Mechanisms underlying spatial control of exit from mitosis.

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Thank you.
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By

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Abstract:
During mitosis, cells must accurately segregate their genome in order to produce healthy daughter cells. In budding yeast, cells align their anaphase spindle along a predetermined axis of division in order to partition their genome into the daughter cells. In the event that the spindle becomes mispositioned, cells will prevent exit from mitosis by inhibiting the mitotic exit network (MEN). The MEN functions to regulate the localization of the essential phosphatase Cdc14. Control of the MEN by spindle position is established through MEN inhibitory signals in the mother cell compartment (such as the kinase Kin4), MEN promoting signals in the bud (such as Lte1) and a GTPase sensor (Tem1) that moves between them. While the molecular functions of Kin4 and Tem1 are well defined, the function of Lte1 has remained unclear. In the first part of this thesis I attribute a function to Lte1 in promoting exit from mitosis. I show that Lte1 functions to prevent Kin4 from inappropriately localizing to SPBs (spindle pole bodies) in the bud cell compartment. I find that these two proteins interact and that the N-terminus of Kin4 mediates this interaction. This work highlights the importance of spatial restriction of Lte1 in the bud and Kin4 in the mother for the proper execution of chromosome segregation in anaphase. In the second part of this thesis I investigate the role of cytoplasmic microtubules in spatial regulation of the MEN. It has been proposed that spatial regulation of the MEN functions as a checkpoint that requires contact between cytoplasmic microtubules (cMTs) and the budneck to arrest cells in anaphase. Loss of cMT-budneck contact was reported to lead to checkpoint failure resulting in anucleate and multinucleate cells. In contradiction to these results, I find that Cdc14 release is responsible for the loss of cMT-budneck interactions that precede inappropriate exit from mitosis. Lastly, through the generation of cells with two nuclei, I show that the coupling of spindle position to exit from mitosis is established in a dual manner through both inhibitory signals in mother cell compartment and through activating signals in the bud.

Thesis Supervisor: Angelika Amon
Title: Professor of Biology
Dedicated to my family for their patience, love and support.
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Chapter I: Introduction
Summary

The function of the cell cycle is to produce healthy daughter cells. At the last stage of the cell cycle, cells partition their genome and then undergo cytokinesis. To ensure that both daughter cells receive one nucleus, budding yeast couple exit from mitosis to nuclear position through the regulation of mitotic cyclins, the regulators of cell cycle progression. Two signal transduction cascades called the Cdc14 early anaphase release network (FEAR) and the mitotic exit network (MEN) regulate cyclin levels at the end of mitosis. In this introduction I give an overview of the budding yeast cell cycle with particular attention to the mechanisms governing spatial control of exit from mitosis. I also review checkpoint regulation of the cell cycle and end by briefly discussing the conservation of the MEN and the centrosome position checkpoint.
An overview of the mitotic cell cycle in budding yeast

The purpose of cell division is to produce viable daughter cells. In order to accomplish this, the cell must grow, duplicate its chromosomes and organelles and then accurately partition them between the mother and daughter cells. The cell cycle is divided into four stages; two rest phases (G1 and G2 respectively) that are separated by a DNA synthesis phase (S-phase) and a mitosis or partitioning stage (M-phase). The passage though these stages is controlled by the essential cell cycle kinase Cdc28. Activity of the Cdc28 kinase is regulated through its binding to small cell cycle-regulated proteins called cyclins (Morgan, 1997) (Figure 1). There are three cyclins in budding yeast that are important for the G1 to S-phase transition (Cln1-3). Two B-type cyclins are important for S-phase progression (Clb5-6) and lastly, four B-type cyclins are required for mitosis (Nasmyth, 1996). In yeast, cyclin function is redundant, which is highlighted by the fact that no single cyclin is required to complete any cell cycle stage. However, the activity of these cyclins is at the core of the cell cycle and their activity is controlled through transcriptional regulation, their interaction with CKIs (cyclin dependent kinase inhibitor) as well as through their proteasome-dependent degradation (Nasmyth, 1996).
Figure 1: Cyclin control of the cell cycle. At the G1 to S phase transition, Cln3 phosphorylates the SBF inhibitor Whi5 which results in the production of Cln1/2. Cln3 also activates MBF, which is the transcription factor complex that is responsible for the production of the major S-phase cyclins Clb5/6. The SCFGrr1 is responsible for the destruction of Cln1/2 cyclins and the SCF^Cdc4 degrades Clb6. In late S-phase, SBF is also indirectly responsible for the production of the mitotic cyclin Clb1-4. Destruction of the mitotic cyclins is performed by both the APC^Cdc20 and APC^Cdh1. Inhibition of the mitotic cyclins is also performed by the CKI Sic1. Sic is activated in late anaphase and is degraded by SCF^CDC4*. Also depicted is the centrosome and chromosome state at each cell cycle stage.
**Entry into the Cell Cycle**

In budding yeast, cells that have returned to G1 wait to enter the subsequent cell cycle until they have achieved a critical size (Rupes, 2002). Once the cell reaches this size, it will commit to the cell cycle and will transit through Start. To do this, the cyclin-CDK complex Cln3-Cdc28 phosphorylates the G1/S inhibitor Whi5, which results in its export from the nucleus. When in the nucleus, Whi5 binds and inhibits the Swi4/6 transcription factor complex (SBF) (Bloom and Cross, 2007). Active SBF results in the production of other late G1 cyclins (Cln1 and Cln2) (Morgan, 1997). The production of Cln1/2 results in spindle pole body (SPB; yeast centrosome) duplication and is required for Cdc42-dependent polarization through the relocation of its exchange factor Cdc24. In a parallel pathway, Cln3 also activates the transcription factor complex MBF (Swi6/Mbp1) that is required to transcribe Clb5 and Clb6, the main cyclins that are responsible for DNA synthesis. Cln1/2 activation results in the SCF\(^{Cdc4}\)-dependent destruction of B-type cyclin inhibitor Sic1 (Verma et al., 1997).

**S-Phase**

Proper DNA synthesis requires the licensing and firing of origins of replication once per cell cycle. Origin licensing happens during G1 (which permits the loading of specific DNA replication factors at origins of replication) and origin firing starts in S-phase (Stillman, 1996). During S-phase the cell also links duplicated chromosomes (sister chromatids) to each other using a torus-shaped complex called the cohesin complex. Loading of the cohesin complex ensures
that sister kinetochores can come under tension and become bioriented during metaphase. (Nasmyth and Haering, 2009)

Cyclin production not only allows for DNA synthesis but also promotes progression into the mitosis. Increasing levels of Clb5/6 inhibit the transcription of Cln1/2 and promote the transcription of the mitotic cyclins. Finally, SBF is responsible for the transcription of the late S-phase transcription factor Hcm1. The Hcm1 transcription factor in turn is required for the production of the Mcm1-Fkh (SFF) transcription factor complex. This complex both activates the transcription of the major mitotic cyclins Clb1-2 and promotes the return of Whi5 into the nucleus where it inhibits Cln1/2 expression until the subsequent G1 (Enserink and Kolodner, 2010; Bloom and Cross, 2007).

**Entry into Mitosis**

After DNA replication, cells must attach each sister chromatid to the mitotic spindle in order to segregate chromosomes to opposite sides of the cell. Formation of the mitotic spindle along with other events in mitosis (such as chromosome condensation) requires the function of the remaining B-type cyclins (Clb1-4) (Miele, 2004). Chromosomes attach to the mitotic spindle via a large protein structure called the kinetochore. The yeast kinetochore has both a structural role in connecting centromeres to microtubules as well as a signaling role to prevent chromosome segregation prior its attachment to the spindle microtubules (discussed in checkpoint control of the cell cycle below). Once all kinetochores have attached to the mitotic spindle, cells sense this and cleave the
Sccl subunit of the cohesin complex (Uhlmann et al., 1999). Cohesin cleavage is performed by the protease Esp1 (Separase). Esp1 is kept from inappropriately cleaving cohesin earlier in the cell cycle by its inhibitor Pds1 (Securin). Esp1 is liberated from its inhibitor at the metaphase to anaphase transition through its regulated destruction by the APC^{Cdc20} (Ciosk et al., 1998). The APC is also regulated, and under certain conditions is kept from degrading Pds1. The APC is prevented from degrading Pds1 (thus causing the cell to arrest in metaphase) in response to DNA damage or unattached kinetochores/lack of spindle tension.

Regulation of Cdc20 in response to these cellular insults is mediated by cell cycle surveillance networks, which ensure that DNA integrity and accurate chromosome segregation are coupled to cell cycle progression (checkpoint regulation of the cell cycle is discussed below).

After cohesin is cleaved, the sister chromatids (attached to their respective kinetochore microtubules) move quickly apart towards their opposite spindle poles. This movement is characterized by the rapid depolymerization of kinetochore microtubules (anaphase A). In a second, slower phase, interpolar microtubules push the spindle poles to the opposite sides of the cell further separating the chromosomes in late anaphase (anaphase B).

**Cyclin Destruction and Control of Exit from Mitosis**

At the same time that cohesin is cleaved, cells activate a network that decreases mitotic CDK activity and primes the cell for exit from mitosis (Stegmeier and Amon, 2002). The cell does this by allowing the regulated
release of the phosphatase Cdc14. Cdc14 is an essential phosphatase that is required for cells to exit from mitosis. Throughout the cell cycle, Cdc14 is kept sequestered in the nucleolus where it is bound by its inhibitor Cfi1/Net1 (Visintin et al., 1999; Shou et al., 1999). Upon anaphase entry Cdc14 is transiently released by the Cdc14 early anaphase release network (FEAR network). This transient release serves to prime the cell in preparation for the execution of anaphase. In late anaphase a second essential pathway, known as the mitotic exit network (MEN), triggers the sustained release of Cdc14 from the nucleolus.

Release of Cdc14 results in cyclin destruction and the reversal of mitotic CDK phosphorylation, both which are required for cell to return to G1 (Stegmeier and Amon 2004). Cdc14 counteracts CDK phosphorylation by 1) directly dephosphorylating Clb-CDK targets, 2) promoting the interaction of the APC with its anaphase specificity factor Cdh1 and 3) by stabilizing the CDK inhibitor Sic1 (Jaspersen et al., 1998; Visintin et al., 1998).

The FEAR Network

The FEAR network was discovered in 2002 when Stegmeier and Amon found that Cdc14 was released into the nucleus (and to a lesser extent the cytoplasm) in cells lacking MEN activity. The factors involved in this transient release are Separase (Esp1), Slk19, Polo kinase (Cdc5), Clb-CDK, Zds1/2, PP2A-Cdc55, Spo12 and Fob1 (Stegmeier et al., 2004; 2002; Queralt et al., 2006; Queralt and Uhlmann, 2008). While all of these factors have been shown to be involved in the early anaphase release of Cdc14, assembling them into a
Pathways controlling the release of Cdc14. The transient release of Cdc14 in early anaphase is regulated by the FEAR network. This network is activated at the metaphase to anaphase transition and results in the release of Cdc14 primarily into the nucleus. The sustained release of Cdc14 is performed by the MEN. MEN-mediated Cdc14 release is essential for exit from mitosis and serves to inactivate Clb-CDK activity as well as reverse Clb-CDK phosphorylations.

framework to describe how the FEAR functions at a molecular level is still unclear. What is known is that at the metaphase to anaphase transition, through an unknown mechanism, the activated FEAR component Esp1 decreases PP2A-Cdc55 dephosphorylation of Cfi1/Net1 (Queralt et al., 2006). The resultant increase in CDK-dependent phosphorylation of Cfi1/Net1 results in the release of Cdc14 (Azzam et al., 2004). In addition, Esp1 and Slk19 (together with Clb-Cdks)
are also required for phosphorylation of the FEAR component Spo12 (Tomson et al., 2009). The purpose of this Spo12 phosphorylation is thought to be to aid in Fob1 dissociation from Cdc14. The function of Fob1 in the FEAR is to enhance the binding of Cdc14 to Cfi1/Net1 in the nucleolus. Lastly, Cdc5 has also been shown to be a FEAR network component. Cdc5 is involved in the phosphorylation of Cfi1/Net1 and Cdc14. Cdc5-dependent phosphorylation of Cdc14 and Cfi1/Net1 is also thought to promote the release of Cdc14 from the nucleolus (Stegmeier and Amon, 2002; Visintin et. al., 2003; Rahal and Amon, 2004; Manzoni et. al., 2010)

While little is known about the molecular underpinnings of the FEAR network, there is information about its function. This early pulse of Cdc14 has been shown to prime the cell in preparation to efficiently return to G1. This transient release of Cdc14 early in anaphase serves to dephosphorylate key MEN components such as Cdc15 (Stegmeier and Amon, 2002; König et. al., 2010). Because many of the MEN components are phosphoproteins, it is also possible that FEAR-mediated release of Cdc14 promotes exit from mitosis by priming additional MEN factors. The FEAR also aids in rDNA compaction, spindle positioning and spindle integrity. (Stegmeier and Amon, 2002; D'Amours et al., 2004; Ross and Cohen-Fix, 2004.)

**The Mitotic Exit Network**

The MEN is a Ras-like signal transduction cascade with the essential GTPase Tem1 at its center. In addition to Tem1, the main MEN components are
the scaffold Nud1, the putative GEF Lte1, the kinase Kin4, the two component GAP Bub2/Bfa1, the kinase Cdc15, the kinase Cdc5 and the kinase complex Dbf2/Mob1.

**Regulation of the MEN by Spindle Position**

Most components of the MEN, with the exception of Ltc1, localize to and function at SPBs (Spindle Pole Bodies). These components are regulated by spindle position (spatial control) as well as by the cell cycle (temporal control). Spatial control of the MEN is described by a model known as the zone model (reviewed in "The Spindle Position Checkpoint" section below). Briefly, Tem1 is both positively regulated by the putative GEF (Guanine nucleotide exchange factor), Lte1, and negatively regulated by a two component GAP (GTPase-Activating Protein) complex Bub2/Bfa1 (Bardin et al., 2000; Pereira et al., 2000). Lte1 localizes to the bud cell cortex and promotes Tem1 enrichment on SPBs in the bud cell compartment (Molk et al., 2004; Bardin et al., 2000). While Lte1 is non-essential, cells lacking Lte1 are delayed in anaphase (Chan and Amon, 2010). The anaphase delay of cells lacking *LTE1* is exacerbated at cold temperatures, possibly because Tem1 cannot efficiently exchange nucleotides in the cold and thus might require Lte1's putative exchange activity.

Tem1 function in promoting exit from mitosis is inhibited by its two component GAP complex Bub2/Bfa1. While Tem1 localizes to SPBs in metaphase, the activity of the GAP keeps Tem1 activity restricted to anaphase. *In vitro* data, show that the GTPase activity of Tem1 is enhanced by the
Bub2/Bfa1 GAP complex, thus increasing the fraction of GDP-bound Tem1 (Geymonat et al., 2002). In vivo the MEN is hyperactivated in the absence of either GAP component. Likewise, either overexpression of Tem1 or expression of a GTP-locked Tem1 (TEM1-1) has also been shown to hyperactivate the MEN (Scarfone and Piatti, 2015; Chan and Amon, 2010). Therefore, it is likely that the GAP restrains MEN activation through Tem1 by increasing the fraction of inactive (presumably GDP-bound) Tem1.

The Bub2/Bfa1 complex is also regulated by the position of the anaphase spindle. If the anaphase spindle is misaligned in the mother cell compartment the GAP prevents Tem1 enrichment on the SPBs in the mother cell compartment (D’Aquino et al., 2005; Pereira and Schiebel, 2005; Maekawa et al., 2007). Once one pole moves out of the mother compartment and into the bud, it is thought that the GAP complex is inhibited by the Polo kinase Cdc5 (Geymonat et al., 2003; Maekawa et al., 2007; Hu et al., 2001). Inhibition of the Bub2/Bfa1 GAP complex promotes Tem1 activation and thus MEN activation. Although it is known that Tem1 activation is restricted anaphase, where and how Tem1 is activated is still under debate.

What keeps the GAP complex from being inactivated in the mother cell compartment? Two groups showed that Bub2/Bfa1 GAP function is regulated by the kinase Kin4 (D’Aquino et al., 2005; Pereira and Schiebel, 2005). Kin4 asymmetrically localizes to the mother cell cortex and mother SPB and functions to phosphorylate Bfa1. Kin4 functions at anaphase SPBs and its phosphorylation
of Bfa1 prevents Cdc5 inhibiting from the GAP (Maekawa et al., 2007).

Therefore, if the spindle becomes mispositioned in the mother cell compartment, Kin4 localizes to those SPBs and keeps the MEN inactive by maintaining Bub2/Bfa1 activity.

Tem1 in turn is required for the localization of the kinase Cdc15 to SPBs (Visintin and Amon, 2001; Rock and Amon, 2011). Tem1 has been shown to bind Cdc15 and this interaction has been mapped to a small, conserved 82 amino acid region on the Cdc15 kinase (Asakawa et al., 2001)

**Temporal Control of the MEN**

The MEN is not only regulated by spindle position but it is also cell cycle regulated. Evidence for this stems from the fact that the anaphase kinetics of cells lacking *TEM1* (and kept alive by overexpression of the downstream kinase Cdc15; *tem1Δ CDC15-UP*) differ little from the anaphase kinetics of wild type cells (Rock and Amon, 2011). Therefore, if MEN activation is still restricted to anaphase in the absence of the spindle position sensor Tem1, there must be factors outside of Tem1 that prevent the MEN from becoming active earlier in the cell cycle (temporal control of the MEN). One factor that was found to be important in limiting MEN activation to anaphase was the polo kinase Cdc5 (Rock and Amon, 2011). This was shown when *tem1Δ CDC15-UP* cells that also overexpressed Cdc5 no longer restricted MEN activation to anaphase. Cdc5 was shown promote exit from mitosis in the absence of Tem1 by recruiting Cdc15 to SPBs. Consistently, when Cdc15 was tethered to the SPB by fusion to the SPB
component Cnm67 (CDC15-SPB), it bypassed the requirement for both Tem1 and Cdc15 for activation of the MEN (Rock and Amon, 2011). This result shows that Cdc15 incorporates both spatial signals from Tem1 and temporal signals from Cdc5 to activate the MEN.

Cdc15 is essential to activate the MEN, so how does it do this? Both biochemical and genetic data have demonstrated that Cdc15 is required to activate the Dbf2 kinase, the most downstream component of the MEN. Cdc15 has been shown to directly phosphorylate Dbf2 and is also required for its localization to SPBs and its kinase activity (Visintin and Amon, 2001; Mah et al., 2001). Dbf2 alone cannot localize to SPBs but requires the protein Mob1 and the scaffold Nud1, both of which are also phosphorylated by Cdc15 (Rock et al., 2013; Gruneberg et al., 2000). The mechanism by which Cdc15 promotes Dbf2 localization has been recently described (Rock et al., 2013). In this model, phosphorylation of Nud1 on T78 by Cdc15 creates a docking site for the kinase complex Dbf2-Mob1 (Rock et al., 2013). After phosphorylating Nud1, Cdc15 then activates Dbf2-Mob by phosphorylating it. Through a largely unknown mechanism, it has been proposed that Dbf2-Mob1 then functions to phosphorylate the nuclear localization sequence of Cdc14. This phosphorylation serves to promote, at least in part, Cdc14 release into the cytoplasm (Mohl et al., 2009).
Figure 3: Spindle positioning pathways in budding yeast. There are two pathways that position the spindle along the mother-bud axis. The first pathway relies on Kar9, Bim1 and Myo2 to pull the nucleus along actin cytoskeleton and serves to position the preanaphase spindle at the budneck. The second pathway relies on Dynein, its associated activators and Num1. This second pathway is primarily active in anaphase and tows the anaphase cMTs into the bud.

Spindle positioning Pathways
Because spindle position and the site of cytokinesis are uncoupled in budding yeast, these cells must ensure that the mitotic spindle is properly aligned along the mother-bud axis before they exit from mitosis.

There are two partially redundant pathways that ensure proper spindle alignment, one that is dependent on the microtubule adaptor protein Kar9 and the other that relies on Dyn1 (Miller and Rose, 1998; Yeh et al., 1995). These pathways take
advantage of both the actin and microtubule cytoskeleton to pull the spindle pole into the future daughter cell (Figure 3).

**Kar9-Dependent Spindle Positioning**

The first pathway functions through the binding of the plus-end microtubule tracking protein Bim1 via its binding partner Kar9 to the kinesin Myo2 (Miller et al., 1999; Beach et. al., 2000; Lee et al., 2000). Because budding yeast actin filaments radiate from the bud tip and bud neck into the mother cell compartment, Myo2, together with its microtubule adaptors, serves to align the pre-anaphase spindle by pulling the yeast nucleus along the actin filaments and toward the bud cell compartment. Kar9 asymmetrically localizes to the bud-bound spindle pole body. This SPB asymmetry is important because it allows cells to effectively align the spindle by towing only one SPB toward the bud. The mechanisms governing Kar9 localization remain unclear however Cdk-dependent phosphorylation has been shown to enhance Kar9 SPB asymmetry by asymmetrically Kar9 SPB localization (Liakopoulos et al., 2003).

**Dynein-dependent Spindle Positioning**

At the onset of anaphase, the spindle is positioned in or adjacent to the budneck due to the activity of the Kar9-dependent spindle-positioning pathway. A second pathway governing spindle position, which is mainly active in anaphase, helps align the anaphase spindle in the bud (Yeh et. al., 1995; Woodruff et. al., 2009). This pathway relies on the microtubule cytoskeleton and
the activity of cytoplasmic dynein. In contrast to most other organisms, the only function for dynein in budding yeast is in nuclear positioning. Dynein, together with the coactivating dynactin complex is delivered, by the kinesin Kip2, to the tips of cMTs (Roberts et al., 2014). Once present on ends of the cMTs, dynein is anchored to the cell cortex by Num1. Num1 is a pleckstrin homology-domain containing protein that is bound to the cell cortex through its interaction with phosphoinositide PI(4,5)P2 (Kormanec et al., 1991; Farkasovsky and Küntzel, 2001). Dynein is a minus-end directed motor protein and serves to pull cytoplasmic microtubules (cMTs) along the cell cortex. So how does this pathway aid in directed spindle movement into the bud? In yeast, dynein appears asymmetrically distributed on the cytoplasmic microtubules in the bud (Grava et al., 2006). This could enhance the daughter-directed pulling forces during anaphase. Additionally, She1 has been proposed to inhibit dynein activity in the mother cell compartment by preventing its interaction with the dynactin complex (Woodruff et. al., 2009; Markus et al., 2012; Bergman et al., 2012). How She1 activity is restricted to the mother cell compartment however remains unclear.

