppress the temperature sensitive lethality of MEN mutants (Jaspersen et al., 1998; Parkes and Johnston, 1992; Stegmeier et al., 2002). We found that overexpression of SPO12 suppressed the proliferation defect of cdc15-2 mutants as well as of cdc15-2 pbs2Δ mutants (Figure 8). Furthermore, cdc15-2 mutants lacking the FEAR network components SPO12 or SLK19 were no longer able to proliferate under hypertonic culture conditions at 37°C (Figure 7B) and Cdc14 release induced by high osmolarity was lost in the cdc15-2 spo12Δ double mutant (Figure 7C). These results indicate that the HOG pathway requires the FEAR network to suppress the mitotic exit defect of MEN mutants. Thus, the HOG pathway either functions upstream of or in parallel to the FEAR network to promote exit from mitosis when the MEN is impaired.

To determine whether the HOG pathway promotes exit from mitosis through the FEAR network we examined whether high osmolarity affected components of this network. Exposure of cdc15-2 cells to hyperosmotic conditions did not increase protein levels of known FEAR network components (Figure 9). We did, however, observe that a hypersomotic environment led to an approximately two-fold increase in the specific activity of the FEAR network component Polo kinase Cdc5 (Figure 7D) and that this increase required protein synthesis (Figure 7D). Cdc5 is a potent inducer of Cdc14 release from the nucleolus. The protein kinase can induce Cdc14 release from the nucleolus in any cell cycle stage and only small increases in Cdc5 activity are necessary during mitosis to suppress the need for the MEN in promoting Cdc14 activation (Visintin et al., 2003). The increase in Cdc5 activity brought about by HOG pathway stimulation was however modest (2 fold). We therefore tested whether a two-fold increase in Cdc5 kinase activity brought about by means other than a high osmotic environment could suppress the temperature sensitive lethality of a cdc15-2 mutant. Introduction of an extra copy of CDC5 into cdc15-2 mutants did not allow the temperature sensitive mutant to proliferate at the restrictive temperature (data not shown), indicating that though the
Figure 8: Suppression of *cdc15-2* by overexpression of *SPO12* does not depend on the HOG pathway.

Cells (from top: A1674, A6895 and A12435) all carrying *SPO12* under the control of the *GAL1-IO* promoter were spotted onto plates containing galactose at either 25°C or 37°C.

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Figure 9: Protein levels of FEAR network components do not change in response to hypertonic stress in *cdc15-2* mutants.

*cdc15-2* cells either carrying a *CDC5-HA* (A6438) or a *SPO12-MYC* (A6438) or a *SLK19-HA* fusion (A9634) were arrested at 37°C (0 minute time point), treated with sorbitol (1.5 M) and then analyzed by Western blot analysis.
increase in Cdc5 activity although likely to contribute to the rescue of the proliferation
defect brought about by high osmolarity it is not solely responsible for it. However,
consistent with the idea that stimulation of Cdc5 kinase activity contributes to HOG
pathway mediated suppression of MEN mutants was the finding, that temperature
sensitive mutants in \textit{CDC5} were not suppressed by hypertonic conditions (Figure 2A,
Table 1). Based on this observation and the fact that Cdc5 kinase is stimulated by
hyperosmotic conditions, we favor the idea that the HOG pathway functions upstream of
the FEAR network rather than in parallel to it.

The HOG pathway could promote exit from mitosis only in response to hypertonic
conditions or function during every cell cycle under non-stress conditions to promote exit
from mitosis. To distinguish between these possibilities we analyzed the localization of
Cdc14 in cells lacking \textit{HOG1} or \textit{PBS2} during an unperturbed cell cycle. Deletion of
either MAP kinase cascade component had no effect on Cdc14 localization either at 25°C
or 37°C (Figure 7E). However, deletion of the HOG pathway components \textit{HOG1}, \textit{PBS2}
or \textit{STE20} (Figure 3A) reduced the restrictive temperature of \textit{cdc15-2} mutants (Figure
7F) and led to a synthetic growth defect when combined with a deletion in the MEN
activator \textit{LTE1} (Figure 7G; Hofken and Schiebel, 2002; note that the growth defect of
\textit{lte1A ste20A} double mutants is more severe than that of \textit{lte1A pbs2A} or \textit{lte1A hog1A}
mutants, which indicates that \textit{STE20} has functions in addition to HOG pathway signaling
during exit from mitosis). These results indicate that under non-stress conditions the
HOG pathway contributes to the regulation of exit from mitosis.