**Checkpoint control of the cell cycle**

The cell cycle is coordinated such that during every cycle there is one round of DNA replication and one round of nuclear and cellular division. The surveillance mechanisms that ensure a cell completes one cell cycle stage before initiating another one is known as a checkpoint pathway. The idea that such mechanisms exist first came from observations in experiments where two cells
were fused together (Rao et al 1970). These experiments showed that in cells where one nucleus was in G2 and the other nucleus was in S phase, entry into mitosis of the cell in G2 was delayed until the other nucleus had completed S phase. One common interpretation of these data is that the nucleus that was in S phase emitted a checkpoint signal that prevented the other nucleus from entering mitosis. Cells will also arrest the cell cycle if they sense the presence of DNA damage, unattached kinetochores/lack of spindle tension or defects in the actin cytoskeleton. Together these cell cycle regulations ensure the viability of cell progeny. Below I discuss two checkpoints, the spindle assembly checkpoint and the spindle position checkpoint.

**Spindle Assembly Checkpoint**

During S-phase sister chromatids are linked together by the cohesin complex. This complex is believed to form a ring structure that holds chromosomes together. Upon entry into metaphase, each chromosome forms an attachment to spindle microtubules. Once each sister chromatid forms a bipolar attachment to the spindle, the cell enters anaphase and chromosomes are segregated to opposite sides of the cell. Through the study of cells that had been exposed to microtubule poisons, it was found that cells prevented progression through the cell cycle in the absence of a bipolar spindle. Two screens identified Mad1, Mad2, Mad3, Bub1, Bub2 and Bub3 as factors that are essential to maintain this cell cycle arrest in the absence of microtubules (Hoyt et al., 1991; Li and Murray, 1991). Further investigation revealed that, while all of the factors listed above are required for the arrest in the absence of spindle microtubules,
Bub2 most likely acts in an independent mechanism to regulate exit from the cell cycle. This was demonstrated, in part, through the kinetic analyses of cells lacking these genes that were grown in the presence of microtubule depolymerizing agents. While all of the cells with single deletions progressed through the cell cycle with similar kinetics, it was observed that these kinetics were delayed in comparison to cells without nocodazole treatment. However when cells lacking BUB2 were combined with cells lacking MAD2 or MAD1, this delay was eliminated indicating that BUB2 functions independently of MAD2 or MAD1. Additionally, unlike the other checkpoint components, BUB2 was shown not to be required for viability in response to low doses of nocodazole. Finally, unlike cells lacking MAD1, MAD2, BUB1 or BUB3, cells without BUB2 were not synthetic sick when combined with the kinetochore mutant ctf13. This indicated that BUB2 is not necessary to inhibit cell cycle progression in response to unattached kinetochores/spindle tension (Wang and Burke, 1995; Li, 1999).

Investigation into the molecular mechanism that mediated this cell cycle arrest revealed that all of these factors, with the exception of Bub2, localized to kinetochores during mitosis. This led to the hypothesis that cells arrest in metaphase in the absence of spindle microtubules because they sense the lack of kinetochore attachment to the spindle (Waters et al., 1998). However, upon kinetochore attachment, the attached chromosome also comes under tension generated by the spindle. Therefore, it is at present unclear whether spindle-
generated tension or microtubule attachment (or both) lead to alleviation of the spindle assembly checkpoint (Musacchio and Salmon, 2007).

At a molecular level the checkpoint functions by regulating Cdc20. Cdc20, an APC specificity factor, is needed for the degradation of Pds1 (reviewed above). In the event that even a single kinetochore does not form a bipolar attachment to the spindle, the kinetochore-localized mitotic checkpoint complex (MCC) Mad1, Bub1 and Mad2, induce a conformational change in Mad2 that is not attached to a kinetochore. This conformational change allows Mad2 to bind and inhibit Cdc20 together with Bub3 and Mad3. (Musacchio and Salmon, 2007; London and Biggins, 2014).

**Spindle Position Checkpoint**

The Bloom lab first described evidence of a checkpoint surveillance mechanism that coupled exit from mitosis to spindle position (Yeh et al., 1995). In this paper, experiments were performed to determine the role of cytoplasmic dynein in budding yeast. What was observed was that cells lacking DYN1 mispositioned their anaphase spindle in the mother cell compartment. Additionally, if the spindle became mispositioned in the mother compartment, it was observed that there was an associated delay in anaphase. Subsequent work has established that this delay is mediated by the inhibition of the mitotic exit network (MEN) (Bardin et. al., 2000; Pereira et. al., 2000). Inappropriate activation of the MEN in cells with a mispositioned spindle results in the generation of anucleate and multinucleate cells. There are several models which describe how cell cycle progression is coupled to spindle position (Figure 4):
Model I: Cytoplasmic Microtubule Contact with The Budneck

As its name underscores, the spatial regulation of the MEN has traditionally been described as a checkpoint response to defects in spindle positioning. When the spindle becomes mispositioned in the mother cell compartment, the cytoplasmic microtubules of budding yeast characteristically extend from the mispositioned spindle and jut through the bud neck into the bud. The cMT checkpoint model posits that the cytoplasmic microtubules of these mispositioned spindles elicit a checkpoint response through their contact with components of the budneck (Adames et al., 2001; Moore et al., 2010). In support of this model, Moore et al. demonstrated that elimination of this cMT-budneck interaction by laser severing, induced exit from mitosis in cells with mispositioned spindles. This analysis revealed that ~45% of cells with mispositioned spindles that lost cMT-budneck contact due to cMT severing exited mitosis in the mother cell compartment. When the laser was targeted to other cell structures (such as the spindle or a cMT not in contact with the budneck) cells did not inappropriately exit from mitosis. This supports the hypothesis that cMT budneck contact is required for the SPoC arrest of a mispositioned spindle. What is unclear about these data is that upon laser severing of the cytoplasmic microtubule, ~35% of these cells only exited mitosis more than 30 minutes thereafter. If loss of a cMT from the budneck causes exit from mitosis, cells with mispositioned spindles in late anaphase should exit mitosis much more rapidly. In comparison, in this study
an aligned spindle took ~20 minutes to exit mitosis once one pole had moved into the bud cell compartment.

In complementary experiments, use of a β-tubulin allele (tub2-401) that lacks cMTs at room temperature also demonstrated that cells lacking cMTs inappropriately exit mitosis in the mother cell compartment. ~20% of cells containing this allele inappropriately exited mitosis in the mother cell compartment. In comparison to cells with mispositioned spindles that lack BUB2 which inappropriately exit mitosis in 100% of cells, contribution of the cMTs to the SPoC is minor (Adames et. al., 2001). Additionally, it is noteworthy to mention that the tub2-401 allele results in constitutive loss of cMTs. Therefore it is possible that this allele lacks checkpoint function not because it is part of a checkpoint but because these microtubules are necessary for the normal turnover of MEN components on the SPBs. Further investigation is needed to understand how the tub2-401 allele affects the exit from mitosis

Lastly, if cMT-budneck interactions generate a checkpoint response to arrest cells in anaphase, it is unclear how these cMTs are sensed at the budneck. Preliminary evidence shows that cells lacking the septin CDC10 also inappropriately break down in the mother compartment (Castillon et al., 2003). However it is unclear if cMTs and Cdc10 actually interact and what the molecular nature of the checkpoint signal at the budneck actually is. Finally it has been shown that this pathway most likely acts by regulating the MEN but there is no evidence indicating how this regulation might occur (Moore et. al., 2009).
Figure 4: Models Describing Spatial Regulation of the MEN. Three models describing how spindle position regulates exit from mitosis. (Top panel) The zone model posits that movement of one SPB out of the MEN inhibitory zone (the mother compartment) and into the MEN promoting zone (the bud compartment) allows Tem1 enrichment on the daughter SPB and triggers exit from mitosis. (middle panel) The checkpoint model proposes that mispositioned spindles inhibit the MEN through the activation of a checkpoint that is triggered by the interactions of cMTs with the budneck. These are specific to the mispositioned...
spindle and so upon spindle realignment, the cMT budneck contact is not present and the MEN can be activated. (bottom panel) The sink model posits that movement of the SPB through the budneck promotes the accumulation of the Bub2/Bfa1 GAP on that SPB. This acts as a sink, eliminating Tem1 on the mother SPB. Active Tem1 on the mother SPB signals exit from mitosis.

**Model II: Disappearance of the GAP from the Mother SPB (Sink Model)**

Another model for how spatial regulation of the MEN is executed was proposed to require the disappearance of the Bub2/Bfa1 GAP complex from the mother spindle pole body (Fraschini et al., 2006). During anaphase, as the spindle pole moves from the mother cell compartment and into the bud, both Tem1 and its GAP complex become enriched on the daughter SPB (dSPB). In the event that the spindle is mispositioned in the mother compartment, the GAP is present on both SPBs. Movement of the Tem1-bearing spindle pole into the bud correlates with exit from mitosis, however it is puzzling that the inhibitor of Tem1, Bub2/Bfa1, also becomes concentrated on this same SPB. One model that attempts to explain these observations proposes that it is the disappearance of the GAP from the mother pole that triggers exit from mitosis.

The main evidence for this model stems from an allele of Bfa1 that symmetrically localizes to both SPBs. This Bfa1 allele contains multiple copies of the Myc epitope tag. Bfa1-9MYC symmetrically localized to both SPBs in anaphase and was shown to have anaphase delay. Based on this evidence, it was concluded that disappearance of Bfa1 (and by extension the GAP) from the mother cell-localized SPB led to activation of Tem1 on that spindle pole body. Movement of the SPB through the bud neck was proposed to be the signal for
Bfa1 relocalization and was shown to require several bud neck-localized factors. Subsequent studies have found however this result is not reproducible (Monje-Casas and Amon, 2009).

Recently, the validity of this model has been called into question. Data refuting this model come from the analysis of additional symmetric alleles of both Bub2 and Bfa1. Fusions of either Bfa1 or Bub2 to SPB proteins (Spc72 or Cnm67) result in a loss of function phenotype for both GAP components (Caydasi and Pereira, 2009; Scarfone et al., 2015). This loss of function phenotype of the GAP-SPB fusions is specific to Bub2/Bfa1’s function in spatial regulation of the MEN because all fusions were functional when tested for activity in the SAC. Thus, these fusions are most likely functional constructs. In addition, tethering Tem1 to both SPBs (by fusing it to the SPB component Cnm67) also results in symmetric localization of the GAP. This Tem1 fusion construct hyperactivates the MEN as shown by an increase in inappropriate exit from mitosis in cells with mispositioned spindles. (Valerio-Santiago and Monje-Casas, 2011)

**Model III: The Zone Model**

A model of spatial regulation of the MEN was first described after the observation that Tem1 became concentrated on one SPB upon movement into the bud cell compartment. It was rationalized that this change in Tem1 localization was due to the influence of positive regulators in the bud such as
Lte1 (Bardin et al., 2000). While Lte1 is nonessential, studies showing that Lte1 increased Tem1 dSPB localization supported this model (Molk et al., 2004).

This model was further refined after to the discovery of the kinase Kin4. The screen for Kin4 was done with the following two observations 1) if spindle becomes mispositioned in the mother cell compartment, cells arrest for very long period of time in late anaphase (Yeh et al., 1995) 2) In contrast, cells with aligned spindles that lack Lte1 are only transiently delayed in anaphase. Therefore it was rationalized that there must be additional asymmetrically localized regulators controlling the MEN. Kin4 inhibits the MEN and is asymmetrically localized to the mother cell cortex and mother spindle pole body (D'Aquino et al., 2005; Pereira and Schiebel, 2005). Likewise, it was also found that when cells lacking \textit{KIN4} misposition their spindles, they inappropriately exit from mitosis to generate a binucleate and anucleate cell (D'Aquino et al., 2005; Pereira and Schiebel, 2005).

Spatial regulation of the MEN which takes into account the functions of Lte1, Kin4 and Tem1 is called the zone model. This model posits that there are two zones which control activity of the MEN through the regulation of the GTPase Tem1 which moves between them (Bardin et al., 2000; Chan and Amon, 2010). There is a mitotic exit-promoting zone in the bud cell compartment where mitotic exit activating factors such as Lte1 are present (Bardin et al., 2000). There is also a mitotic exit inhibitory zone in the mother cell compartment where negative regulators of the MEN such as Kin4 are localized (D'Aquino et al., 2005; Pereira and Schiebel, 2005). If the spindle becomes mispositioned in the mother cell
compartment, Kin4 localizes to both spindle pole bodies and the MEN is kept inactive (Pereira and Schiebel, 2005; Chan and Amon, 2009). If one pole escapes the Kin4 inhibitory zone and moves into the bud cell compartment (where Kin4 is largely no longer preset) Tem1 is enriched on the daughter spindle pole body and the MEN can become active.

This model is supported by experiments where Lte1 was targeted into the mother cell compartment resulting in inappropriate exit from mitosis in cells with mispositioned spindles. Mislocalizing Lte1 to the mother cell compartment has been done in several ways: 1) by overexpression Lte1 2) by using a hypermorphic allele of LTE1 (LTE1-8N) which localizes to both mother and bud cortices and 3) by eliminating a component of the septin diffusion barrier (SEP7) allowing Lte1 to diffuse into the mother cell compartment (Bardin et al., 2000; Castillon et al., 2003; Geymonat et al., 2009). In all three cases, allowing Lte1 into the mother cell compartment resulted in inappropriate exit from mitosis in cells with mispositioned spindle.

The zone model is also supported by experiments in which Kin4 is targeted to the dSPB. If Kin4 is on the dSPB, this was shown to result in an arrest in late anaphase even when the spindle is properly positioned (D'Aquino et al., 2005; Maekawa et al., 2007; Chan and Amon, 2010). Targeting Kin4 to the dSPB has also been done in several ways. Eliminating KAR9 or the cyclin CLB4 resulted in the localization of Kin4 to the dSPB and a delay in anaphase (Chan and Amon, 2010). Likewise tethering Kin4 to both SPBs also resulted in an
anaphase delay (Maekawa et al., 2007). This model also predicts that Kin4 acts in every cell cycle, not just those in which the spindle is mispositioned. This prediction is supported by evidence showing that cells with aligned spindles are delayed in exit from mitosis in cells lacking Lte1 (Chan and Amon, 2010).

**Conservation of the MEN and the FEAR**

**Septation Initiation Network**

Similar counterparts to many of the core components of the MEN have also been found to regulate mitosis in S. pombe (Figure 5). In S. pombe the SIN (Septin Initiation Network) is required for cell division. In the absence of the SIN, fission yeast undergo nuclear divisions in the absence of septation. If the SIN is hyperactivated, these cells initiate septation in the absence of nuclear division (Krapp and Simanis, 2008).

Similar to the MEN GTPase Tem1, at the heart of the SIN is the GTPase Spg1. Like Tem1, Spg1 is regulated by both a two component GAP (Cdc16/Byr4) as well as a putative GEF (Etd1) (Furge et al., 1998; García-Cortés and McCollum, 2009). Spg1 localizes to SPBs and in its GTP-bound form it recruits the Cdc15 homolog Cdc7. Cdc7 then (directly or indirectly) activates Sid2-Mob1 which has been shown to activate the Cdc14-like phosphatase Clp1. Clp1, like Cdc14, is kept inactive in cell cycle stages outside of anaphase. When the SIN becomes active, Clp1 is released into the cytoplasm where it dephosphorylates factors to inhibit mitotic CDK activity as well as factors that are required for cytokinesis (Bardin and Amon, 2001)
Similar to the components of the MEN, many SIN factors asymmetrically localize to SPBs. In late anaphase, the GAP complex localizes specifically to the older SPB and Cdc7 coordinately disappears from that same pole (Sohrmann et al., 1998). This GAP-generated asymmetry is thought to result in the SIN being active only on the younger SPB (Cerutti and Simanis, 1999; Li et al., 2000). Consistent with the SIN being active only on the younger SPB, an antibody that is specific to SPG1-GDP was shown to be enriched on the older SPB (Sohrmann et al., 1998). SPB asymmetry of the SIN components has been suggested to be important for the efficient inactivation of the SIN. This was proposed when cells with symmetric SIN activity were shown to form multiple septa indicating that the SIN was not efficiently inactivated (García-Cortés and McCollum, 2009).

**HIPPO**

The HIPPO pathway was first identified in *Drosophila* and regulates cell proliferation, differentiation, growth and death. The corresponding pathway has been also shown to exist in mammals and regulates similar functions. The HIPPO pathway controls the activity of the transcriptional co-activator Yorkie. In the absence of HIPPO activity, Yorkie activity is unrestrained and transcription of a suite of genes results in tissue overgrowth due to production of proliferation and anti-apoptosis signals (Yu et al., 2015). Interestingly, many of the regulators of
**Figure 5: MEN, SIN and HIPPO.** Many components of the MEN are conserved in other eukaryotes. The equivalent pathway in *S. pombe* is the septation initiation network (SIN). In *D. melanogaster*, the HIPPO pathway, which regulates growth, has factors that are also highly similar to the core components of the MEN.

the HIPPO pathway are involved in cell polarity and the cytoskeleton. One such example is F-actin. The connection between the HIPPO pathway and F-actin was found when mutations in actin capping proteins resulted in activation of the HIPPO (Janody and Treisman, 2006). This was interpreted to be due to an overabundance of F-actin because the increasing of F-actin also resulted in activation of the HIPPO (Fernández et al., 2011).
Many of the components of the HIPPO pathway also have highly similar counterparts in yeast (Figure 5). While the output of this signaling pathway is completely different than the MEN, regulation of the HIPPO pathway components is remarkably similar (Staley and Irvine, 2012). Akin to Nud1 in budding yeast, the Sav protein in mammalian cells likely acts as a scaffold for the Cdc15-like kinase Hippo to promote its interaction with the Wts (Dbf2/Mob in budding yeast). To date, however there is no evidence that a GTPase functions in the HIPPO pathway.

**Coupling cell cycle progression to spindle position in higher eukaryotes**

In budding yeast, spindle position is important because the site of cytokinesis is set well before mitosis. Thus yeast must coordinate spindle position with cytokinesis. In many metazoans cell types however, the position of the mitotic spindle largely dictates the site of cytokinesis. Classical experiments done by Ray Rappaport in fertilized sea urchin eggs demonstrated that signals from the mitotic spindle govern the position of the cleavage furrow (Rappaport, 1961). By coupling spindle position to cytokinesis, the cell ensures that each daughter cell will inherit one genomic complement.

Despite the intimate coupling of cytokines to spindle position, additional mechanisms ensure that the spindle is properly centered during the metazoan cell cycle. The mechanisms that position the mitotic and/or meiotic apparatus vary between cell types as does the final position of the mitotic spindle (Oliferenko et al., 2009; Siller and Doe, 2009). For example, in HeLa cells, the
spindle is positioned in the middle of the cell whereas in mammalian oocytes the spindle is positioned asymmetrically at the cell periphery. Thus despite the linkage between spindle position and cytokinesis, where the spindle is positioned in the cell is also very important for cell function. Listed below are two cases in which metazoan cell types employ mechanisms to position the spindle. These cell types also appear to sense spindle position because, like budding yeast, these cells delay the cell cycle in the event that the spindle becomes misaligned.

**Drosophila Germline Stem Cells**

Male *Drosophila* germline stem cells (GSCs) divide asymmetrically to produce one germ cell and one stem cell. The stem cell’s identity is maintained by its proximity to the stem cell niche in the apical tip of the testis. Somatic cells in the niche called hub cells secrete the ligand Unpaired (Upr) which in turn regulates the JAK-STAT (Janus kinase/signal transducer and activator of transcription) pathway to specify stem cell identity (Drummond-Barbosa, 2008). The spatial constraints dictating stem cell identity make it necessary for GSC’s to divide in a defined polarity. In contrast to most cell types, these cells duplicate and segregate their centrosomes in interphase and by mitosis the spindle is always aligned with respect to the polarity of the hub (Yamashita et al., 2007).

Like budding yeast, it also appears that the GSC cell cycle might be coordinated with centrosome position. If the GSC centrosomes are not positioned perpendicularly to the hub cells, they are delayed in G2 until the centrosomes
realign with respect to the hub cells (Venkei and Yamashita, 2015). Interestingly, there has been at least one kinase which has been shown to be important for the cell cycle delay of GSCs with a mispositioned spindle (Yuan et al., 2012). This kinase localized to the spectrosome, an asymmetrically localized organelle that involved in spindle positioned and is present in germline stem cells. This kinase (Par-1) couples cell cycle progression to spindle orientation through the regulation of cyclin A (Yuan et al., 2012). While little is known about the detailed molecular mechanisms by which Par-1 regulates cyclin A, it is intriguing to note that Par-1 is a member of the same family of kinase as budding yeast Kin4. Thus while the cell cycle appears quite different in these cells in comparison to budding yeast, it appears that GCSs, like yeast, link centrosome position to progression through the cell cycle.

**Mammalian Cells**

Spindle position in cells that undergo a largely symmetric division also appears to be coupled to the cell cycle progression. Experiments using cultured rat kidney cells demonstrated that misposition of the mitotic spindle by micromanipulation resulted in a delay in the start of anaphase (O'Connell and Wang, 2000). Subsequent studies, which investigated the role of tissue architecture in spindle position in HeLa cells, revealed that perturbation of the actin cytoskeleton resulted in spindle mispositioning (Charnley et al., 2013). Spindle misposition in these cells led to a delay in chromosome alignment at the metaphase plate. (Charnley et al., 2013; Zhu et al., 2013) Not surprisingly, cells with mispositioned
spindles exhibited a SAC-dependent prometaphase delay. These studies introduce the idea that correct alignment of the spindle in mitosis is required for accurate chromosome segregation.

Conclusions and Perspectives:

A tremendous amount of work has been generated to establish how cells transition from mitosis back to G1. This work has shown that it is the activity of the essential phosphatase Cdc14 that is required for mitotic cells to return to G1. Two complex networks, the FEAR and the MEN, collaborate in the regulation of this phosphatase. While the nonessential FEAR network primes the MEN in preparation for exit from mitosis, the MEN is required for full activation of Cdc14 and return to G1.

Here I investigate the spatial control of the MEN. In the first chapter, I determine that one function of the positive regulator of the MEN, Lte1, is to inhibit Kin4 from localizing to the daughter SPB. This work supports a model in which both negative regulators in the mother compartment and positive regulators in the bud compartment function to spatially control exit from mitosis. In the third chapter I show that, in contradiction to previous findings, contact between cytoplasmic microtubules and the budneck is not required to inhibit the MEN in cells with mispositioned spindles. I also find, through the generation of cells with two nuclei, that spatial regulation of the MEN is not governed by a checkpoint. Rather, I find that it is the movement of one MEN-bearing SPB into the bud that triggers exit from mitosis.
References


Chapter II: Controlling the localization of the spindle position checkpoint kinase Kin4

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Dr. Leon Chan and Jill Falk contributed equally to all figures with the following exceptions: LYC alone performed the following experiments: Fig3, Fig5 E and F.

JEF alone performed the following experiments: Fig7, Fig 6F, Fig9A and C, Fig11.

Fig 3 was adapted from Leon Chan's PhD thesis at MIT (L.Y.Chan, Mechanisms of regulation of the spindle position checkpoint kinase, Kin4. (2010)). This figure was used with permission in the PNAS manuscript.
Introduction

Polarized cell division or the division of the cell along a predetermined axis is critical to building higher-order biological structures. Eukaryotic cells that divide in this manner must position the mitotic spindle along this defined axis to ensure that each mitotic product receives a complete genomic copy. The spindle position checkpoint (SPOC) is a feedback mechanism that delays cell-cycle progression in response to defects in spindle position, and it has been found to be operative during the division of budding yeast, fruit fly stem cells, and cultured mammalian cell (Yeh et al., 1995; Cheng et al., 2008; O'Connell and Wang, 2000). This surveillance mechanism is best understood in budding yeast, where every cell division is polarized and requires proper positioning of the mitotic spindle along the mother–bud axis to produce euploid daughter cells. The SPOC prevents exit from mitosis when the spindle is not aligned along the mother–bud axis (Yeh et al., 1995). When the SPOC is defective, cells with mispositioned spindles inappropriately exit from mitosis, giving rise to abnormal mitotic products with either zero or two nuclei (Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Pereira et al., 2000).

In cells with mispositioned spindles, the SPOC delays cell cycle progression by inhibiting the mitotic exit network (MEN). This pathway triggers exit from mitosis by bringing about the inactivation of mitotic cyclin-dependent kinases (CDKs), which are responsible for promoting entry into and progression through mitosis (reviewed in ref. (Stegmeier and Amon, 2004)). MEN signaling commences with the activation of the MEN GTPase Tem1. The GTPase then
signals through a kinase cascade to activate the protein phosphatase, Cdc14, the major antagonist of mitotic cyclin-CDK activity.

The SPOC controls mitotic exit by regulating the activation of Tem1. When the spindle is mispositioned, the SPOC kinase, Kin4, activates the GTPase-activating protein complex (GAP) of Tem1, the Bub2-Bfa1 complex, through phosphorylation of Bfa1 on residues S150 and S180 (Maekawa et al., 2007). Kin4 itself is regulated at multiple levels. For example, phosphorylation by the protein kinase Elm1 is critical for kinase activity, whereas the protein phosphatase 2A bound to its targeting subunit Rts1 regulates Kin4 localization (Moore et al., 2010; Caydasi et al., 2010; Chan and Amon, 2009).

How does the SPOC sense spindle position and translate this spatial information into a chemical signal to regulate the MEN? The work by Adames et al. (Adames et al., 2001) proposed a model in which interactions between microtubules and the bud neck inhibit the MEN, but how this could lead to Kin4 activation, if indeed Kin4 is activated by spindle misposition, is not known. We previously proposed a model termed the zone model, which posits that the budding yeast cell is divided into a MEN inhibitory zone in the mother cell and a MEN activating zone in the daughter cell and that a sensor, the GTPase Tem1, moves between them.

Tem1 as well as most other components of the MEN reside at spindle pole bodies (SPBs; yeast centrosomes). An activator of the MEN, Lte1, localizes to the bud; the MEN inhibitor, Kin4, is enriched in the mother cell and at the mother
**Figure 1**

The zone model Depicted are Lte1 (green), Kin4 (red) and Tem1 (blue). 
**A)** shows a cell with an aligned spindle. **B)** shows a cell with a mispositioned spindle. See text for discussion of the model.

SPB (Bardin et al., 2000; Pereira et al., 2000; D' Aquino et al., 2005; Pereira and Schiebel, 2005). The asymmetric localization of these two key Tem1 regulators allows the cell to trigger the MEN only when at least one SPB has escaped the zone of inhibition in the mother cell, where Kin4 resides, and enters the zone of activation in the bud where Lte1 resides (Fig. 1A). When the spindle is mispositioned, neither SPB escapes the mother cell, and the MEN remains inhibited (Fig. 1B). Importantly, in the zone model, spindle position is coupled to MEN activation during every cell cycle and is not a regulatory mechanism that is only elicited in response to spindle misposition. Although the role of Kin4 in MEN regulation is well-defined, the role of Lte1 is not clear. The protein is essential for exit from mitosis at low temperatures and has homology to guanine nucleotide exchange factors (GEFs). Despite this homology, Lte1 GEF activity has,
however, not been detected in vitro (Shirayama et al., 1994; Geymonat et al., 2009). Here, prompted by the observation that when Kin4 and Lte1 reside in the same compartment, Lte1 activation of the MEN prevails over Kin4 inhibition (Bardin et al., 2000; Geymonat et al., 2009; Castillon et al., 2003; Chan and Amon, 2010), we investigate the possibility that Lte1 inhibits Kin4. Indeed, we find that Lte1 prevents Kin4 from associating with the SPB in the daughter cell during anaphase. Our results indicate that a key aspect of spindle position surveillance is to allow Kin4 to inhibit MEN signaling from SPBs that reside in the mother cell but not from SPBs that reside in the daughter cell. At least two control mechanisms ensure that this finding is the case: (i) Kin4 is restricted to the mother cell, and (ii) bud-restricted Lte1 inhibits any Kin4 that enters the bud from loading onto the SPBs in the bud. By these mechanisms, inappropriate inhibition of mitotic exit by Kin4 is prevented.

**Results**

**LTE1 Prevents Kin4 from Associating with SPBs in the Bud.**

Genetic studies have placed *LTE1* upstream of or in parallel to *KIN4* in regulating the MEN. The mitotic exit defect of *lte1Δ* cells is suppressed by deletion of *KIN4* (D'Aquino et al., 2005; Pereira and Schiebel, 2005; Chan and Amon, 2010).

Owing to the homology of Lte1 with GEFs, we previously proposed that Lte1 promotes mitotic exit by functioning as a GEF for Tem1 and thus, in parallel to Kin4. However, Lte1 does not harbor GEF activity in vitro (Geymonat et al., 2010), indicating that Lte1 might function in mitotic exit through a different mechanism. Insight into a potential function for Lte1 in promoting mitotic exit
Figure 2: Kin4 loads onto dSPBs in the absence of LTE1.

(A and B) WT (A19900) or lte1Δ (A26156) cells expressing GFP-tagged Kin4 and mCherry-tagged Tub1 were grown to exponential phase in yeast extract peptone dextrose (YEpd) medium and imaged live. Serial sections...
spanning the entire cell were collected to ensure imaging of all SPBs. Loading of Kin4-GFP onto the SPBs was judged by Kin4-GFP foci colocalizing with the ends of the anaphase spindle. Any cells with Kin4-GFP at the dSPB were categorized as dSPB. Cells with Kin4-GFP only on the mSPB were categorized as mSPB. Cells lacking Kin4-GFP at SPBs were categorized as 0 SPB. Arrowheads indicate SPBs. \( n \geq 50 \) cells, and error bars represent the SEM.

(C and D) Cells expressing GFP-tagged Kin4 and mCherry-tagged Tub1 with the genetic backgrounds \( \textit{bub2A} \) (A26941) or \( \textit{bub2A} \ \textit{lte1A} \) (A26940) were arrested in the G1 phase of the cell cycle in YEPD medium using 5 \( \mu \)g/mL \( \alpha \)-factor and released into pheromone-free medium at room temperature. Cells were collected every 15 min post-release and analyzed as in Fig. 1 A and B. \( n > 50 \) cells, and error bars represent the SEM.

(E) \( \textit{KIN4-3HA} \) (A11779), \( \textit{kin4-T209A-3HA} \) (A22119), and \( \textit{KIN4-3HA} \ \textit{lte1A} \) (A26158) were grown to midexponential phase and arrested with 15 \( \mu \)g/mL nocodazole for 2 h. Kin4 was immunoprecipitated, and the kinase activity was measured against a maltose binding protein-BFA1 (MBP-BFA1) substrate. Kin4-associated kinase activity (Kin4 kinase), immunoprecipitated Kin4 (IPed Kin4), total amount of Kin4-3HA in cell extracts (input), and levels of Bfa1 substrate (MBP-Bfa1) are shown. The band that is shown for associated Kin4 kinase activity is the first major degradation product of MBP-Bfa1 as described in ref. 9.

(F) Cells in A and B were grown to exponential phase, shifted to 12 °C for 22 h, and analyzed as in B. \( n > 50 \) cells, and error bars represent the SEM.

came from the observation that when Lte1 and Kin4 are in the same compartment—either by targeting Lte1 to the mother cell or by targeting Kin4 to the bud—Lte1 activation of the MEN prevails over Kin4 inhibition of this signaling pathway. These findings raise the possibility that Lte1 is an inhibitor of Kin4. Because Kin4 inhibits MEN signaling at SPBs (Maekawa et al., 2007), we first tested this hypothesis by examining the effects of deleting \( \textit{LTE1} \) on Kin4 localization.

Kin4 localization is dynamic. In G1 cells, the protein localizes to the cell
**Kin4 is an insoluble protein.**

**(A)** Cells expressing a Kin4-3HA fusion (A11779) were lysed as described in Materials and Methods and split into three aliquots. The first aliquot was boiled in SDS sample buffer and analyzed (total), the second aliquot was fractionated by a 13,000×g centrifugation, and the third aliquot was fractionated by a 100,000×g centrifugation. Soluble (S13 and S100) and pelletable (P13 and P100) fractions were analyzed by Western blot analysis. Pep12 served as a vacuolar marker that is known to pellet in 100,000×g fractionations. Pgap1 was used as a cytoplasmic marker that is known to remain soluble during 100,000×g fractionations.

**(B)** Cells expressing a Kin4-3HA fusion (A11779) were lysed as described in Materials and Methods, and the cell lysate was treated with the indicated conditions for 1 h. The lysates were fractionated by centrifugation and analyzed by Western blot analysis.

cortex. When cells enter the cell cycle, the protein transiently localizes to the emerging bud. Shortly thereafter, Kin4 predominantly localizes to the mother cell cortex. During late anaphase, the protein also binds the SPB in the mother cell (mSPB). The fact that Kin4 associates with the mSPB only during late and not during early anaphase is reflected in the quantification of Kin4 localization in anaphase cells. Only 40–50% of anaphase cells harbor Kin4 at mSPBs (Pereira and Schiebel, 2005)(Figs. 2A, 2B, 2D, 5C, and 6D). Shortly before cytokinesis,
mother cortex enrichment of Kin4 becomes less pronounced, and Kin4 is also seen at the bud neck and adjacent bud cortex (Pereira and Schiebel, 2005; D'Aquino et al., 2005; Chan and Amon, 2010).

To determine whether LTE1 affects Kin4 localization, we analyzed the localization of the protein kinase in cells lacking LTE1. Deletion of LTE1 did not seem to affect the association of Kin4 with the mother cell cortex or the mSPB. However, whereas Kin4 was only detected on the daughter SPB (dSPB) in 2.0 ± 1.1% (SEM) of WT cells during anaphase, the protein was detected on the dSPB in 47.3 ± 4.1% (SEM) of ite1Δ cells (Fig. 2 A and 2 B). Kin4 loading onto the dSPB in ite1Δ mutants was not caused by the cell-cycle delay displayed by cells lacking LTE1 (Chan and Amon, 2010; Jensen et al., 2004). Cells that lack both LTE1 and BUB2 exit mitosis with WT kinetics (Bardin et al., 2000; Adames et al., 2001; Pereira and Schiebel, 2005; Jensen et al., 2004; Yoshida et al., 2003), and in these cells, Kin4 was still observed to load onto dSPBs, albeit with reduced efficiency (Fig. 2 C and D). Taken together, these data indicate that LTE1 prevents Kin4 from loading onto the dSPB in the bud, thereby preventing spurious inhibition of the MEN.

In addition to preventing Kin4 from binding to SPBs, Lte1 could also inhibit Kin4 kinase activity. To test this theory, we examined the consequences of deleting LTE1 on Kin4 kinase activity. Kin4 is a highly insoluble protein (Fig. 3A and B), but small amounts of Kin4 can be immunoprecipitated; additionally, the ability of immunoprecipitated Kin4 to phosphorylate recombinant Bfa1 can be
determined (Maekawa et al., 2007). In this assay, deletion of LTE1 did not affect Kin4-associated kinase activity (Fig. 2E). However, possible effects of Lte1 on the kinase activity of insoluble Kin4 cannot be excluded (Discussion).

Lte1 is essential at low temperature (at and below 18 °C). lte1Δ mutant cells grown at low temperature arrest in anaphase (Shirayama et al., 1994). If inhibiting Kin4 association with dSPBs is the critical function of LTE1 in promoting exit from mitosis, we hypothesized that Kin4 association with dSPBs would be increased at low temperature. This hypothesis seems to be the case. Kin4 was found on dSPBs in 69.1 ± 4.9% (SEM) of lte1Δ cells (Fig. 2F). This result suggests that the ability of LTE1 to prevent Kin4 association with the dSPB is especially important at low temperatures.

**Lte1 Functions Together with Mother Cell Restriction to Prevent Kin4 dSPB Binding.**

Kin4 predominantly localizes to the mother cell (D'Aquino et al., 2005; Pereira and Schiebel, 2005). This mechanism likely contributes to preventing Kin4 from associating with dSPBs. To test this possibility, we examined the consequences of losing mother cortex restriction of Kin4 in lte1Δ cells. The Kin4-S508A mutant protein localizes to both the mother cell and bud cortices. Because this allele causes lethality when expressed in cells lacking LTE1 (Chan and Amon, 2010), we examined Kin4-S508A localization in lte1Δ cells that also lack BUB2, which allows the lte1Δ KIN4-S508A mutant to grow (Chan and Amon, 2010). The fraction of bub2Δ lte1Δ cells harboring Kin4-S508A on dSPBs was
significantly higher than the fraction of \textit{bub2}\Delta \textit{lte1}\Delta cells with WT Kin4 on dSPBs [55.2 ± 4.1 vs. 20.8 ± 5.2% (SEM), respectively] (Fig. 4 A and B). In fact, Kin4-S508A was detected on dSPBs in the same percentage of anaphase \textit{bub2}\Delta \textit{lte1}\Delta cells as on the mSPB in \textit{bub2}\Delta cells (Fig. 2 D). Similar results were obtained in cells where Lte1 was depleted but \textit{BUB2} was functional (Fig. 4C and D). Our data indicate that Lte1 and the restriction of Kin4 to the mother cell represent two parallel mechanisms that prevent Kin4 from associating with dSPBs.

\textbf{Lte1 Can Prevent Kin4 from Binding SPBs in the Mother Cell.}

Is the ability of Lte1 to inhibit Kin4 SPB loading restricted to the bud, or could this function also be operative in the mother cell? To test this possibility, we examined the consequences of targeting Lte1 to the mother cell on Kin4 localization. The Lte1-8N mutant protein associates with both the mother cell and bud cortices, because the mutant protein binds more tightly to its cell cortex anchor, Ras (Geymonat et al., 2010). However, Lte1-8N is also found in the cytoplasm of both the mother cell and bud (Fig. 5A). In WT cells, Kin4 was observed to localize at mSPBs in 50.0 ± 4.0% (SEM) of anaphase cells, whereas in \textit{LTE1-8N} cells, Kin4 binding to mSPBs was significantly reduced [5.6 ± 2.2% (SEM)] (Fig. 5B and C). We conclude that Lte1 prevents Kin4 from associating with SPBs. In WT cells, this activity is directed only to the dSPB, because Lte1 is restricted to the bud.

Cells expressing the LTE1-8N allele are defective in the SPOC but not the
LTE1 acts in parallel to the mechanisms that restrict Kin4 to the mother cell.

(A and B) Cells expressing GFP-tagged Kin4-S508A and mCherry-tagged Tub1 with the genetic back-grounds bub2Δ (A25847) or bub2Δ lte1Δ (A26170) were analyzed as in (2C and D). n > 50 cells, and error bars represent the SEM.

(C and D) WT (A21557) and pGAL1-10-URL-3HA-LTE1 (A24247) cells expressing Kin4-S508A-GFP and mCherry-Tub1 were grown in yeast extract peptone (YEP) medium supplemented with 2% raffinose and galactose to mid-exponential phase. Cells were then transferred to yeast extract peptone dextrose (YEPD) medium, and samples were collected 7 hrs after the medium shift for live microscopy and analyzed as in Fig. 2 A and B; n ≥ 50 cells, and error bars represent the SEM.
spindle assembly checkpoint (Geymonat et al., 2009)(Fig. 5D and E). Could this defect be caused by an inability to localize Kin4 to SPBs in cells with mispositioned spindles? Kin4 binds to both SPBs in cells where spindle misposition is triggered by the inactivation of dynein (dyn1Δ), but binding was greatly reduced in dyn1Δ cells expressing the LTE1-8N allele (Fig. 5F). We conclude that the LTE1-8N allele causes SPOC defects at least in part by preventing Kin4 from loading onto SPBs in cells with mispositioned spindles.

In both DYN1 and dyn1Δ cells, LTE1-8N affected the localization of Kin4 not only at SPBs but also at the cell cortex. Kin4 was found at both the mother and bud cortices and also in the cytoplasm in LTE1-8N cells. Overall, the localization of Kin4 in the LTE1-8N mutant strongly resembled the localization of Lte1- 8N itself (compare Fig. 5A with 5B). How the LTE1-8N allele affects Kin4 at the cortex is not yet clear. The protein could bind Kin4 tightly and thus, could determine Kin4 localization. Alternatively, the localization defect of Kin4 in the LTE1-8N mutant could be caused by the effects of Lte1 on overall cell polarity (lte1Δ mutants display defects in polarity cap localization) (Pereira et al., 2000). Based on the observation that overexpressed Lte1 determines the localization of Kin4 (see below), we favor the aforementioned binding model.

**Lte1 Physically Interacts with Kin4**

To begin to determine the mechanism used by Lte1 to prevent the association of Kin4 with SPBs, we examined the consequences of overexpressing LTE1 from
Mother-targeted Lte1 can inhibit Kin4 from loading onto mSPBs.

(A) Cells expressing GFP-tagged Lte1 (A22632) or Lte1-8N (A28043) were grown to exponential phase in YEPD medium. Cells were imaged with identical acquisition conditions.

(B and C) WT (A19900) and LTE1-8N (A27414) cells expressing GFP-tagged Kin4 and mCherry-tagged Tub1 were collected and analyzed as in Fig. 2 C and D. n > 50 cells, and error bars represent the SEM.
(D) dyn1Δ (A17349), dyn1Δ kin4Δ (A17351), dyn1Δ LTE1-8N (A26130), and LTE1-8N (A26131) cells were grown to mid-exponential phase in YEPD medium. The cultures were then shifted to 14 °C for 24 h, and samples were collected for tubulin indirect immunofluorescence and DAPI staining. Gray bars represent cells with the arrested morphology, which are defined as large-budded cells with an anaphase nucleus located in the mother cell. The white bars represent cells with the bypassed morphology defined as unbudded cells with more than one nucleus or anucleate cells. n ≥ 100 cells.

(E) WT (A2587), bub2Δ (A1863), kin4Δ (A17865), and LTE1-8N (A26131) cells were arrested and released as in Fig. 2 C and D., except that the release medium contained 15 μg/mL nocodazole. Samples were collected every 20 min and examined for the presence of cells with more than one bud (rebudded). n ≥ 100 cells. Note the formation of an additional bud “rebudded” indicates that spindle assembly checkpoint bypass occurred.