The HOG pathway consists of two branches each defined by a trans-membrane protein at
the top of the pathway (reviewed in O'Rourke et al., 2002; Figure 3A). Although
inactivation of both the Sln1 and Sho1 branch is necessary to eliminate the response to
osmotic stress, it is thought that the two branches of the pathway respond to different
signals. Turgor pressure is thought to activate the Sln1 branch (Reiser et al., 2003). An
osmotic signal controlling the Sho1 branch of the pathway has not been identified. We
examined the ability of hypertonic conditions to suppress the proliferation defect of
\textit{cdc15-2} mutants in the absence of components of the two HOG pathway branches.
Whereas the cdc15-2 ssk1Δ strain (the SLNI branch is inactive) displayed a similar proliferation rate as cdc15-2 strain in the presence of sorbitol at the restrictive temperature, the cdc15-2 sho1Δ mutant did not form colonies (Figure 3B). Moreover, the cdc15-2 strain deleted for either STE20 or STE11, both of which encode protein kinases acting in the Sho1 branch of the HOG pathway also did not proliferate (Figure 3B). Furthermore, we found that overexpression of STE20 was able to alleviate the growth defect of cdc15-2 mutants (Figure 3C). This suppression depended on downstream components in the HOG pathway (PBS2, HOG1, RPD3 and SIN3) as well as the FEAR network components SLK19 and SPO12 (Figure 3C). These data indicate that the Sho1 branch of the HOG pathway is responsible for promoting exit from mitosis in MEN mutants. Consistent with this idea was the observation that activation of the SHO1 branch in cdc15-2 mutants in response to hypertonic stress branch occurred over an extended period of time, which was in stark contrast to the transient activation of the SLNI branch (Figure 3D). Our results demonstrate an essential role for the Sho1 branch of the HOG pathway in promoting mitotic exit in MEN mutants after osmotic stress and advocate for a functional diversification between individual signaling branches of the HOG pathway in osmo-protection.

We show here that hypertonic stress allows efficient exit from mitosis in mutants defective in the Mitotic Exit Network. The exit promoting activities of a hypertonic environment were not observed in an unperturbed cell cycle indicating that high osmolarity does not accelerate exit from mitosis when this cell cycle transition occurs with wild-type kinetics. A hypertonic environment is however very capable of promoting exit from mitosis when this transition is compromised. Our results further show that the suppression of MEN mutants by a hypertonic environment is mediated by the HOG pathway. This signaling cascade promotes the release of Cdc14 from the nucleolus in a manner that depends on the FEAR network. Intracellular signals such as onset of chromosome segregation and nuclear position have been shown to regulate exit from mitosis through controlling Cdc14 activity (Ross and Cohen-Fix, 2004; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). High osmolarity is, to our knowledge, the first extracellular signal shown to affect the activity of this key phosphatase. Why would high
osmolarity promote exit from mitosis? Perhaps stress survival is higher in G1 than in mitosis and one function of the HOG pathway is to push cells that are delayed in anaphase into G1 by promoting the activation of Cdc14 and stabilization of the CDK inhibitor Sic1 (Escote et al., 2004) to increase their survival chance. It is also possible that the answer to this question lies in the localization pattern of Sho1. Sho1 localizes to the bud neck (Raitt et al., 2000; Reiser et al., 2000), the region of the cell that may be most sensitive to osmotic stress due to cell-wall remodeling and contraction of the actomyosin ring during cytokinesis. Osmotic imbalance in this region would then signal through the HOG pathway to the mitotic exit machinery to promote exit from mitosis. The Hog1 MAP kinase as well as components of the FEAR network, such as the polo kinase Cdc5 are highly conserved among eukaryotes. The recent finding that the stress pathway regulates Polo function in S. pombe (Petersen and Hagan, 2005) raises the interesting possibility that stress-activated MAP kinase pathways regulating Polo kinase family members is conserved across species.

Experimental Procedures
Strains
All strains are derivative of W303 (K699). Deletions of genes were performed by the PCR-based method described in Longtine et al. (1998).

Cell synchronization
Cells were arrested in G1 in YPD medium containing α-factor (5 μg/ml) and subsequently released into α-factor-free YPD medium at 37 °C. To release cells from a nocodazole block, cells were first arrested by addition of nocodazole (15 μg/ml, 1% DMSO) in YPD medium for 2-3 h, then washed and released into fresh YPD containing 1% DMSO.

Microscopy
Immunofluorescence (tubulin and HA-tagged Cdc14), fluorescence (SPC42-CFP) and light microscopy techniques were as described previously in Visintin et al. (1999) and
Visintin and Amon (2001), respectively. Images were captured using an Axioplan 2 microscope (Zeiss) and analyzed using the Openlab software package (Improvision).

**Protein techniques**

Western blot analysis, Dbf2 and Cdc5 kinase assays were performed as described previously (Visintin and Amon, 2001). The following antibodies were used: anti-HA (HA-11, Babco), anti-myc (9E10, Babco), anti-α-tubulin (Oxford Biotechnology) and anti-Hog1 antibodies (Santa Cruz). Hog1 phosphorylation was measured using an anti-phospho-p38 antibody (Cell Signaling).
Literature Cited


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