(F) dyn1Δ (A26165) and dyn1Δ LTE1-8N (A26450) cells expressing Kin4-GFP and mCherry-Tub1 were grown to exponential phase in YEPD medium, shifted to 14 °C for 24 h, and then, analyzed as in Fig. 2A and B. n > 50 cells, and error bars represent the SEM.

the methionine repressible MET25 promoter on Kin4 localization. Overexpressed Lte1 mostly associates with the bud cortex, but some protein is also found in the cytoplasm and at the mother cell cortex (Farkasovsky and Küntzel, 1995)(Fig. 6A and B). Like the LTE1-8N allele, overproduced Lte1 prevented Kin4 association with the mSPB (Fig. 6C and D). Interestingly, overproduced Lte1 also affected cortical Kin4. Whereas Kin4 displayed the expected enrichment at the mother cell cortex in WT cells, a fraction of Kin4 associated with the bud cortex in cells that
**Figure 6**

**A** Localization of *LTE1-GFP*

- **unbudded**
- **small budded**
- **large budded**

**B** Localization of *pMET25-LTE1-GFP*

- **unbudded**
- **small budded**
- **large budded**

**C** Localization of *pMET25-LTE1* with Kin4-GFP and tubulin

- **wild-type**
- **pMET25-LTE1**

**D** Percent Anaphase Cells

- mother cortex
- bud cortex
- symmetric

**E** Percent Anaphase Cells

- wild-type
- pMET25-LTE1

**F** Kinase Mutations and Protein Complexes

- **Kin4-3HA**
- **Kin4-S508A-3HA**
- **Lte1-13MYC**
**Kin4 and Lte1 interact.**

**(A and B)** Cells expressing Lte1-GFP from (A) the endogenous promoter (A22632) or the methionine repressible *MET25* promoter (B) (A28096) were grown to exponential phase in YEPD supplemented with 8 mM methionine and then transferred to synthetic complete medium lacking methionine to induce *LTE1* expression for 4.25 h. Cells were imaged with identical acquisition conditions to illustrate differences in Lte1 levels and localization between cells harboring WT Lte1 or overexpressed Lte1. A, Bottom is a contrast-enhanced version of the Lte1-GFP image (A, Middle).

**(C–E)** WT (A19900) and *pMET25-LTE1* (A27806) cells expressing GFP-tagged Kin4 and mCherry-tagged Tub1 were collected and analyzed as in Fig. 2 C and D with the following modifications. Cells were grown overnight in YEPD medium supplemented with 8 mM methionine, arrested in synthetic complete medium lacking methionine, and released into synthetic complete medium lacking methionine and pheromone. SPB loading of Kin4-GFP (D) as well as cortex localization (E) was determined. Cortex association was assessed using the following categories: bud cortex, asymmetrically localized to bud cortex; mother cortex, asymmetrically localized to the mother cell cortex; symmetric, symmetrically localized to both cortices. The percent of *pMET25-LTE1* cells with or without Kin4-GFP at SPBs is indicated in C, Right. n > 100 cells for D, and n ≥ 100 cells for E. Error bars represent the SEM.

**(F)** Cells expressing Lte1 tagged with 13 Myc epitopes (A22668), Kin4-S508A tagged with three HA epitopes (A20608), and both tagged proteins (A26157) as well as cells expressing Lte1 tagged with 13 Myc epitopes and Kin4 tagged with three HA epitopes (A28273) were grown to exponential phase and then lysed. Lte1-13Myc was immunoprecipitated using a Myc antibody, and its ability to coimmunoprecipitate Kin4-S508A-3HA or Kin4-3HA was examined. The asterisk indicates a cross-reacting band.

_overexpress L*TE1* (Fig. 6C and E). The ability of Lte1 to recruit a mother cortex-associated protein to the bud cortex was specific to Kin4. Overexpression of Lte1 showed little to no effect on the localization of the mother cortex-associated proteins Num1 and Sfk1 (Farkasovsky and Küntzel, 1995; Audhya and Emr, 2002) (Fig. 7 A and B) or the localization of the bud cortex-associated proteins
This finding indicates that Lte1, when overproduced, can determine the localization of Kin4 but not other mother cell cortex-associated proteins. Overexpression of LTE1 allows cells with mispositioned spindles to inappropriately exit from mitosis(Bardin et al., 2000). The ability of overexpressed Lte1 to prevent Kin4 from associating with SPBs in the mother cell provides an explanation for this phenotype.

The observation that overexpressed Lte1 or Lte1-8N can recruit Kin4 to sites in the cell where they localize raises the possibility that Lte1, directly or indirectly, interacts with Kin4. To test this possibility, we asked whether Lte1 can be co-immunoprecipitated with Kin4. This possibility seems to be the case; the two proteins are found in a complex (Fig. 6F). Thus, although Kin4 and Lte1 are primarily located in different cellular compartments, the proteins can interact with each other when this compartmentalization is disrupted in cell extracts. Consistent with this idea is the observation that the interaction between Kin4-S508A and Lte1 was not increased compared with the interaction between WT Kin4 and Lte1 (Fig. 6F). We conclude that Lte1 can bind to Kin4 in a direct or indirect manner and when overexpressed, determines the localization of Kin4. This finding seems to be critical for preventing the association of Kin4 with dSPBs.

**The N Terminus of Kin4 Mediates Regulation by Lte1**
We next wished to identify the region of Kin4 that mediates its interaction with Lte1. Kin4 consists of an N-terminal kinase domain (amino acids 1–341) and a regulatory C-terminal domain (amino acids 342–800), which harbors a cortex-targeting domain in the extreme C terminus and a mother cell-targeting region in the middle of the protein (amino acids 503–511) (Fig. 8A)(Chan and Amon, 2010). When the C-terminal regulatory domain is deleted, the N-terminal kinase domain localizes to the bud cortex from S-phase to late anaphase (Chan and Amon, 2010). This observation raises the possibility that an Lte1 interaction domain is located in this part of the protein. Indeed, whereas kin4(1–341) localized to the bud cortex in WT cells, the protein no longer associated with the bud cortex in cells lacking LTE1 (Fig. 8 B and C). Furthermore, Lte1 is sufficient to direct the localization of this Kin4 fragment to other sites in the cell. In cells expressing the LTE1-8N allele, kin4(1–341) was now no longer restricted to the bud, but it was, as the Lte1-8N protein, present at the mother cell and bud cortices (Fig. 8 D and E). Coimmunoprecipiation analyses also confirmed an interaction between kin4(1–341) and Lte1 (Fig. 9A).

To determine whether additional Lte1-interacting domains are present in Kin4, we examined the ability of overproduced Lte1 to recruit the C-terminal Kin4 fragment kin4(342–800) into the bud. kin4(342–800) remained evenly associated with both the mother in cells overexpressing LTE1 (Fig. 9B and C). We conclude that the 341 N-terminal amino acids of Kin4 harbor an Lte1 interaction domain.
Figure 7

A  
unbudded  small  budded  large  budded  
wild-type  
unbudded  small  budded  large  budded  
pMET25-LTE1  

B  
unbudded  small  budded  large  budded  
wild-type  
unbudded  small  budded  large  budded  
pMET25-LTE1  

C  
unbudded  small  budded  large  budded  
wild-type  
unbudded  small  budded  large  budded  
pMET25-LTE1  

D  
unbudded  small  budded  large  budded  
wild-type  
unbudded  small  budded  large  budded  
pMET25-LTE1  

Figure 7
Localization of mother-associated protein and polarity factors.

**A–D** WT or pMET25-LTE1 cells expressing GFP-tagged (A) Num1 (A26168 or A28047), (B) Sfk1 (A26169 or A28048), (C) Kel1 (A26550 or A28050), or (D) Spa2 (A26554 or A28049) were grown and imaged as in Fig. S4A.

The N-terminal domain of Kin4 contains the Kin4 kinase domain and a short 40-aminoacid N-terminal extension. Deletion of the first 40 amino acids abolished the bud cortex association of the Kin4 kinase domain (Fig. 8F). This region of the protein is, however, not the only Lte1-interacting domain. Kin4 lacking the first 40 amino acids [Kin4(41–800)] was still targeted to the bud cortex by high levels of *LTE1*; however, the targeting was not as efficient, because some Kin4 remained at the mother cell cortex as well (Fig. 8G, symmetric category). This finding indicates that the first 40 amino acids of Kin4 represent an Lte1-interacting domain but that additional domains exist in the context of full-length Kin4. Could Lte1 be an integral part of the dynamic localization pattern of Kin4? Kin4 transiently localizes to the nascent bud early in the cell cycle (Chan and Amon, 2010). We were not able to reliably address whether *LTE1* was required for this transient localization, but we were able to examine the role of Lte1 in the localization of Kin4-S508A. Localization of this Kin4 mutant protein at the cortex
Figure 8

A

<table>
<thead>
<tr>
<th>41</th>
<th>314</th>
<th>S508</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-341</td>
<td>-</td>
<td>342-800</td>
</tr>
</tbody>
</table>

B

S-phase | metaphase | anaphase

bub2Δ

lce1Δ; bub2Δ

kin4(1-341)-GFP + tubulin

C

Percent Cells

bub2Δ | lce1Δ; bub2Δ

bud cortex | not cortical

D

DIC | kin4(1-341) | GFP | tubulin

wild-type

LTE1-8N

E

Percent Anaphase Cells

mother cortex | bud cortex | both cortices cortical | not cortical

wild-type | LTE1-8N

F

Percent Large-budded Cells

bud cortex | not cortical

kin4(1-341) | kin4(41-800)

G

Percent Anaphase Cells

bud cortex | symmetric | mother cortex

kin4(41-800) | pMET25-LTE1

KIN4 pMET25-LTE1
The N-terminal 341 aa of Kin4 mediate interaction with Lte1.

(A) Domain structure of Kin4. The kinase domain and cortical targeting domain are filled in, and the residues that define the kinase domain and serine 508 are indicated. The positions of N- and C-terminal truncations are indicated by the double-headed arrows below the diagram.

(B and C) bub2Δ (A26565) or bub2Δ lte1Δ (A26567) cells expressing kin4(1–341)-GFP and mCherry-Tub1 were grown as in Fig. 2A. Cortex association was assessed by scoring S-phase, metaphase, and anaphase cells for whether kin4(1–341)-GFP seemed enriched at the bud cortex. n > 50 cells, and error bars represent the SEM.

(D and E) WT (A23410) or LTE1-8N (A27959) cells expressing kin4(1–341) tagged with GFP and Tub1 tagged with mCherry were grown to exponential phase in YEPD medium, and the presence or absence of cortex association was assessed. n > 100 cells, and error bars represent the SEM.

(F) Cells expressing 3HA-kin4(41–341)-GFP (A28315) or kin4(1–341)-GFP (A23247) were grown as described in D and E, and the presence or absence of cortex association was assessed. n > 50 cells, and error bars represent the SEM.

(G) 3HA-kin4(41–800) (A25646), pMET25-LTE1 3HA-kin4 (41–800)-GFP (A27996), or pMET25-LTE1 KIN4-GFP (A27806) cells expressing mCherry-Tub1 were grown to exponential phase in YEPD medium supplemented with 8 mM methionine. Cells were then induced to express Lte1 in synthetic complete medium lacking methionine for 4.25 h. Cortex association was assessed using the following categories: bud cortex, asymmetrically localized to bud cortex; mother cortex, asymmetrically localized to mother; symmetric, symmetrically localized to both cortices. n ≥ 50 cells, and error bars represent the SEM.

of small buds is more pronounced (presumably because its subsequent enrichment at the mother cell cortex is impaired), and Kin4 localization to small buds can be assessed more easily. Kin4- S508A localizes to the bud cortex in small-budded WT cells (Chan and Amon, 2010) (Fig. 10A and B). This enrichment in small buds was dramatically reduced in lte1Δ mutants (Fig. 10A and B). We conclude that LTE1 is necessary for early bud cortex localization of
kin4(1–341) interacts with Lte1, but localization of kin4(342–800) is unaffected by Lte1.

(A) Cells expressing Lte1 tagged with 13 Myc epitopes (A3717), kin4(1–341) tagged with three HA epitopes (A14052), or both tagged proteins (A28146) were grown to exponential phase and then lysed. Lte1-13Myc was immunoprecipitated using an Myc antibody, and its ability to coimmunoprecipitate kin4(1–341) was examined.

(B) Strains expressing mCherry-Tub1 with the genetic backgrounds KIN4-GFP pMET25-LTE1 (A27806), 3HA-kin4(342–800)-GFP (A27988), or 3HA-kin4(342–800)-GFP pMET25-LTE1 (A27986) were analyzed as in Fig. 4 A and C. Cells were grown overnight in YEPD medium supplemented with 8 mM methionine and then incubated for 1 h in synthetic complete medium lacking methionine. Presence, absence, and asymmetry of cortex association of the Kin4 truncations were assessed. n > 50 cells, and error bars represent the SEM.

(C) Cells expressing Kin4-GFP (A19900) and 3HA-kin4(342–800)-GFP (A27988) were lysed and analyzed for expression of the GFP fusion protein by Western blot. Levels of Kar2 were monitored to control for loading.
Kin4-S508A. This result, furthermore, raises the possibility that Lte1 is an integral part of Kin4 localization control during early stages of the cell cycle.

**Discussion**

Keeping Kin4 off the daughter spindle pole is essential for cell cycle progression. This finding is evident from the analysis of a KIN4 allele (KIN4-S508A) in cells that lack LTE1. In such cells, Kin4 localizes to the daughter spindle pole, and cells arrest in anaphase. It is, thus, not surprising that multiple mechanisms are in place that prevent Kin4 from associating with dSPBs (Chan and Amon, 2010). Throughout most of the cell cycle, most of Kin4 is restricted to the mother cell (D’Aquino et al., 2005; Pereira and Schiebel, 2005). Furthermore, Lte1 prevents any Kin4 that has escaped the mother cell from binding to the dSPB. The escape of Kin4 from the mother cell compartment happens every cell cycle. Although Kin4 is greatly enriched in the mother cell, low levels of Kin4 are also observed in the bud (Chan and Amon, 2010). Loss of LTE1 leads to these low levels of Kin4 associating with the dSPB, and exit from mitosis is delayed. Importantly, this cell-cycle delay is suppressed in its entirety by deleting KIN4 (D’Aquino et al., 2005; Pereira and Schiebel, 2005; Chan and Amon, 2010), indicating that the main function of Lte1 in promoting mitotic exit is to prevent Kin4 from associating with the dSPB.
Enrichment of symmetric Kin4 in early buds requires LTE1.

(A and B) Cells expressing GFP-tagged Kin4-S508A and mCherry-tagged Tub1 with the genetic backgrounds bub2Δ (A25847) or bub2Δ lte1Δ (A26170) were grown as in Fig. 2 C and D, and small bud cortex enrichment was assessed. n > 50 cells, and error bars represent the SEM.

The ability of Kin4 to associate with dSPBs in cells lacking LTE1 is more pronounced at low temperature, providing an explanation for why LTE1 is essential at low temperatures. However, the basis for this observation is not clear. Mother cortex enrichment of Kin4 is not obviously altered at low temperature, and loading of Kin4 is not only increased at dSPBs, but it is also modestly increased at mSPBs at low temperatures (Fig. 2). This finding raises the interesting possibility that Kin4 associates more stably with SPBs at low temperatures, and therefore, Lte1 is especially needed to prevent Kin4 from binding to dSPBs. We also do not yet know whether preventing Kin4 from associating with dSPBs is the sole function of Lte1 in mitotic exit. The
observations that (i) the mitotic exit defect of \( lte1\Delta \) mutants is completely suppressed by the deletion of \( KIN4 \) and (ii) Kin4 binding to dSPBs is increased at low temperature when \( LTE1 \) is essential suggest that this is the case. However, Lte1 plays an important role in regulating aspects of cell polarity, and cell polarity factors have been implicated in MEN regulation (Geymonat et al., 2010; Höfken and Schiebel, 2002). Thus, additional MEN regulatory roles cannot be excluded. Clearly, it will be necessary to compare \( lte1\Delta \) mutants with \( KIN4 \) mutants that have lost their ability to interact with Lte1.

Our data provide insights into the mechanism used by Lte1 to prevent Kin4 from associating with dSPBs. Lte1 and Kin4 are found in a complex, and Lte1 seems to determine the localization of Kin4. When Lte1 is overproduced and high levels of Lte1 are present at the bud cortex, a fraction of Kin4 is recruited from the mother cell cortex to the bud cortex. A Kin4 truncation that retains its Lte1 interaction domain, kin4(1–341), coimmunoprecipitates with Lte1 and is partly redirected from the bud cortex to the mother cell cortex by an Lte1 mutant protein (Lte1-8N) that associates with the mother cell and bud cortices. Together, these data suggest that Lte1 binds directly or indirectly to Kin4 and prevents its association with SPBs. The mechanism used by Lte1 to prevent Kin4 from binding to SPBs is not yet known. Our previous studies indicated that the ability of Kin4 to associate with the cell cortex is tightly linked to its ability to bind SPBs (Chan and Amon, 2010). This tight correlation led to the proposal that cell cortex association of Kin4 is a prerequisite for its SPB loading. It is tempting to
Kin4 phosphorylation during anaphase requires LTE1.

(A and B) Cells expressing GFP-tagged Kin4-S508A and mCherry-tagged Tub1 with the genetic backgrounds bub2Δ (A25847) or bub2Δ lte1Δ (A26170) were grown as in Fig. 2 C and D. Cell-cycle stage was determined by spindle morphology, and samples were collected at the indicated time points to determine Kin4-GFP mobility on SDS/PAGE by Western blot. Kar2 served as a loading control. The arrows indicate the phosphorylated forms of Kin4.

speculate that Lte1 prevents Kin4 from translocating from the cell cortex to SPBs, but simple models, where binding of Lte1 to the N-terminus of Kin4 occludes an SPB-targeting domain, are equally possible.

The interaction between Kin4 and Lte1 is mediated in part by the extreme N terminus of Kin4, which is located immediately adjacent to the kinase domain of Kin4. Could Lte1, thus, also inhibit Kin4 kinase activity? Although deleting LTE1 did not affect Kin4 activity assayed by immunoprecipitation kinase assays, it may
be premature to conclude that LTE1 does not affect Kin4 activity. Cortical Kin4, which presumably is a significant constituent of the insoluble Kin4 pool, could very well be inhibited by Lte1. Although it is not yet known whether Lte1 binding inhibits Kin4 kinase activity, it is clear that Lte1 binding to Kin4 affects the ability of Kin4 to associate with SPBs and the phosphorylation state of Kin4 (Fig. 11). Kin4 is hyperphosphorylated during late stages of mitosis but is hypophosphorylated during S-phase and early mitosis. Based on the correlation between phosphorylation status and presumptive time of KIN4 function during the cell cycle, we previously suggested that dephosphorylated Kin4 was active (Chan and Amon, 2009). We find that Kin4 is hypophosphorylated in cells lacking LTE1, which is consistent with the idea that Lte1 regulates Kin4 by controlling its phosphorylation status. Whether it does so by regulating Kin4 activity itself, the Kin4 phosphatase PP2A-Rts1, the Kin4 kinase Elm1, other Kin4 kinases, or the availability of Kin4 itself to be phosphorylated remains to be determined.

Our data not only show that LTE1 plays a key role in preventing Kin4 that escapes the mother cell from associating with the dSPB but also raise the possibility that Lte1 regulates aspects of the normal localization pattern of Kin4. Kin4 very transiently localizes to the bud early in the cell cycle. This localization is enhanced in the KIN4-S508A mutant that lacks mother cell-targeting signals. Interestingly, the transient enrichment of this Kin4 mutant protein at the bud cortex depends on LTE1. Based on this observation and previous studies, we propose the following model for how Kin4 localization is controlled. As the bud
emerges and all vectorial transport is directed to the growing bud, Kin4 is transported into the bud and transiently anchored there by Lte1. This initial association is disrupted by the mechanisms that direct Kin4 to the mother cell cortex, which is mediated by the C-terminal regulatory domain of Kin4, and could involve selective degradation of bud-anchored Kin4 or a retrograde transport mechanism along the cell cortex into the mother cell. After Kin4 reaches the mother cell cortex, the protein is no longer able to associate with the dSPB. However, this spatial segregation mechanism is not completely effective. Some Kin4 either remains in the bud or escapes from the mother cell into the bud. Lte1 together with other mechanisms, such as Clb4-CDK–controlled SPB loading mechanisms, prevent this pool of Kin4 from binding to SPBs (Chan and Amon, 2010). The importance of Lte1 in this control mechanism is illustrated by the fact that deletion of LTE1 causes a cell-cycle arrest/delay in anaphase that depends on KIN4.

Finally, our data have important implications for our understanding of SPOC function. The isolation of a genetic condition—KIN4-S508A lte1Δ—that inhibits exit from mitosis even in cells with correctly positioned spindles indicates that spindle misposition is not a prerequisite for the ability of Kin4 to inhibit the MEN, and thus, Kin4 is not activated by spindle misposition. Rather, our results indicate that SPOC function is based on the spatial restriction of the MEN regulators Kin4 and Lte1 and inhibition of Kin4 by Lte1. In fission yeast, a signaling pathway homologous to the MEN, known as the septation initiation network (SIN), governs
the final stages of cell division. Fission yeast contains a functional homolog of Lte1 (Etd1), and the protein kinase Ppk1 is similar in sequence to Kin4 (García-Cortés and McCollum, 2009). It will be interesting to determine whether a similar regulatory relationship exists between these two proteins in fission yeast.
Experimental Procedures

Yeast Strains and Growth Conditions.

All strains are derivatives of W303 (A2587) and are listed in Table S1. LTE1-8N was generated with a two-step gene replacement strategy using the URA3 gene from Kluyveromyces lactis and a PCR product containing the 8N mutation derived from plasmid pA1843 [YIplac128-LTE1-8N-GFP (pMG180); gift from Marco Geymonat, Gurdon Institute, University of Cambridge, Cambridge, United Kingdom]. Plasmid pA1946 was digested with Swal replacing the LEU2 locus to generate the pMET25-LTE1 allele. KEL1-GFP, SPA2-GFP NUM1-GFP, SFK1-GFP, LTE1-GFP, and LTE1-8N-GFP were generated by standard PCR-based methods (Longtine et al., 1998). 3HA-kin4(342–800)-GFP, 3HA-Kin4(41–341)-GFP, and 3HA-KIN4(41–800)-GFP were generated by similar PCR-based methods using plasmid pA1517 and standard PCR-based methods (Longtine et al., 1998). Growth conditions are detailed in Figs. 1–5.

Plasmid Construction.

pA1946 was generated by digesting pNH605-pCYC1 (gift from the Lim laboratory, University of California, San Francisco, CA) with PspOMI and BamHI, a PCR fragment containing pMET25 with PspOMI and XhoI, and a PCR fragment containing the coding sequence of LTE1 with XhoI and BamHI and ligating all three fragments together. pA1517 (pFA6a- KanMx6-pKIN4-3HA) was generated by amplifying the promoter of KIN4 (1,000 bp upstream of the KIN4 ORF),
digesting this fragment with BstYI and PacI, digesting pFA6a-KanMx6-pGAL1-10–3HA (Longtine et al., 1998) with BglII and PacI, and ligating these two fragments together.

**Differential Centrifugation and Solubility.**

Differential centrifugation and differential solubility analyses were performed as described in (Kaiser et al., 2002). Cells were grown to midexponential phase, harvested, washed with 10 mM Tris-Cl, pH 7.5, and 10 mM sodium azide, and resuspended in 10 mM Tris-Cl, pH 7.5, 100 mM EDTA, and 0.5% β-mercaptoethanol. Cells were incubated for 20 min at 30 °C and pelleted by gentle centrifugation followed by spheroplasting in 40 mM Hepes-KOH, pH 7.5, 1.2 M sorbitol, 0.5 mM MgCl2, and 8 μg/mL oxalyticase at 30 °C. Cells were then washed with the spheroplasting buffer without oxalyticase, washed again in 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 0.2 M sorbitol, and lysed by dounce homogenization. The lysate was centrifuged for 5 min at 500 × g. The supernatant was then subjected to a 13,000 or 100,000 × g centrifugation for 10 min or 1 h, respectively. To determine if various denaturing or solubilizing treatments could solubilize P100 Kin4, the clarified cell lysates were treated with 500 mM NaCl, 1% Triton X-100, 100 mM sodium carbonate to change the pH to 11.5 or 2 M urea for 1 h at 4 °C before centrifugation. Samples were centrifuged for 1 h at 100,000 × g to separate soluble and pelletable fractions.

**Other Methods.**

Indirect in situ immunofluorescence methods to stain Tub1 were as described
(Kilmartin and Adams, 1984). Live cell imaging of Kin4-GFP, Lte1-GFP, and mCherry- Tub1 was as previously described (Chan and Amon, 2009). Num1-GFP, Sfk1-GFP, Kel1-GFP, and Spa2-GFP were imaged as Kin4-GFP.

Immunoprecipitations were performed as previously described (Chan and Amon, 2009). Immunoblot analysis of Kin4-3HA, Kin4-GFP, Lte1-13Myc, and Kar2 was as previously described (Chan and Amon, 2009; D'Aquino et al., 2005; Chan and Amon, 2010; Seshan et al., 2002). Kin4 kinase assays were performed as previously described (Chan and Amon, 2009).
Table 1: Yeast Strains

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A28273  MATa KIN4-3HA::KanMx6 LTE1-13MYC::KanMx6
A28315  MATa KanMx6::3HA-kin4(41-341)-GFP::His3Mx6
References


Chapter III: Spatial signals link exit from mitosis to spindle position.

Submitted for publication as a manuscript: Jill E. Falk, Dai Tsuchiya, Jolien Verdaasdonk, Soni Lacefield, Kerry Bloom and Angelika Amon. 2015.

Jill Falk performed all experiments with the exception of the following figures: Dai Tsuchiya and Soni Lacefield performed Figure 10. Figure 4 was done with together with Jolien Verdaasdonk and Kerry Bloom.
Summary

Cells must partition their genome during mitosis in order to produce viable progeny. In budding yeast, if the spindle is mispositioned so that anaphase spindle elongation occurs only in the mother cell compartment, exit from mitosis is prevented through the inhibition of the mitotic exit network (MEN). The MEN is an essential signaling cascade that localizes to spindle pole bodies (SPBs) and promotes exit from mitosis by activating the phosphatase Cdc14. There are two competing models to explain how MEN activity is regulated by spindle position. According to the “zone model” the cell is partitioned into a MEN inhibitory zone in the mother cell and a MEN activating zone in the bud. Exit from mitosis occurs when a MEN-bearing SPB enters the bud during anaphase. The “cMT checkpoint model” posits that stable contacts between cytoplasmic microtubules (cMTs) and the bud-neck, which only occur in cells with mispositioned spindles, prevent MEN activity. Here we test both models. We find that eliminating cMT – bud neck interactions does not trigger exit from mitosis and show that cMT loss from the bud neck does not precede Cdc14 activation but is rather a consequence thereof. Furthermore, through the generation of cells containing two nuclei we show that exit from mitosis occurs as long as one MEN-bearing SPB moves into the bud during anaphase even when a mispositioned spindle is also present in cells. We conclude that it is proper spindle elongation that triggers exit from mitosis rather than improper spindle position that inhibits it.
Introduction

Asymmetric cell division is a common characteristic of development and is seen in diverse cell types ranging from *Drosophila* neuroblasts to mammalian oocytes. In order to produce viable progeny with distinct cell fates, asymmetrically dividing cells must coordinate nuclear position with the site of cytokinesis. When the spindle is mis-positioned with respect to the cleavage plane cell cycle progression is delayed until the spindle is correctly aligned. In the budding yeast *Saccharomyces cerevisiae*, coupling exit from mitosis with spindle position is particularly important because the site of cytokinesis forms independently of mitotic spindle position (Pruyne et al., 2004). Thus budding yeast has evolved mechanisms that align the spindle and that ensure that cytokinesis only occurs after the nucleus has been partitioned between the mother cell and bud, the future daughter cell (Bardin et al., 2000; Miller et al., 1999; Pereira et al., 2000; Yeh et al., 1995).

Budding yeast employs two redundant mechanisms to position the spindle along the mother – bud axis. The first positioning mechanism relies on the type V myosin motor Myo2. Myo2, together with its adaptor Kar9 and the plus-end microtubule binding protein Bim1, positions the pre-anaphase spindle at the bud neck by pulling cytoplasmic microtubules (cMTs) along actin cables (Beach et al., 2000; Kopecká and Gabriel, 1998; Miller et al., 1999; Miller and Rose, 1998; Palmer et al., 1992). The second pathway is active in anaphase and requires the minus-end microtubule motor protein dynein, together with its associated coactivating complex dynactin. Dynein, when anchored to the cell cortex by
Num1, aligns the spindle by essentially towing it along the cortex of the cell (Farkasovsky and Küntzel, 2001; Heil-Chapdelaine et al., 2000; Muhua et al., 1994; Yeh et al., 1995). The consequences of eliminating either positioning pathway are minor but cells lacking both pathways are inviable (Miller and Rose, 1998). Cells that fail to segregate the nucleus into the bud will arrest in late anaphase with the spindle mispositioned in the mother cell compartment. If the cell manages to correct this positioning defect and threads the nucleus through the bud neck into the bud, it will then disassemble the anaphase spindle and exit from mitosis (Yeh et al., 1995).

Yeast cells link spindle position and exit from mitosis through the regulation of the essential phosphatase Cdc14 (Bardin et al., 2000; D'Aquino et al., 2005; Pereira et al., 2000; Pereira and Schiebel, 2005). Cdc14 functions to reverse mitotic cyclin-CDK (cyclin dependent kinase) activity by dephosphorylating cyclin-CDK targets as well as by targeting cyclins for degradation (Jaspersen et al., 1998; Visintin et al., 1998; Zachariae et al., 1998). These Cdc14 functions cause exit from mitosis, the final cell cycle transition that encompasses disassembly of the mitotic spindle and cytokinesis (Stegmeier and Amon, 2004). Cdc14’s essential role in exit from mitosis requires that its activity is tightly regulated. Cdc14 is kept inactive from G1 to metaphase by its inhibitor Cfi1/Net1, which functions by sequestering the phosphatase in the nucleolus. It is only upon anaphase entry that Cdc14 is released from Cfi1/Net1 to spread throughout the cell where it antagonizes mitotic CDK activity and so returns the cell to G1 (Shou
et al., 1999; Visintin et al., 1999).

Two pathways control the activity of Cdc14: the Cdc14 early anaphase release network (FEAR) and the mitotic exit network (MEN). The FEAR network, whose components are not essential, serves to ensure anaphase spindle stability, spindle midzone assembly and proper rDNA segregation by transiently releasing Cdc14 from its inhibitor in the nucleolus during early anaphase (reviewed in (Rock and Amon, 2009)). In contrast, the MEN is responsible for sustained Cdc14 release and is essential for cells to exit from mitosis (Jaspersen et al., 1998; Lee et al., 2001; Stegmeier et al., 2002; Visintin et al., 1999; 1998).

The MEN is a GTPase signaling pathway whose constituents primarily localize to spindle pole bodies (SPBs; yeast centrosomes). Regulation of the GTPase Tem1 is central to MEN regulation. When Tem1 is in its GTP-bound state, the MEN is active and cells will exit from mitosis (Scarfone and Piatti, 2015). Likewise when Tem1 is inactive, the MEN is off and cells will arrest in anaphase (Geymonat et al., 2002; Shirayama et al., 1994). Tem1 regulates a kinase cascade comprised of the PAK-like kinase Cdc15 and the protein kinase Dbf2. Tem1 activates Cdc15 by recruiting it to the spindle pole body (Rock and Amon, 2011; Visintin and Amon, 2001). Cdc15 in turn recruits Dbf2 to spindle poles by creating a phospho-peptide binding domain on the SPB component and MEN scaffold Nud1. In its phosphorylated state, Nud1 docks the Dbf2-activating subunit Mob1 (Rock et al., 2013). Activated Dbf2-Mob1 together with Cdc5 then promote the sustained release of Cdc14 from the nucleolus through a largely
uncharacterized mechanism (Manzoni et al., 2010; Mohl et al., 2009).

Tem1 itself is controlled by two opposing factors, Bub2/Bfa1 and Lte1.
Bub2/Bfa1 functions as a GTPase-activating protein complex (GAP) for Tem1 and so inhibits the MEN (Bloecher et al., 2000; Geymonat et al., 2002; Li, 1999; Shirayama et al., 1994). The GAP complex in turn is regulated by the protein kinase Kin4. Kin4 localizes to the mother cell cortex as well as the mother cell-localized SPBs and functions to maintain GAP activity by preventing the inactivation of Bub2/Bfa1 by the Polo kinase Cdc5 (D’Aquino et al., 2005; Maekawa et al., 2007; Pereira and Schiebel, 2005). Lte1 localizes to the bud cell compartment and promotes exit from mitosis by preventing Kin4 localization to SPBs in the bud (Bertazzi et al., 2011; Falk et al., 2011). Lte1 displays homology with guanine nucleotide exchange factors (GEFs); however, whether Lte1 also functions as a GEF for Tem1 remains unknown.

Spindle position regulates MEN activity. When the spindle is mispositioned, the MEN is inactive: Cdc14 is sequestered in the nucleolus and cells arrest in anaphase (Bardin et al., 2000). This regulatory mechanism that prevents exit from mitosis in response to spindle misposition is called the spindle position checkpoint (SPoC; (Yeh et al., 1995)). Two models have been proposed to explain how spindle position signals MEN activity. The “zone model” proposes that the cell is partitioned into a MEN inhibitory zone in the mother cell compartment and a MEN activating zone in the bud (Figure 1A)(Chan and Amon, 2010). The MEN inhibitor Kin4 localizes to the mother cell, the MEN activator
Figure 1: Models describing spatial control of exit from mitosis.

(A) Zone model of exit from mitosis. The anaphase yeast cell is partitioned into two zones: a MEN inhibitory zone in the mother cell compartment and a MEN promoting zone in the bud cell compartment. If the spindle becomes misaligned in the inhibitory zone, MEN inhibitors such as Kin4 prevent Tem1 enrichment on SPB and therefore inhibit exit from mitosis. It is only once one SPB escapes the inhibitory zone and moves into the bud cell compartment that Tem1 can become enriched on the SPB and the cell can exit mitosis.

(B) cMT checkpoint model of exit from mitosis. If the spindle becomes misaligned in the mother compartment, cytoplasmic microtubules activate a checkpoint response through their interactions with factors at the bud neck. Once the spindle has realigned, the cytoplasmic microtubules are no longer in contact with the bud neck, the checkpoint signal is eliminated and cells can exit from mitosis.

Lte1 to the bud (Bardin et al., 2000; D’Aquino et al., 2005; Pereira et al., 2000; Pereira and Schiebel, 2005). In the event that anaphase spindle elongation occurs only in the mother cell, the spindle poles (where Tem1 resides) cannot escape the negative regulation of Kin4 and the MEN is kept inactive. Only once the spindle realigns along the mother-bud axis and one spindle pole exits the Kin4 inhibitory zone is inhibition of Tem1 is relieved and the MEN is active.

The bud cell compartment promotes Tem1 activation through redundant
mechanisms: 1) Kin4 is largely not present in the bud and 2) the Kin4 inhibitor Lte1 prevents any small amount of Kin4 present in the bud from localizing to the daughter SPB. Support for the zone model comes from studies in which the localization of Kin4 or Lte1 has been switched. Targeting Lte1 to the mother cell leads to inappropriate mitotic exit in cells with misaligned spindles (Bardin et al., 2000; Bertazzi et al., 2011; Castillon et al., 2003; Geymonat et al., 2009). Targeting Kin4 to the bud and simultaneously inactivating its inhibitor, Lte1, causes an anaphase arrest in cells with correctly positioned spindles (Chan and Amon, 2010; Falk et al., 2011).

A second model proposes that MEN activity is controlled by a microtubule-based checkpoint mechanism (Figure 1B; henceforth the “cMT checkpoint model”) (Adames et al., 2001; Moore et al., 2009; Muhua et al., 1998). The model posits that stable contact between cytoplasmic microtubules and the bud-neck activates a checkpoint response that prevents cells from exiting mitosis. The hypothetical cMT checkpoint sensor would, according to this mode, localize to the mother side of the septin ring (Castillon et al., 2003). The model derives from studies showing that cytoplasmic microtubule loss from the bud neck precedes anaphase spindle disassembly and exit from mitosis (Adames et al., 2001; Moore et al., 2009). Laser ablation of cytoplasmic microtubules was reported to trigger exit from mitosis (Moore et al., 2009), a finding consistent with this model.

Here we describe several experimental approaches aimed at distinguishing between the zone model and the cMT checkpoint model. These analyses refute
the cMT checkpoint model and support the zone model. In the first approach we conducted live cell imaging to investigate the relationship between the presence of cMTs in the neck and exit from mitosis in cells with mispositioned spindles. As previously reported, we found that cMT loss from the bud neck does indeed precede exit from mitosis in cells that inappropriately breakdown their spindle in the mother cell compartment. However, our data show that loss of cMTs from the bud neck is not a cause but rather a consequence of exit from mitosis. We find in cells that exit from mitosis despite a mispositioned spindle, Cdc14 release precedes the disassembly of cytoplasmic microtubules and subsequently the mitotic spindle. Second, we show that severing cytoplasmic microtubules does not lead to inappropriate exit from mitosis in cells with mispositioned spindles. Finally, we developed a method that allowed us to create cells containing two nuclei. We find that as long as one nucleus enters the bud, cells will exit from mitosis, irrespective of whether the other nucleus is correctly or incorrectly positioned. Our data are inconsistent with the model that cMT-bud-neck interactions prevent exit from mitosis in cells with mispositioned spindles. Instead, they support the conclusion that spatial regulation of the MEN is controlled through the delivery of a SPB into the bud.

Results

A system to monitor spindle misposition by live cell microscopy
Inactivation of either Kar9 or Dyn1 causes a small fraction of cells to transiently misposition their spindles (Fig. 2A). Such cells will then delay in anaphase until
the spindle position has been corrected (Fig. 2B) (Miller and Rose, 1998). The relatively low penetrance and transient nature of the spindle positioning defect of \textit{kar9}\textsuperscript{Δ} and \textit{dyn1}\textsuperscript{Δ} cells has impeded the investigation of the consequences of spindle misposition on cell cycle progression. To overcome this limitation we developed a system to conditionally inactivate both the Kar9 and Dyn1 spindle positioning pathways. We depleted Dyn1 using the Indole-3-acetic acid (IAA; auxin) depletion system (Nishimura et al., 2009). IAA is a naturally occurring plant hormone that promotes the degradation of proteins containing an AID degron sequence by targeting them for ubiquitinylation by the SCF-\textit{Tir1} ubiquitin ligase (Dharmasiri et al., 2005; Gray et al., 2001; Kepinski and Leyser, 2005; Teale et al., 2006). We generated a strain carrying a \textit{DYN1-AID} fusion and a deletion of \textit{KAR9}, henceforth the \textit{DYN1-AID kar9}\textsuperscript{Δ} strain. Live cell imaging showed that 92\% of \textit{DYN1-AID kar9}\textsuperscript{Δ} cells initially mispositioned their spindles (i.e. had spindles greater than 2\,\mu m in length in the mother cell compartment) upon IAA addition in contrast to 13\% seen in the wild type controls. This finding indicated that the \textit{DYN1-AID kar9}\textsuperscript{Δ} system effectively inactivates spindle-positioning systems in the cell.

To characterize the effects of spindle mispositioning on exit from mitosis we compared anaphase duration of cells with correctly aligned spindles to those with mispositioned spindles. Wild-type cells with correctly aligned spindles underwent anaphase within 19.2 ± 4.8 minutes (Figure 2C and 2E). In contrast,
Figure 2

A

B

C

D

E

F

G

Wild Type kar9Δ dyn1Δ

E

F

G

DYN1-AID

kar9Δ

Wild Type

DYNI-AID

kar9Δ

80

60

40

20

0

Tub1-GFP

DIC
Figure 2. A new system to monitor mispositioned spindles.

**A-B** Wild type (A33138), kar9Δ (A33729) and dyn1Δ (A32922) cells harboring GFP-tagged alpha tubulin were grown to mid-log in YEPD and arrested in G1 with 10μg/mL of the alpha-factor pheromone at 25° C. The cultures were released into the cell cycle in YEPD and then loaded onto a Y04C CellASIC flow cell. Cells were imaged on the flow cell in Synthetic Complete pH 6.0 medium. **A** Quantification of the percent of anaphase cells which misposition their anaphase spindle. Anaphase was defined as any spindle measuring >2μm. Aligned spindles were defined as those that entered anaphase with one spindle pole in the bud cell compartment. Mispositioned spindles were defined as those that entered anaphase with both spindle poles in the mother cell compartment. **B** Time-lapse analysis of anaphase length. n =100 cells for each strain.

**C-G** osTIR1 (A35699) and osTIR1 DYN-AID kar9 (A35707) cells expressing GFP-tagged alpha tubulin were grown in YEPD medium at 25° C and arrested in the G1 phase of the cell cycle with 10μg/mL of the alpha-factor pheromone. Cells were released into the cell cycle in YEPD pH 6.0 medium and then monitored by live cell microscopy. Depletion of dyn1-AID was induced on a Cellasic flow cell with 100μM auxin in SC pH 6.0 medium at 25°C. **C** Time-lapse analysis of anaphase length. Open squares indicate cells arrested greater than 200 minutes in anaphase. **D** Analysis of ploidy. Cells that were arrested and contained a misaligned spindle or cells that exited mitosis that contained an aligned spindle were categorized as “euploid”. Cells that inappropriately exited mitosis and broke down the spindle in the mother cell compartment were categorized as multinucleate. n=100 cells. **E-G** Montage of representative time-lapse images. The numbers at the top of the GFP images are time in minutes.

*DYN1-AID kar9Δ* cells spent 65.9 ± 51.7 minutes in anaphase. This anaphase delay was highly variable. Most cells (85%) eventually were able to pull the mispositioned spindle into the bud (see Figure 2F for an example), which was followed by exit from mitosis. Only 6% of cells arrested with a mispositioned spindle for the duration of the movie analysis (longer than 200 minutes, open squares in Figure 2C). The fact that the majority of *DYN1-AID kar9Δ* cells
eventually managed to correctly align their spindles along the mother – bud axis suggests that cells harbor residual dynein activity, perhaps because the depletion is not complete. It is also possible that additional minor spindle positioning pathways exist in these cells (Segal et al., 2002).

Although exit from mitosis was prevented in the majority of cells with mispositioned spindles, we observed inappropriate exit from mitosis in 9% of such cells, leading to the formation of anucleate and binucleate cells (Figure 2D and 2G). This incomplete penetrance of the cell cycle arrest caused by spindle misposition has been observed previously (D’Aquino et al., 2005; Pereira and Schiebel, 2005). The reason why a small fraction of cells escape the anaphase arrest caused by spindle misposition was, however, not understood.

**Loss of cMT- bud neck contacts does not cause inappropriate mitotic exit in cells with mispositioned spindles**

One hallmark of cells with mispositioned spindles is long cMTs that emanate from one or both SPBs through the bud neck and into the bud (Fig 2F; 100 minute time point). In the small fraction of cells that escape the anaphase arrest caused by spindle misposition, inappropriate mitotic exit is preceded by retraction of cytoplasmic microtubules from the bud neck (Moore et al., 2009).

Having established a tool to induce spindle misposition in many cells, we decided to reinvestigate this correlation. As reported previously, we found that cells with mispositioned spindles frequently display cMTs that contact the bud neck (Figure 3, cells 1-30; Supplemental Movie 1).
Figure 3

- cMT absent from budneck
- cMT in budneck
- Spindle pole entry into bud
- Spindle breakdown
- Re budding
- No longer analyzed
Figure 3. Analysis of cytoplasmic microtubules in the bud neck:

Cells harboring osTIR1 DYN-AID kar9 and expressing GFP-labeled tubulin (A35707) grown and imaged as described in Figure 1. A table summarizing cMT-bud neck contact for cells that contained a mispositioned spindle for 60-minutes (cells 1-30) or exited mitosis within that time frame (cells 31-40). Each row shows the color-coded fate of one cell for the given time period, as well as whether it had a cMT in contact with the bud neck. cMT analysis was performed by assessing if a cMT was present or absent from the bud neck. Cells with a cMT end that was in the bud neck or bud was categorized as “cMT in bud neck” (grey boxes) Cells lacking any cMT in the bud neck are described as “cMT absent from bud neck” (black boxes). Movement of one spindle pole into the bud is “spindle pole movement into bud” (blue boxes). Inappropriate exit from anaphase was determined by the spindle morphology and is described as “spindle breakdown” (red boxes). A second category of inappropriate exit from mitosis was scored based on whether the cell rebudded without spindle collapse or cytokinesis (yellow boxes). Due to the low frequency of inappropriate spindle breakdown in the mother compartment, this table shows all cells that inappropriately exit mitosis from 2 experiments (cells 31-40). The cells that remain euploid (cells 1-30) are from experiment 1.

Also consistent with previous studies, we found in cells that exit from mitosis despite harboring a mispositioned spindle, that this cell cycle transition was preceded by the loss of cMT-bud neck interactions. Contact was lost approximately 2-8 minutes before spindle breakdown (Figure 3, cells 31 – 37; Supplemental Movie 2). Additionally, we found a small number of cells that did not lose cMT-bud neck contact but entered the next cell cycle as assessed by budding (Figure 3, cells 38 – 40; Supplemental Movie 3). These cells also did not completely breakdown their spindle and did not complete cytokinesis.

Importantly, our analysis also revealed that inappropriate mitotic exit was not an obligatory consequence of loss of cMT-bud neck interactions. The majority of cells with mispositioned spindles lacked cMT-bud neck contacts for significant
Figure 4. cMT laser ablation in cells with mispositioned spindles.

A-B) Cells with the osTIR1 DYN-AID kar9 genetic background that expressed GFP-tagged alpha tubulin, mCherry-tagged Cdc3 and an NLS-mCherry (A35143) were grown overnight to mid-log in YEPD at 25°C. They were resuspended in synthetic complete medium supplemented with 100uM IAA and incubated 2-3 hours at 25°C. The cells were prepared on an agar pad for live cell microscopy. Two pre-ablation images were taken
(only one is shown) before the cMT was cut. Cells were then imaged for 1 hour at 15-minute intervals to follow cell cycle status. The arrowheads indicate the approximate laser-targeting site. A) A montage of a cell with a mispositioned spindle where cMT bud neck interactions were disrupted due to microtubule severing. The DIC channel is optimized to enhance contrast. B) A montage of a control cell with an aligned spindle where the laser was targeted to the cytoplasm. The DIC channel is optimized to enhance contrast.

C) Table summarizing cell cycle status of aligned and misaligned spindles 69 minutes post ablation. The culturing conditions for cells in the first (A35143) and second rows (A34832: the same as A35143 but lacking Cdc3-mCherry) of the table are the same as described above. In the third row, cells lacking DYN1 and expressing GFP-tagged alpha tubulin and NLS-mCherry (A34722) were grown overnight at 25°C to mid-log and then shifted to 16°C for 2-5 hours to enrich for cells with mispositioned spindles. The cells were prepared on an agar pad for live cell microscopy. One set of pre-ablation images was taken before the cMT was cut. Post ablation cells were monitored as described above.

periods of time yet stayed arrested in anaphase (Supplemental Movie 4; Figure 3, cells 1 – 30). We conclude that loss of cMT–bud neck interactions does not necessarily cause inappropriate mitotic exit in cells with mispositioned spindles.

A previous study reported that eliminating cMT–bud neck interactions by ablating GFP-labeled microtubules causes exit from mitosis in cells with mispositioned spindles (Moore et al., 2009). We conducted a similar analysis and found this not to be the case. We used a laser to ablate microtubules in cells either lacking both the KAR9 and DYN1 spindle positioning pathways or just lacking the DYN1 positioning pathway. We ablated the cMT that interacted with the bud neck in 15 DYN1-AID kar9A cells, but exit from mitosis was not observed in the mother cell compartment within the time that we monitored cells post severing (69 minutes; Figure 4A). We also assessed exit from mitosis following
laser ablation in these cells using a marker for cytokinesis. None of the 10 cells with mispositioned spindles in which we ablated the bud neck-interacting cMT exited mitosis as judged by loss of septin Cdc3 from the bud neck (Figure 4A and 4C). To ensure that exposure of cells to the laser pulse did not cause cell cycle arrest, we ablated the cytoplasm of cells with correctly positioned spindles. In the 20 cells analyzed in this manner, exit from mitosis occurred within 2-24 minutes following laser ablation (Figure 4B; 4C, aligned category). Furthermore, when laser ablated cells with mispositioned spindles managed to realign their spindle, they also exited mitosis (Figure 4C, spindle breakdown in the bud category).

Lastly, we ablated cMTs in dyn1Δ cells. Exit from mitosis did not occur in 8/10 cells with mispositioned spindles in which cMTs were ablated for the duration of the analysis (69 minutes). 1/10 cells succeeded in positioning its spindle correctly along the mother – bud axis and promptly exited mitosis thereafter. In 2 cells exit from mitosis followed ablation of cMTs. One cell exited mitosis 8 minutes post ablation and the other cell exited mitosis 69 minutes post ablation. The latter instance seems unlikely to be the consequence of loss of cMTs from the bud neck because cMTs rapidly regrow following ablation (see Figure 4A, 9 minute time point post-ablation). The one cell that exited mitosis shortly after cMT ablation is well in line with the fraction of wild-type cells that exit from mitosis despite harboring a misaligned spindle (Figure 2D). In summary, our results show that ablation of cMTs does not promote exit from mitosis in the vast majority (33/35) of cells analyzed (Figure 4C, mispositioned spindles category). We
conclude that although cMT retraction from the bud neck is a hallmark of cells with misaligned spindles that inappropriately exit mitosis, it is not the cause of exit from mitosis.

**Cdc14 release from the nucleolus precedes loss of cMT – bud neck interactions in cells that exit mitosis despite containing a mispositioned spindle.**

If loss of cMT-bud neck interactions does not induce inappropriate exit from mitosis in cells with misaligned spindles, what does? To begin to address this question we asked whether inappropriate exit from mitosis in cells with mispositioned spindles relied on the same regulatory pathways that promote exit from mitosis in cells with correctly positioned spindles.

Cdc14 is the key trigger of exit from mitosis (reviewed in (Stegmeier and Amon, 2004)). Cdc14 release from the nucleolus during anaphase activates the phosphatase to trigger exit from mitosis. We used Cdc14 localization as the criterion to determine whether cMT retraction from the bud neck occurred before or after exit from mitosis. To examine Cdc14 localization we used a Cdc14-tdTomato fusion. This allele is slightly
Figure 5. Cdc14 Release precedes cMT retraction in cells that inappropriately breakdown in the mother cell compartment

A-B) A diploid strain (A37463) homozygous for osTIR1 dyn1-AID kar9 Δ and heterozygous for GFP-Tub1 and Cdc14-tdTomato was grown to midlog in Synthetic Complete medium. Cycling cells were imaged on a flow cell in Synthetic Complete medium supplemented with 100μM IAA. 

A) Representative images are shown for Cdc14-tdTomato release and GFP-Tub1 cMT retraction. B) The coefficient of variation (the standard deviation divided into the mean) was measured for Cdc14 pixel intensity for the cell pictured in the Fig 4A montage. Time (in minutes) is displayed on the X-axis and the zero time point reflects anaphase onset.
hypermorphic (it causes inappropriate exit from mitosis in a small fraction of cells with mispositioned spindles after a substantial anaphase delay, data not shown) but nevertheless accurately reflects the changes in Cdc14 localization during the cell cycle. Live cell imaging showed that Cdc14 release from the nucleolus preceded both, cMT retraction from the bud neck and mitotic spindle breakdown in cells that exited mitosis despite harboring a misaligned spindle. Visual quantification revealed that in $77.0 \pm 3.7\%$ of cells ($n \geq 37$ cells per replicate), Cdc14 release occurred approximately 5 minutes prior to loss of cMT–bud neck interactions and approximately 5-15 minutes prior to mitotic spindle breakdown (Figure 5A). Of these cells, $72.7 \pm 3.5\%$ released Cdc14 from the nucleolus during anaphase (Figure 5A). However in a small fraction of these cells, $4.3 \pm 4.1\%$, Cdc14 appeared to be fully released already at the metaphase to anaphase transition (Figure 6A). To more precisely analyze when Cdc14 was released from the nucleolus relative to cMT retraction, we calculated the coefficient of variation (standard deviation/mean) of Cdc14-tdTomato pixel intensity in the whole cell over time (Lu and Cross, 2010). As Cdc14 is released from the nucleolus and spreads throughout the nucleus and later the cytoplasm, the standard deviation of pixel intensities will decrease as cells progress through anaphase. In this analysis it was evident that the coefficient of variation of Cdc14-tdTomato pixel intensity decreased before cMT retraction from the bud neck.
Figure 6: Analysis Full Cdc14 Release at the Metaphase to Anaphase Transition

A-B) The diploid strain (A37463) homozygous for osTIR1 dyn1-AID kar9 Δ and heterozygous for GFP-Tub1 and Cdc14-tdTomato was grown to midlog in Synthetic Complete medium. Cycling cells were imaged as in Figure 4. A) Representative images are shown for Cdc14-tdTomato release and GFP-Tub1 cMT retraction. B) The coefficient of variation (the standard deviation divided into the mean) was measured for Cdc14 intensity for the cell pictured in Supplemental Figure 2A. Time (in minutes) is displayed on the X-axis and the zero time point reflects anaphase onset.

Not all cells showed release of Cdc14 from the nucleolus prior to cMT retraction from the bud neck. In 10.9 ± 4.8% of cells Cdc14 release from the nucleolus occurred concomitantly with cMT retraction. In the remaining 12.1 ± 1.3% of cells that did not release Cdc14 prior to cMT retraction, Cdc14 release from the nucleolus was not detected prior to anaphase spindle breakdown. This was most likely because the fraction of Cdc14 that was released
from the nucleolus was too small to detect by imaging. Importantly we never observed that Cdc14 release followed cMT retraction from the bud neck. Consistent with the idea that Cdc14 triggers inappropriate exit from mitosis in cells with mispositioned spindles we find that depletion of Cdc14 suppressed exit from mitosis observed in 4% of wild type cells with mispositioned spindles (Figure 7A). We conclude that inappropriate mitotic exit in cells with mispositioned spindles is not due to loss of cMT–bud neck interactions. Rather, cMT retraction is a consequence of Cdc14 release from the nucleolus in these cells. We further propose that the reason why loss of cMT–bud neck interactions always precedes mitotic spindle breakdown in cells with mispositioned spindles that exit from mitosis is because disassembly of a single cMT occurs more quickly than disassembly of the mitotic spindle following Cdc14 activation.

**The FEAR network is required for Cdc14 release from the nucleolus in cells that exit from mitosis despite a mispositioned spindle.**

Two signaling pathways control Cdc14 release from the nucleolus. The FEAR network promotes the transient release of the phosphatase from the nucleolus during early anaphase. The MEN is needed for the sustained release of the phosphatase during later stages of anaphase (Stegmeier and Amon, 2004). Our data showing that Cdc14 is inappropriately released in some cells with mispositioned spindles led us to investigate which pathway regulating Cdc14
Figure 7

A. Euploid vs. Multinucleate

B. Euploid vs. Multinucleate

C. Euploid vs. Multinucleate

D. cM in budneck: cM absent from SPB entry into budneck

E. Cdc14 Released vs. Cdc14 Sequestered
Figure 7. Inhibition of Cdc14, the MEN and the FEAR Network Suppresses Spindle Breakdown in the mother cell compartment

A) dyn1-AID kar9\(\Delta\) (A35707) and dyn1-AID kar9\(\Delta\) cdc14-1-AID (A37895) cells harboring GFP-tagged alpha tubulin were grown as described in Fig 1A. Cells were monitored by live cell microscopy and scored for inappropriate spindle breakdown in the mother cell compartment. \(n=100\) for dyn1-AID kar9\(\Delta\) and \(225\) for dyn1-AID kar9\(\Delta\) cdc14-1-AID.

B) dyn1-AID kar9\(\Delta\) (A35707), dyn1-AID kar9\(\Delta\) cdc15-AS1 (A36264) expressing GFP-tagged alpha tubulin, were analyzed as in 5A but with the addition of 20uM NAPP1 to the flow cell medium. \(n=100\) for each strain.

C) dyn1-AID kar9\(\Delta\) (A35707), dyn1-AID kar9\(\Delta\) spo12\(\Delta\) (A35700), dyn1-AID kar9\(\Delta\) sk19A (A36028) expressing GFP-tagged alpha tubulin, were analyzed as in 5A. \(n \geq 274\) for each strain.

D) cMT analysis was performed on dyn1-AID kar9\(\Delta\) spo12\(\Delta\) (A35700) as described in Fig 2. Each row shows the color-coded fate of one cell for the given time period, as well whether it had a cMT in contact with the bud neck. cMT analysis was performed by assessing if a cMT was present or absent from the bud neck. Cells with a cMT end that was in the bud neck or in the bud was categorized as “cMT in bud neck” (grey boxes) Cells lacking any cMT in the bud neck are described as “cMT absent from bud neck” (black boxes). Movement of one spindle pole into the bud is “spindle pole movement into bud” (blue boxes). Note: Cell #25 transiently moves a spindle pole into the bud cell compartment but does not exit mitosis. This is most likely due the inefficient activation of the MEN in cells lacking the FEAR network.

E) Wild type (A37753) or spo12\(\Delta\) (A37610) diploid strains homozygous for osTIR1 dyn1-AID kar9\(\Delta\) and heterozygous for GFP-Tub1 and Cdc14-tdTomato were grown to mid log in synthetic complete medium. Cells with mispositioned spindles that had two distinct nucleoli were scored based on whether Cdc14-tdTomato was released from the nucleolus in late anaphase. Cells that did not exit mitosis in the mother cell compartment were monitored for 60 minutes. \(n \geq 22\) cells was responsible for promoting inappropriate Cdc14 release in these cells. Not surprisingly, inhibition of the MEN, a pathway which is essential for exit from mitosis in cells with correctly positioned spindles, also suppressed the inappropriate mitotic exit that is observed in the 11% of cells that exit from mitosis.
when their spindle is mispositioned (Fig. 7B). We were, however, surprised to find that inactivation of the FEAR network, either by deleting SPO12 or SLK19 also prevented the inappropriate exit from mitosis in cells with mispositioned spindles (Fig. 7C). Consistent with the idea that cMT–bud neck interactions are not regulating mitotic exit, we found that deleting SPO12 largely did not affect them (compare Figures 3 and 7D) despite completely suppressing inappropriate mitotic exit in cells with mispositioned spindles. Finally, in cells with mispositioned spindles, we found that the late anaphase release of Cdc4 was prevented in cells lacking SPO12 (Fig. 7E).

Together, our data lead to the following two conclusions. First, cMT–bud neck interactions are not responsible for preventing Cdc4 activation and exit from mitosis in response to mispositioned spindles. Instead, activation of Cdc4 causes cMT retraction from the bud neck and exit from mitosis in cells with mispositioned spindles that inappropriately exit from mitosis. Second, Cdc4 activation in the cells that exit from mitosis despite harboring a mispositioned spindle depends on the FEAR network. This observation raises the interesting possibility that it is high FEAR network activity in some cells that causes the leakiness of the anaphase arrest triggered by spindle misposition.

**Spindle elongation into the bud signals correct spindle position and triggers exit from mitosis.**

A central tenet of the cMT checkpoint model is the prediction that as long as a spindle is mispositioned, mitotic exit is inhibited. The “zone model” or any
Figure 8

A) Zone Model: Activating signal from aligned spindle → exit
Inhibitory signal from misaligned spindle → arrest

B) Checkpoint Model: 

C) Time in Anaphase (min)

D) Class I

E) Class II

F) Class II delayed

G) DIC GFP-Tub1 and Cdc3-mCherry
Figure 8. Analysis of Exit from Mitosis in **prm3Δ** heterokaryons:

**A)** Cartoon depicting the predictions for heterokaryon cell cycle states based on the zone model and cMT-checkpoint model. In the cartoon of the zone model, Lte1 is shown in green, Tem1 in gold and Kin4 in red.

**B)** Cartoon of the **prm3Δ** heterokaryon system showing the two classes of heterokaryons obtained.

**C-G)** Heterokaryons were obtained by mating cells lacking **PRM3** (see Materials and Methods for details). Briefly, G1 cells isolated by centrifugal, elutriation were mated at 30° for 2 hours and then loaded onto a Y04D CellASIC flow cell for imaging. Synthetic complete pH 6.0 medium was used in the flow cell during imaging and was supplemented with 100uM IAA to induce spindle mispositioning. **C)** Montages of binucleate zygotes created by mating cells lacking **PRM3**. Binucelate diploids are homozygous for **prm3Δ** and **osTIR1** and heterozygous for **DYN-AID, kar9Δ**, mCherry-labeled Cdc3 and GFP-labeled α-tubulin (A37892 x A35570). The DIC channel was adjusted to maximize contrast. **D-E)** Analysis of anaphase kinetics of cells described in Figure 6B. Class 1: n= 16. Class II: n= 21 **F)** Binucelate diploids that were homozygous for **prm3Δ, osTIR1, DYN-AID, kar9Δ** and heterozygous for mCherry-labeled Cdc3 and GFP-labeled α-tubulin (A35570 x A35571) were analyzed for anaphase duration. Black diamonds indicated permanently arrested cells (permanently arrested ≥ 320 minutes) n= 52 cells. **G)** Montage of cells from Figure 6F. The DIC channel was adjusted to maximize contrast.

other model that posits a mitotic exit-activating signal in the bud predicts the opposite. As long as a spindle is correctly positioned along the mother-bud axis exit from mitosis will occur, even if the cell also harbors a spindle that is mispositioned (Figure 8A).

To determine whether a MEN inhibitory signal caused by a misaligned spindle prevents MEN activation and hence exit from mitosis or whether a correctly aligned spindle activates the MEN and hence triggers mitotic exit, we
generated cells with two nuclei (henceforth, heterokaryons). When these cells undergo anaphase two main classes of cells are observed (Figure 8B, C):

Class I cells: both spindles correctly align along the mother (Figure 8C, top panel).
Class II cells: one spindle aligns whereas the other one is misaligned (Figure 8C, bottom panel).

We generated heterokaryons using two different methods. In the first method, we generated cells with two nuclei by mating two cells that lacked the nuclear fusion gene PRM3 (Figure 8B) (Heiman and Walter, 2000; Shen et al., 2009). In cells in which both spindles were correctly aligned along the mother – bud axis (Class I cells) both spindles entered the bud in quick sequence during anaphase and exit from mitosis (as judged by Cdc3 loss from the bud neck) occurred promptly thereafter (Figure 8D). Class II cells also exited mitosis even though only one spindle entered the bud during anaphase. Average anaphase duration for these cells was 15.7 ± 2.3 minutes, which was comparable to the average anaphase duration in cells in which both spindles were correctly positioned (16.6 ± 3.7 minutes; compare Figure 8D, E). Furthermore the time of entry of one spindle into the bud until exit from mitosis was the same in cells in which both spindles aligned correctly and cells in which one spindle was correctly aligned and the other was misaligned (compare Figure 8D, E column “bud entry to exit from mitosis”). Importantly, we were also able to obtain many
Figure 9: Analysis of Exit from Mitosis in *prm3Δ* heterokaryons:
Montage of binucleate zygotes created by mating homozygous for *prm3Δ*, *osTIR1*, *DYN-AID*, *kar9Δ* and heterozygous for mCherry-labeled Cdc3 and GFP-labeled alpha tubulin (A35570 x A35571). Binucleate diploids express mCherry-labeled Cdc3 and GFP-labeled alpha tubulin. The montage depicts a zygote with one aligned anaphase spindle and a second spindle in metaphase in the mother compartment. Both spindles exit mitosis at the same time with the metaphase spindle never going through anaphase. The DIC channel was adjusted to maximize contrast.

heterokaryons where both anaphase spindles were mispositioned for prolonged periods of time (henceforth “Class II delayed”). These cells were severely delayed in anaphase (Figure 8F, G). In the vast majority of these cells it was only once one spindle moved from the mother compartment into the bud that cells promptly exited mitosis (44/50 cells). Of the remaining cells, three (6%) permanently arrested in anaphase with both spindles in the mother cell compartment, two inappropriately exited mitosis with both spindles in the mother cell (4%) and one cell arrested in anaphase even though one pole had entered the bud (2%). Strikingly, we also noticed that in a very small fraction of cells (2/50), exit from mitosis occurred even when the mispositioned spindle in the
mother cell had not yet initiated anaphase (Figure 9). We conclude that movement of one spindle pole into the bud triggers exit from mitosis.

The second method to generate heterokaryons took advantage of the fact that cells undergoing meiosis can be returned to vegetative growth (Simchen, 2009). Diploid yeast cells sporulate in response to nutrient deprivation. Budding is suppressed and cells progress through premeiotic S-phase. When glucose-containing medium is supplied to cells that lack the CDK inhibitory kinase Swe1 following premeiotic S phase (in pachytene of meiotic prophase I) these cells will return to vegetative growth and undergo mitosis producing cells with two nuclei (Fig. 10A) (Tsuchiya and Lacefield, 2013). We analyzed the mitotic cell cycle after the formation of a binucleate cell. In this method of generating heterokaryons, a third class of cells was observed: one spindle is pulled into the bud generating cells with a misaligned spindle in the mother and bud cell compartments (Figure 10A). As in the heterokaryons generated by mating, cells in which both spindles were correctly aligned along the mother–bud axis (Class I cells) exited from mitosis as judged by the average anaphase spindle breakdown 15.8 ± 5.5 minutes after the two spindles had entered the bud (Figure 10B).

Class II cells also exited mitosis even though only one spindle entered the bud during anaphase. Anaphase duration was similar for both spindles and both spindles broke down soon after one spindle entered the bud (Figure 10C).
Figure 10

A

[Diagram showing meiotic cell cycle without budding and stages of meiotic entry and progression through cell cycle.

Mata/a, swe1Δ/ swe1Δ ZIP1-GFP]

Class I

Class II

Class III

B

Time in Anaphase (min)

Class I

C

Time in Anaphase (min)

Class II

D

Time in Anaphase (min)

Class III
Figure 10. Analysis of Exit from Mitosis in swe1Δ heterokaryons:

A) Cartoon of swe1Δ return to growth heterokaryon system and depictions of cell type classes that were analyzed. Briefly, diploid cells lacking SWE1, also harboring the prophase marker Zip1, tagged with GFP as well as GFP-tagged Tub1 (LY1043), were induced to enter meiotic prophase through nutrient starvation. Upon entry into meiotic prophase (as judged by the presence of Zip1-GFP positive cells), cells were returned to glucose-containing complete medium in microfluidic chambers and thus induced to grow mitotically. These cells were monitored by live cell microscopy.

B) Anaphase kinetics of Class I cells (depicted in Figure 7A). Anaphase duration is classified as the time the cell spends with a spindle >2μm to spindle breakdown. Bud entry to exit from mitosis is defined as the time that at least one anaphase spindle pole is in the bud to exit from mitosis

C) Anaphase kinetics of Class II cells (depicted in Figure 7A). Anaphase duration and bud entry to exit from mitosis are defined as in 7B.

D) Anaphase kinetics of Class III cells (depicted in Figure 7A). Anaphase duration and bud entry to exit from mitosis are defined as in 7B. n=50 cells for each class.

Spindle disassembly of the nucleus in the mother cell also occurred concomitantly with spindle disassembly in the nucleus in the bud of class III cells (Figure 10D). This result indicates that exit from mitosis is not triggered by a correctly positioned spindle but rather a spindle that is in the bud, as exit from mitosis occurred in class III cells even though the spindle in the bud was mispositioned. It is important to note however, that a spindle simply being in the bud is not sufficient to bring about exit from mitosis. Exit from mitosis only occurred after the bud localized spindle had undergone anaphase. This observation is consistent with previous findings showing that anaphase entry is
required for MEN activation and exit from mitosis (Rock and Amon, 2011) (data not shown).

In summary, our heterokaryon analyses do not support the hypothesis that a dominant inhibitory signal originating from a mispositioned spindle prevents MEN activation and exit from mitosis. Instead, our data show that movement of the spindle into the bud as occurs during a cell cycle with a correctly positioned spindle activates the MEN and exit from mitosis.

Discussion

In budding yeast, the site of cytokinesis is established long before cells even undergo mitosis. Division by budding also means that the connection between mother cell and bud is small and the nucleus and other organelles must be squeezed through the bud neck to be accurately partitioned. Therefore division by budding not only requires sophisticated mechanisms to position the nucleus along the mother-bud axis but it also requires mechanisms to prevent cells from exiting mitosis and undergoing cytokinesis until the nuclei are partitioned between the mother and bud cell compartments. In 1995, Yeh et al. demonstrated the existence of such a mechanism. They showed that cells with mispositioned spindles arrest in late anaphase and fail to exit from mitosis. They termed this regulatory mechanism the spindle position checkpoint (SPoC). Subsequent studies showed that exit from mitosis is prevented in cells with misaligned spindles through the inhibition of the Mitotic Exit Network, the GTPase signaling cascade that promotes anaphase spindle disassembly, chromosome...
decondensation and cytokinesis by activating Cdc14 (D’Aquino et al., 2005; Pereira and Schiebel, 2005).

Two models have been proposed to explain how MEN activity is inhibited in response to spindle misposition: the cMT checkpoint model and the zone model. The former posits that a MEN inhibitory activity is generated by a misaligned spindle, the latter that a MEN activating activity is produced by a correctly aligned spindle (Adames et al., 2001; Bardin et al., 2000; Chan and Amon, 2010; Moore et al., 2009). In this paper we took advantage of a new inducible system to study mispositioned spindles to distinguish between these two models. Our data support the conclusion that a correctly aligned spindle promotes exit from mitosis. Our data, together with previous studies, further indicate that it is the movement of the MEN component-carrying SPB into the bud that signals exit from mitosis.

cMT – bud neck interactions do not prevent mitotic exit

Cytoplasmic microtubules continuously interact with the bud neck during spindle positioning prior to anaphase. However, once the nucleus traverses the bud neck during anaphase these interactions are lost. In cells that misposition their spindles and undergo anaphase in the mother cell, cMT–bud neck interactions persist. The proposal that it is these cMT–bud neck interactions that prevent exit from mitosis in cells with misaligned spindles stems from the analysis of cells that exit mitosis despite harboring a misaligned spindle (Adames
et al., 2001; D’Aquino et al., 2005; Pereira and Schiebel, 2005). Previous work by Adames and Cooper demonstrated that cMT retraction from the bud neck precedes mitotic spindle breakdown in cells that exit mitosis with a mispositioned spindle (Adames et al., 2001). This correlation led them to propose that cMT–bud neck interactions emit an inhibitory signal that prevents exit from mitosis. This hypothesis was supported by the observations that 1) elimination of cMT–bud neck interactions by microtubule ablation or 2) loss of cMTs brought about by the cold sensitive β-tubulin allele (tub2-401) increased the frequency with which cells with mispositioned spindles inappropriately exit from mitosis (Adames et al., 2001; Moore et al., 2009).

We also observed this striking correlation between cMT retraction from the bud neck and mitotic spindle breakdown, but several additional analyses demonstrate that this correlation does not indicate causality. First, retraction of cMTs occurs frequently and often for extended periods of time also in cells with mispositioned spindles that do not inappropriately exit mitosis in the mother cell compartment. Second, in our cMT ablation studies we did not observe exit from mitosis following the loss of cMT–bud neck interactions. We cannot explain why our ablation results differ from those of Moore et al. but we note that mitotic exit that followed ablation of cMTs took a long time (approximately 18 minutes) in this previous study and regrowth of cMTs into the bud neck was also observed during the time it took until cells exited mitosis.
The most conclusive demonstration that loss of cMT-bud neck interactions does not trigger exit from mitosis was the analysis of Cdc14 localization. It clearly showed that cMT retraction from the bud neck did not precede Cdc14 release from the nucleolus but was a consequence thereof. We further propose that the reason why cMTs invariably disassemble prior to the mitotic spindle in such cells is that disassembly of a single cMT upon mitotic CDK inactivation takes less time than the disassembly of a mitotic spindle which is composed of many microtubules. An inherently higher instability of cMTs compared to spindle microtubules could of course also explain this difference in disassembly timing. Together, our studies disfavor a mitotic exit inhibitory function of cMT–bud neck interactions.

MEN activating and inhibitory zones couple exit from mitosis to spindle position.

MEN signaling takes place at SPBs (Maekawa et al., 2007; Valerio-Santiago and Monje-Casas, 2011; Visintin and Amon, 2001). The GTPase Tem1 and Polo kinase recruit the MEN kinase Cdc15 to SPBs where it is activated by an unknown mechanism (Rock and Amon, 2011). Regulators of the GTPase are strategically placed in the cell. Kin4, the GTPase inhibitor localizes to the mother cell, the Kin4 inhibitor and hence MEN activator Lte1 localizes to the bud (Bardin et al., 2000; D'Aquino et al., 2005; Pereira and Schiebel, 2005). These localization patterns led us to propose that spindle position controls exit from
mitosis through the establishment of a MEN activating compartment in the bud, a MEN inhibitory compartment in the mother cell and a sensor, the MEN component bearing SPB that shuttles between them. When both of the spindle pole bodies are in the MEN inhibitory mother cell compartment, the cell cannot exit from mitosis and arrests in anaphase. It is only once one MEN component-bearing spindle pole body moves into the mitotic exit-activating zone in the bud does the MEN become active and exit from mitosis occurs. It is important to emphasize that the zone model takes into account that not only are there MEN inhibiting factors in the mother cell compartment, but that there are also factors in the bud that promote exit from mitosis. Evidence that cells have both a negative zone in the mother cell compartment and a positive zone in the bud comes from the analysis of cells in which the MEN activating and inhibitory zones were switched. When the Kin4 inhibitor Lte1 is targeted to the mother cell, cells with mispositioned spindles inappropriately exit from mitosis (Bardin et al., 2000; Bertazzi et al., 2011). When Kin4 is targeted to the bud and its inhibitor Lte1 is inactivated, cells with correctly positioned spindles cannot exit from mitosis and arrest in anaphase (Chan and Amon, 2010; Falk et al., 2011). The analysis of heterokaryons presented here also supports the zone model. Irrespective of whether or not a cell harbors a mispositioned spindle, exit from mitosis occurs once one anaphase spindle enters the bud. Our analysis of heterokaryons in which one of the two spindles gets pulled into the bud in its entirety further shows that it is the presence of a spindle pole in bud and not a correctly positioned
spindle that leads to MEN activation. In cells that harbor one nucleus in the bud and one in the mother cell, both spindles are mispositioned, yet exit from mitosis occurs once the spindle in the bud has initiated anaphase. This finding further demonstrates that two signals are necessary for MEN activation in anaphase: (1) a spatial signal – the movement of a MEN bearing SPB into the bud and (2) a temporal signal that indicates that anaphase chromosome segregation has occurred (Bardin et al., 2000; Chan and Amon, 2010; Manzoni et al., 2010; Rock and Amon, 2011). Dissecting the molecular details of how these two signals interact to control Tem1 activity will be a critical next step in understanding how the MEN integrates spatial and temporal cues to control exit from mitosis.

**Why is the anaphase arrest of a mispositioned spindle leaky?**

It has long been known that a small fraction of cells exit from mitosis despite the presence of a mispositioned spindle (D'Aquino et al., 2005; Pereira and Schiebel, 2005). We show here that this event is preceded by the release of Cdc14 from the nucleolus. Our data further indicate that this Cdc14 activation requires FEAR network function because inappropriate mitotic exit in cells with mispositioned spindles is completely prevented when FEAR network component encoding genes are deleted. FEAR network activity is under the control of the regulatory mechanisms governing the metaphase – anaphase transition (Stegmeier et al., 2002). At this cell cycle transition, a checkpoint known as the spindle assembly checkpoint (SAC) inhibits entry into anaphase until all
chromosomes have attached correctly to the mitotic spindle (reviewed in (Musacchio and Salmon, 2007)). Once this has occurred, the SAC inhibition of a protease known as Separase is alleviated and the protease initiates chromosome segregation by cleaving cohesins, the protein complexes that hold sister chromatids together (Nasmyth, 2002). As Separase is also a component of the FEAR network (Stegmeier et al., 2002), SAC activity also governs the release of Cdc14 from the nucleolus during early anaphase.

It will be interesting to determine why there are cell-to-cell differences in Cdc14 release from the nucleolus when spindles are mispositioned. Metaphase duration could be a factor. Difficulties in mitotic spindle formation and correctly attaching chromosomes to the mitotic spindle, may lead to prolonged metaphase delays, during which FEAR network component levels could increase leading to a burst of FEAR network activity once the checkpoint is satisfied and cells enter anaphase. This could also explain why the cell cycle arrest following spindle misposition is especially leaky in the tub2-401 mutant, in which the SAC is activated. It is also possible that mitotic CDK activity, which inhibits FEAR network-mediated Cdc14 release from the nucleolus varies between individual cells. Especially high levels of activity could cause a more sustained FEAR-network-dependent release from the nucleolus.

Irrespective of where this variability originates from, the fact that the cell cycle arrest is not absolute in cells with mispositioned spindles is interesting. It not only indicates that FEAR network activity exhibits cell to cell variability with
biologically meaningful consequences, it also raises the question of whether the leakiness of the arrest serves a biological function. Is it possible that under conditions where spindle misposition occurs at higher frequency (i.e. in the cold) that a not universally permanent cell cycle arrest provides an advantage? Could binucleation and hence polyploidization provide a reservoir of cells with increased adaptability? Further investigation is needed to better understand the molecular basis and importance of these cell-to-cell differences.

**Is the spindle position checkpoint a checkpoint?**

Classically, checkpoint pathways are defined as surveillance mechanisms that monitor the proceedings of a (cell cycle) event and prevent the next one from occurring until the preceding event is completed or defects therein have been corrected (Hartwell and Weinert, 1989). If a mispositioned spindle were to emit such a diffusible cell cycle inhibitory checkpoint signal it should act in a dominant fashion (Rao and Johnson, 1970), preventing exit from mitosis even when the cell also contains a correctly positioned spindle. We tested this prediction by examining the fate of binucleate cells in which one spindle was mispositioned in the mother and the other spindle properly positioned along the mother-bud axis. We observed that delivery of one SPB into the bud compartment is sufficient to trigger exit from mitosis regardless of the position of the other spindle. These data argue against a checkpoint model to explain the anaphase arrest in
response to spindle misposition. Instead they support a model where the delivery of a MEN bearing SPB into the bud activates the MEN.

Checkpoint regulation has been described for other asymmetric divisions (Cheng et al., 2008; O'Connell and Wang, 2000). In Drosophila male germline stem cells with mispositioned centrosomes, cell cycle progression is delayed until the centrosomes properly align with respect to the mother-daughter axis of division (Cheng et al., 2008). Additionally, the AMP-related kinase family member Par-1 (of which Kin4 is also a member) has been shown to be important in delaying cell cycle progression in response to mispositioned spindles in male GSCs (Pereira and Yamashita, 2011; Yuan et al., 2012). Given these recent findings it is intriguing to speculate that a mechanism similar to the one described for spatial control of the MEN by nuclear position, rather than a checkpoint mechanism, also operates in these stem cells. The analysis of cells with multiple centrosomes analogous to what has been described here could address this question.

Experimental Procedures

Strains and Plasmids

Yeast Strains are derivatives of W303 (A2587) and are described in Supplemental Table 1. GFP-Tub1 is described in (Straight et al., 1997). The pCTS1-2xmCherry-SV40NLS plasmid was a gift from Drew Endy’s lab. The Ylp211-CDC3-mCherry plasmid was a gift from the Erfei Bi’s lab and is described
in (Fang et al., 2010). *pFA6a-link-tdTomato-SpHis5* was a gift from Kurt Thorn (Addgene plasmid # 44640). Leon Chan, Thomas Eng, and Vinny Guacci constructed the *pGPD1-OsTIR1-LEU2* and *pFA6-3V5-IAA17-KanMx6* plasmids and these were received as gifts from the D. Koshland and K. Weis labs. All gene deletions and C-terminal tags were constructed by the standard PCR-based procedures (Longtine et al., 1998; Sheff and Thorn, 2004).

**Statistics**

All reported statistical error calculations are standard deviations.

**Live Cell Microscopy**

Growth conditions for live cell imaging are described in their respective figure legends with the exception of Figure 6 (see *prm3Δ* Heterokaryon protocol below). All imaging was done at 25°C. Imaging for the return to growth experiment (Figure 7) was performed with a Nikon Ti-E inverted microscope equipped with a 60X Plan APO 1.4NA objective, a GFP filter, and a CoolSNAPHQ2 CCD camera (Photometrics), controlled by Nikon Elements software. Z stacks of four to eight sections were acquired in 10 min intervals for 12 hr with a 12.5% ND filter and exposure times of 30-500ms.

Imaging performed for Fig 5B was done using Nunc™ Lab-Tek™ II Chambered Coverglass incubation chambers, on a DeltaVision Elite microscope platform (Applied Precision). This microscope platform consisted of an InsightSSI™ solid state light source, an UltimateFocus hardware autofocus system and a model IX-
71, Olympus microscope controlled by SoftWoRx software (Applied Precision).

Time-lapse images were acquired with a 60X Plan APO 1.42NA objective and a CoolSNAP HQ2 camera (Photometrics).

The rest of the live cell imaging, with the exception of the microtubule ablation experiment (see below), was done on a Zeiss Observer.Z1 inverted microscope with a Heliophor™ Pumped Phosphor Light Engine (89 North). Imaging data was collected using a Hamamatsu ORCA-ER C4742-80 CCD camera run by Molecular Devices Metamorph Software. Cells were imaged in a CellASIC Y04C or Y04D flow cell chambers.

**Cytoplasmic Microtubule Severing**

Microtubules were cut using a Coherent OBIS 405LX laser. Two pre-ablation images were acquired to confirm that a cytoplasmic microtubule was present in the bud neck. The microtubule was cut with one 250ms, 405nm laser pulse and severing was confirmed by acquiring a Z-series of nine images spaced at 0.6 um. Microtubule contact with the bud neck was following at 1 min intervals for 8 mins post ablation. Images of the ablated cells were acquired for up to one hour at 15 ± 1 min time intervals to determine cell cycle status.

**Generation of Heterokaryons**

See figure legend for culture methods to generate the *swe1Δ* heterokaryons. To generate the *prm3Δ* heterokaryons, both MATa and MATα cells were grown overnight to mid-log in YEPD at room temperature. Cells were centrifuged and resuspended in YEP and the briefly sonicated using a Branson 250 Sonifier.
These cells were loaded into a Beckman elutriation rotor JE 5.0 (Beckman Coulter, Brea, CA) which was cooled to 4°C and equilibrated with YEP at 2400 rpm. Cells were loaded into the system at a speed to 20mL/min and then equilibrated in the elutriator for 20-30 minutes at a pump speed of ~10mL/min. Cells in G1 were collected at a pump speed of ~23mL/min. Harvested G1 cells were concentrated using a Konte filtration system and then resuspended to a final OD of 5.0 in YEPD. 200 uL of these cells were then plated on a YEPD agar plate and incubated at 30°C for ~2 hours. The resultant population that contained zygotes was washed off of the agar plate using YEPD medium and loaded into a CellASIC Y04D flow chamber for time-lapse analysis.
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LY1043  MATa/alpha, swe1::kanMX/swe1::kanMX, PTUB1-GFP-TUB1-LEU2/TUB1, ZIP1-GFP(700)/ZIP1, Gal4-ER:URA3/Gal4-ER:URA3
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Chapter IV: Discussion
Key Findings in this Thesis:

One function of Lte1 is to inhibit Kin4 in the bud cell compartment

Regulation of the MEN by spindle position has been described in a model known as the zone model. In this model, there is an inhibitory zone in the mother cell compartment where factors such as Kin4 are present, a promoting zone in the bud where positive regulators such as Lte1 localize and the GTPase sensor Tem1 that moves between them. If the spindle is mispositioned in the mother cell compartment, Kin4, which functions at SPBs in the mother compartment prevents Tem1 accumulation on SPBs in the mother cell. Once one pole moves out of the mother cell compartment and into the bud cell compartment where positive regulators such as Lte1 are present, Tem1 becomes enriched on the daughter SPB and the MEN becomes active. While the molecular functions of the Kin4 kinase and the GTPase Tem1 are clear, Lte1 function in promoting exit from mitosis has remained unclear. Here I find that in the absence of Lte1, the small amount of Kin4 that is present in the bud localizes to the daughter SPB. Using alleles of Kin4 that localized symmetrically to both mother and bud cortices I found that Kin4 (Kin4-S508A) was present on the dSPB in a large fraction of cells in the absence of Lte1. Previous data have shown that the symmetric allele of KIN4, KIN4-S508A, is lethal in the absence of LTE1 demonstrating when Kin4 is present on the dSPB that this causes cells to arrest in late anaphase (Chan and Amon, 2010). Consistent with its role in preventing Kin4 from localizing to SPBs in the bud, I show that Kin4 and Lte1 interact. I also map this interaction domain
to the N-terminus of Kin4. Finally, I show that this interaction might be especially important at low temperatures because Kin4 localization to the SPB in the bud was increased in the cold.

**Spatial signals and not cytoplasmic microtubules couple exit from mitosis to spindle position.**

Many alternative models to the zone model have been proposed to explain why budding yeast cells arrest in late anaphase with mispositioned spindles. One such model posits that a checkpoint response is elicited in cells with mispositioned spindles (Adames et al., 2001). It was proposed that this checkpoint signal is generated by the interaction of cytoplasmic microtubules with the budneck. To determine whether cMT contact with the budneck regulates MEN activity, I generated a new system to cause cells to misposition their spindles. Using this system I found that cells with mispositioned spindles which arrest in anaphase often have a cMT in contact with the budneck but that this interaction is not necessary to maintain the anaphase. Consistent with previously published observations, I found that cells that inappropriately exited mitosis in the mother compartment retracted their cMTs from the budneck shortly before spindle disassembly. To more directly test whether cMTs were required to maintain the arrest of cells with mispositioned spindles, I transiently eliminated cMT-budneck interactions by laser severing. Ablation of microtubules that interacted with the budneck did not induce exit from mitosis in the vast majority of ablated cells. Analysis of Cdc14 release showed that in the majority of cells, Cdc14 release preceded cMT retraction from the budneck showing that cMT
retraction cannot be a cause of Cdc14 release. Lastly through the use of cells harboring two nuclei, I found that spatial regulation of the MEN is established by MEN inhibitory signals in the mother cell, MEN activating signals in the bud and the MEN-bearing spindle pole that moves between them.

**Future Directions:**
The understanding of how spindle position regulates exit from mitosis has come a long way since the discovery that cells link spindle position to exit from mitosis (Bardin et al., 2000) (Pereira et al., 2000). Below I discuss open questions pertaining to spatial regulation of exit from mitosis.

**What Regulates Spatial Asymmetry of Lte1 and Kin4?**
Spatial regulation of the MEN is composed of a MEN inhibitory zone in the mother cell compartment, a MEN activating zone in the bud and a GTPase sensor that moves between them. Kin4 is the only known negative regulator of the MEN that primarily localizes to the mother cell compartment. Likewise, Lte1 is the only known positive regulator of the MEN that primarily localizes to the bud. Therefore, in order for spatial regulation of the MEN to function, Kin4 must be asymmetrically distributed in the mother cell compartment and Lte1 must be kept in the bud. If Lte1 is misexpressed in the mother compartment, cells inappropriately exit mitosis if their spindle becomes mispositioned (Bardin et al., 2000; Geymonat et al., 2009). Likewise, if Kin4 is overexpressed and is present in abundance in the bud cell compartment, cells will arrest in late anaphase even when their spindle is correctly aligned (Falk et al., 2011; Bertazzi et al., 2011).
Thus spatial regulation of the MEN is twofold: establishment both a mitotic exit promoting zone in the bud cell compartment and a mitotic exit inhibiting compartment in the mother cell compartment is essential for proper regulation of nuclear inheritance during mitosis.

While there is a multitude of information on which proteins are involved in the regulation of the MEN, how the localization of these components affects their function is less clear. Components of the MEN that govern its spatial regulation are no exception to this trend. For example, localization Lte1 to the bud cell cortex has been shown to be complex, requiring Ras2, the septins and polarity factors such as Kel1, Cdc42 and the Cdc42 effector kinase Cla4. In a model that has yet to be described in molecular detail Lte1, which is present in the bud, is phosphorylated by Cla4 (Seshan et al., 2002; Höfken and Schiebel, 2002). This phosphorylation then thought to promote Lte1 interaction with Ras2. Once bound to Ras2, Lte1 is present at the bud cortex throughout most of the cell cycle and is only released from the cortex by Cdc14 when the cell returns to G1 (Seshan et al., 2002; Jensen et al., 2002). This release results in the distribution of Lte1 throughout the cytoplasm and into both mother and bud compartments.

What remains to be investigated is whether localization of Lte1 to the cortex is required for its function in the MEN, which domains are required for Lte1 to promote exit from mitosis and whether phosphorylation of Lte1 is necessary for this function. To this end, it will be useful to generate an Lte1 chimera that is tethered to the cortex via a Ras2 palmitoylation sequence (Lte1-CAAX).
Assessment of the functionality of Lte1-CAAX will aid in determining whether Lte1 can promote the MEN when it is tethered to the cortex. If Lte1-CAAX can function in promoting exit from mitosis, use of this allele will also be important in determining the role of Lte1 phosphorylation in Lte1 regulation. If the sole purpose of Lte1 phosphorylation is in its localization, then Lte1-CAAX should function to promote MEN even in the absence of Cla4.

Localization of Kin4 to the mother cell compartment is essential for its function in the MEN. Like Lte1, Kin4 is asymmetrically localized throughout cell cycle. It is localized to the mother cell cortex from late S-phase to late anaphase at which point it cortical localization decreases. Kin4 is also asymmetrically localized to the mSPB in late anaphase and this localization is critical for its inhibitory function in mitotic exit. What anchors Kin4 at the cell cortex is not known. Indeed, in very small budded cells, Kin4 is briefly seen localize to the cortex of the new bud in an Lte1-dependent manner (Chan and Amon, 2010; Falk et al., 2011). It is also known that Lte1 can bind Kin4 and so it is not surprising to see both proteins co-localize briefly in the bud. However this interaction is transient because Kin4 localization quickly changes from the bud to the mother cell cortex. What mediates this change in the localization of Kin4 and how Kin4 is anchored in the mother cell compartment is not known. Additionally, whether the pool of Kin4 that localizes to small buds is the same pool of Kin4, which localizes to the mother cortex is not known. Finally, it is also not known whether the transient interaction between Kin4 and Lte1 in these small budded cells is
important for either Kin4 or Lte1 function. To answer these questions, alleles of Kin4 that do not interact with Lte1 and vice versa will need to be generated.

But what anchors Kin4 at the mother cell cortex? Correlative evidence suggests that loss of Kin4 from the cortex perturbs its function (Chan and Amon, 2010). Therefore, screens that identified Kin4 based the fact that its deletion rescues the synthetic lethality of cells lacking both LTE1 and SPO12 should also have identified its anchor but they have not. What this evidence suggests is that the Kin4 anchor is either essential or that there are redundant factors that are sufficient to anchor Kin4. Visual screens to identify the factors that are required to anchor Kin4 at the cortex have not been performed in the past most likely due to the fact that Kin4 is an especially difficult protein to visualize by live cell microscopy. However, overexpression the small GFP-tagged domain of the C-terminus of Kin4 (kin4(655-800)) robustly localizes to the cortex. kin4(655-800) is both necessary and sufficient for localization to the cortex (Chan and Amon, 2010). Therefore to identify potential cortical anchors for Kin4, performing a visual screen of the deletion collection using this kin4(655-800)-GFP might identify such proteins. If the anchor is essential, using a library of cells harboring temperatures sensitive alleles of essential genes could prove useful (Ben-Aroya et al., 2008).

If there are multiple factors that interact with Kin4 to keep it anchored at the cortex, the approaches listed above will not identify the cortical anchor for Kin4. If this proves to be the case, affinity purification of kin4(655-800) followed
by analysis by mass spectrometry to identify interacting partners might be more fruitful.

**How is Kin4 SPB localization restricted to anaphase?**

As described in Chapter 1, Kin4 SPB localization is restricted to mid-anaphase. What prevents Kin4 from localizing to SPBs at other cell cycle stages? One possibility is that it is phosphorylation of Kin4 that regulates its localization to SPBs (D'Aquino et. al., 2005; Maekawa et. al., 2007; Chan and Amon 2009) Kin4 is phosphorylated throughout the cell cycle and is transiently dephosphorylated in early anaphase. This dephosphorylation depends on the phosphatase complex PP2A-Rts1 and has been shown to affect Kin4 localization to SPBs (Chan and Amon, 2009). In the absence of PP2A-Rts1, Kin4 does not localize to SPBs. Could dephosphorylation of Kin4 by PP2A-Rts1 allow Kin4 to bind SPBs? To answer this question, identifying the residues on Kin4 that are targets of PP2A-Rts1 dephosphorylation and then generating a Kin4 phosphomutant will be informative. This tactic has proved difficult because Kin4 is an insoluble protein and identification of phosphorylation sites is technically challenging (Leon Chan personal communication). While a phosphomimetic mutant could answer many questions concerning Kin4 SPB localization, an alternative approach to address this question would be to identify the PP2A-Rts1 counteracting kinase. If phosphorylation of Kin4 inhibits its SPB localization, then Kin4 should localize to SPBs in cell cycle stages outside of anaphase in cells lacking such a kinase.
PP2A-Rts1 is required for Kin4 localization to SPBs but it is also required for Kin4 localization to the cell cortex. Because of this, it is at present unknown whether Kin4 localization to the cell cortex is a prerequisite for its localization to SPBs. If this is the case, then what keeps Kin4 off the SPB in earlier stages of the cell cycle (despite Kin4 being on the mother cortex) is still unknown. Because Kin4 localization to SPBs does not require the actin or microtubule cytoskeleton (Pereira and Schiebel, 2005) it is possible that Kin4 localization to SPB either depends on 1) the localization of another SPB component to the anaphase SPB or on 2) the modification of an SPB component whose early anaphase modification allows the recruitment of Kin4 to the SPB. Factors, which have been shown to be required for Kin4 localization to the SPB, include Nud1, Cdc5 and Spc72. Both Spc72 and Nud1 are phosphorylated in a cell cycle dependent manner at similar times in mitosis (Pereira and Schiebel, 2005; Maekawa et al., 2007). The timing of this phosphorylation coincides with Kin4 localization to SPBs. Therefore it will be interesting to test whether phosphorylation of either of these components is required for Kin4 localization to SPBs.

**Regulation of the GTPase Tem1**

The small GTPase Tem1 is at the core of both spatial and temporal signaling of the mitotic exit network. This GTPase is most similar to Ras-like GTPases and is essential for MEN function. Tem1 activity is regulated by a bipartite GAP (Bub2/Bfa1) as well as Lte1, a putative GEF. Biochemical evidence suggests that Bub1/Bfa1 acts as a GAP: when both Bub2 and Bfa1 are
expressed together, they have been shown to increase GTPase activity of Tem1. Likewise, genetic and cell biological evidence shows that elimination of \textit{BUB2} or \textit{BFA1} hyperactivates Tem1 and that Bub2 and Bfa1 are reciprocally required for each other's localization to SPBs. While these data appear consistent with Bub2/Bfa1 acting as a GAP for Tem1, in vitro analysis of Bfa1 alone on Tem1 GTPase activity adds additional complexity to this model.

Overexpression of Bfa1 inhibits exit from mitosis. Given the role of Bfa1 in regulating Tem1 GTPase activity, it is logical to assume that Bfa1 alone promotes the GTPase activity of Tem1. However, this was not shown to be the case. \textit{In vitro} experiments demonstrated that Bfa1 alone (without Bub2) functions as a guanosine nucleotide dissociation inhibitor (GDI) and prevents the GTPase activity of Tem1 (Geymonat et. al 2002). Thus, the role of Bfa1 and Bub2 in regulating Tem1 is still unclear. Due to the fact that whether Bub2 and Bfa1 are in a complex or not appears to affect their function on Tem1, it will be important to determine where in the cell Bub2 and Bfa1 actually are associated. Because of the mutual dependence of Bub2 and Bfa1 for each other's localization to SPBs it is tempting to speculate that these proteins only affect Tem1 as a complex. Nevertheless, it will be important to determine where and when Bub2 and Bfa1 act as a complex. To this end, it would useful to generate a Bub2-Bfa1 FRET pair to help resolve these issues.

Genetic evidence has shown that the putative GEF, Lte1, also regulates Tem1 function. Lte1 has two GEF domains but has not been shown to harbor \textit{in
vitro GEF activity towards Tem1. What is known is that one function for Lte1 (discussed in Chapter II) is to bind and inhibit Kin4. However whether this is the sole function of Lte1 remains unclear. Truncation mutant analysis of the GEF domains revealed the cells, which harbored only the GEF domain still partially retained Lte1 function (Geymonat et. al., 2009). Despite this fact, whether these domains are responsible for binding Kin4 or indeed functioned to promote Tem1 as an exchange factor is still unclear. More biochemical work, namely purification of active Lte1, will need to be done in order to test whether it functions as a GEF. An additional role for Lte1 in promoting exit from mitosis is discussed at the end of this chapter.

One longstanding question is whether active Tem1 signals in its GTP- or GDP-bound state. Genetic evidence suggests that GTP-bound Tem1 is the active form because both elimination of either of its GAP components hyperactivates the MEN. Recently, in support of these findings, an allele of Tem1 (tem1-1) that has been shown to be GTP “locked” was also shown to hyperactive the MEN. Rigorous characterization of tem1-1 has yet to be done and determining where and when GTP-bound Tem1 acts has also yet to be determined.

**Is Kin4 Inhibition the sole function for Lte1?**

One function of Lte1 has been shown to be inhibition of Kin4. (Chapter II). However whether this is the sole function of Lte1 in promoting exit from mitosis is unknown. Historically, Lte1 has been proposed to act as a GEF due to its positive role upstream of the GTPase Tem1. Assessment of Lte1 function has been
exceptionally difficult due to the fact that it has not been shown to harbor GEF activity in vitro. (Geymonat et al., 2009) Lte1 localizes at the cell cortex and its cortical localization is correlated with its activity. Several cortically localized proteins and membrane-anchored components have been shown to be required for Lte1’s cortex localization. Therefore, when Lte1 was isolated from yeast to determine its GEF activity, it was unclear whether the purified Lte1 was even biochemically active (Seshan and Amon, 2005; Höfken and Schiebel, 2002).

Other assays used to measure Lte1 activity are fairly crude, resorting to growth at low temperatures (when Tem1 GDP exchange is possibly decreased) or using the synthetic lethality of SPO12 and LTE1 to judge Lte1 activity (Geymonat et al., 2009; Yoshida et al., 2003). These methods of measuring Lte1 activity are indirect and can only report gross defects in Lte1 function. Therefore, it remains unknown whether Lte1 acts as a GEF or has other roles in promoting exit from mitosis outside of inhibiting Kin4.

Attempts have been made to determine which domains of Lte1 are important for its function in the MEN. While the majority of these truncation mutants appear to be complete loss of function alleles (due to loss of localization and also possibly phosphorylation) there is one particularly interesting truncation mutant whose analysis might prove informative. This truncation mutant, lte1-ΔEcoRI, appears to localize normally, and can complement some functions of wild type Lte1 (Geymonat et al., 2009). Interestingly, lte1-ΔEcoRI consists primarily of just the two N- and C-terminal GEF domains. To determine if Lte1...
has a function as an exchange factor for Tem1, it will be interesting to use the
\textit{Ite1-\Delta EcoRI} truncation allele to determine if these GEF domains alone can
promote exit from mitosis outside of regulating Kin4 (Geymonat et al., 2009).

\textbf{Concluding Remarks}
The work in this thesis has shed light on the regulation of the MEN by spindle
position. This spatial control of the MEN does not appear to be checkpoint-
regulated but rather is controlled by regulation of the phosphatase Cdc14.
Regulation of Cdc14 is mainly established through a MEN inhibitory zone in the
mother compartment, a promoting zone in the bud and the MEN GTPase spatial
sensor Tem1. These zones are crucial for proper spatial regulation of the MEN it
is now evident that one function for the bud-localized MEN promoter, Lte1, is to
inhibit Kin4 in the bud. Three important questions that remain to be investigated
are: 1) how are the MEN inhibitory and promoting zones are established
2) which SPB does Tem1 signal from and 3) is inhibition of Kin4 is the sole
function for Lte1?
References:


Appendix I: Regulation of Kin4 cortical localization by the ergosterol pathway

The experiments performed in this appendix were done together with Emily Do.

Summary
The asymmetric localization of MEN inhibitory components in the mother compartment and MEN promoting components in the bud is essential for the function of spatial regulation of the MEN. Kin4 is the only known negative regulator of exit from mitosis that asymmetrically localizes to the mother cell cortex and mother-localized SPBs. Kin4’s function is to prevent cells with mispositioned spindles from inappropriately exiting mitosis in the mother cell compartment. Therefore, determining what factors anchor Kin4 in the mother cell compartment is central to the understanding of spatial regulation of the MEN. Recently, a high throughput screen identified Kin4 as a protein that interacts with the membrane sterol ergosterol (Li et al., 2010). Ergosterol is an essential sterol that is unique to fungi and its production is a common target of antifungal drugs. Therefore we chose to investigate whether ergosterol is required for Kin4 localization to the mother cell cortex.

Results and Discussion
To test whether ergosterol is an anchor for Kin4 at the cortex, live cell imaging was performed in cells lacking ERG6. Erg6 is a methyltransferase, which is a downstream component in the ergosterol biosynthetic pathway (Nohturfft and Zhang, 2009). Cells lacking ERG6 are viable and produce the sterol intermediate
Figure 1. Kin4 localizes to the mother cell cortex in the absence of ergosterol. Wild type (A19900) or erg6Δ (A29196) cells expressing GFP-tagged Kin4 were grown in YEPD medium and then imaged live.

zymosterol. Elimination of ergosterol in yeast had little effect on Kin4 localization to the cell cortex. This could indicate that the putative interaction between Kin4 and ergosterol is not critical for localization of Kin4 to the cortex (Fig 1). An alternative explanation is that Kin4 can also bind the sterol intermediate (zymosterol) that is produced in cells lacking ERG6. Like ergosterol, zymosterol is incorporated into the plasma membrane and these cells are viable indicating that zymosterol can substitute for some functions of ergosterol (Zinser et al., 1993).

To test whether Kin4 might also bind zymosterol, we monitored Kin4 localization in cells in which the squalene epoxidase Erg1 was inhibited using the drug terbinafine (i.e. Lamisil) (Leber et al., 2003). Erg1 is an enzyme that functions
Figure 2

**Figure 2. Inhibition of Erg11 by Terbinafine Treatment Reduces Kin4 Localization to the cortex.** Wild type (A19900) cells expressing GFP-tagged Kin4 were grown in YEPD medium and then imaged live. These cells were then treated with terbinafine (1mg/mL) or the equivalent volume of ethanol (mock) for 8 hours and then imaged by live cell microscopy.

early in the ergosterol biosynthesis pathway and cells lacking *ERG1* are inviable.

Surprisingly, we found that Kin4 was largely absent from the cortex in cells treated with terbinafine (Figure 2). We also found that while Kin4 appeared to be largely depleted from the plasma membrane, it still localized to SPBs (data not shown).

While these preliminary data point to a function for the ergosterol pathway in the cortical localization of Kin4, much remains to be investigated. It appears that Kin4 does not localize robustly to the cortex of cells treated with terbinafine, however the concentration of drug used to treat these cells was extremely high.
Therefore it will be necessary to orthogonally test these results using a terbinafine-resistant allele of *ERG1* (Leber et al., 2003). This will rule out the possibility that the change in Kin4 localization is due to off target effects of the drug terbinafine. Additionally, several temperature sensitive alleles of essential enzymes that function in the ergosterol pathway have already been generated (e.g. *erg9, erg12, erg13*) and it will be interesting to see if inactivation of these ergosterol pathway intermediates also leads to loss of Kin4 cortex localization.

Ergosterol has not been reported to be asymmetrically distributed in budding yeast so the while it may participate in anchoring Kin4 to the cortex, the mechanisms which keep Kin4 in the mother cell compartment are still not understood. Interestingly, even though ergosterol appears to be symmetrically localized, several proteins that localize to ergosterol-rich domains on the plasma membrane (eisosomes) appear asymmetrically distributed (Moreira et al., 2012).

Finally, whether ergosterol-mediated cortical localization is important for Kin4 function has also not yet been tested. There is correlative evidence suggesting that cortical localization of Kin4 is also required for its localization to SPBs. Rough mapping of the domain that is both necessary and sufficient for Kin4 mother cortical localization has also been done. It will be interesting to determine if this domain specifically interacts with ergosterol. Finally, whether the Kin4-ergosterol interaction is direct has also not been tested.
Experimental Procedures

All strains are derivatives of W303. Live cell imaging is described in the figures legends and was performed at 25°C. Imaging was performed on a DeltaVision Elite microscope platform (Applied Precision). This microscope platform consisted of an InsightSSI™ solid state light source, an UltimateFocus hardware autofocus system and a model IX-71, Olympus microscope controlled by SoftWoRx software (Applied Precision). Time-lapse images were acquired with a 60X Plan APO 1.42NA objective and a CoolSNAP HQ2 camera (Photometrics).

Table 1: Yeast Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic Composition</th>
</tr>
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<tbody>
<tr>
<td>A19900</td>
<td>MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, ura3::pRS306-mCherry-TUB1::URA3, KIN4-GFP::His3Mx6</td>
</tr>
<tr>
<td>A29196</td>
<td>MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, KIN4-GFP::His3Mx6, erg6::TRP1</td>
</tr>
</tbody>
</table>
References


Appendix II: Epistasis Analysis of *LTE1* and *RTS1* in regulating Kin4

**Summary**

While many of the factors that are involved in the spatial regulation of the MEN have been identified (Table 1), integrating these factors into a more complete view of how spatial regulation of the MEN occurs remains largely unfinished. Therefore to deepen our understanding of how spatial signaling of the MEN works, investigating the interplay of these factors will be important. One example of this is the regulation of the kinase Kin4 by both Rts1 and Lte1. Lte1 has been shown to inhibit Kin4 localization at SPBs. In contrast, the phosphatase complex *PP2A-RTS1* has been shown to be required for Kin4 localization to SPBs. In an effort to determine how these factors might regulate one another we performed epistasis analysis between *RTS1* and *LTE1*.

**Results and Discussion**

To perform epistasis analysis of *LTE1* and *RTS1*, we chose to look at the localization of Kin4-GFP. In the absence of *LTE1*, Kin4 localizes to the mother cortex as well as mother SPB. It also seen on the dSPB in a fraction of cells (see Chapter II). In contrast, Kin4 cortical localization and SPB localization is largely absent in cells lacking *RTS1* (Fig 1A)(Chan and Amon, 2009). We therefore asked whether Kin4 localized to SPBs and the cortex in cells lacking both *LTE1* and *RTS1*. Surprisingly, in the *lte1Δ rts1Δ* double mutant, Kin4-GFP localized to
the mother cortex and mother SPBs. This indicates that $LTE1$ functions downstream or in parallel to $RTS1$.

To further investigate the relationship between $LTE1$ and $RTS1$, we examined the growth of these cells at low temperatures. Cells lacking $LTE1$ are inviable at low temperatures but deletion of Kin4 rescues this phenotype (Shirayama et al., 1994; Chan and Amon, 2010). We therefore asked whether
Figure 1

A) Live cell microscopy of GFP-tagged Kin4 in cells also expressing mCherry-labeled α-tubulin. Cells were grown overnight in YEPD medium to mid-log at 25°C. Depicted are representative images of wild type (A25847), \textit{lte1Δ} (A26170), \textit{rts1Δ} (A31129) and \textit{lte1Δ rts1Δ} (A27684) cells.

B) Viability of wild type (A2587), \textit{lte1Δ} (A18597), \textit{rts1Δ} (A20312) and \textit{lte1Δ rts1Δ} (A32150) cells grown on YEPD medium supplemented with at 25°C or 15°C.

\textit{lte1Δ rts1Δ} double mutant was also inviable in the cold. As has been previously published, we found that cells lacking \textit{LTE1} were inviable when grown at 15°C whereas the growth of cells lacking \textit{RTS1} was indistinguishable from the growth of wild types cells (Fig. 1B). We also found that cells lacking both \textit{LTE1} and \textit{RTS1} were viable in the cold although they did have a slight growth defect in comparison to wild type cells. In contrast to the localization-based epistasis data, these analyses indicate that \textit{RTS1} acts downstream or in parallel to \textit{LTE1}. Based
on these results, it is likely that Lte1 and Rts1 regulate Kin4 in parallel mechanisms.

From these analyses, it is clear that Lte1 regulates Kin4 localization to SPBs. However it is unclear how Lte1 (which is restricted to the bud cell compartment) regulates Kin4 localization to SPBs in the mother cell compartment (Fig1 A bottom panel). Further study of Lte1 will be needed to determine how it affects Kin4 localization in the mother compartment.

From these epistasis data, it is also possible that RTS1 may regulate Lte1 function however preliminary analysis indicates that RTS1 does not effect on Lte1 localization (Chan and Amon, 2009; JEF unpublished observations). RTS1 may regulate LTE1 outside of controlling its localization however this remains to be investigated.

The potential role of RTS1 in regulating LTE1 does not explain the epistasis analysis of low temperature viability (Figure 1B). These data indicate that RTS1 is epistatic to LTE1 and therefore acts downstream or in parallel to LTE1. Therefore, the relationship between LTE1 and RTS1 appears to be complex and cannot be explained by simple epistasis analysis. Additional work will have to be performed to uncover how Lte1 and Rts1 are integrated into a more comprehensive view of spatial regulation of the MEN.

**Experimental Procedures**

All strains are derivatives of W303. Live cell imaging is described in the figure legends and was performed at 25°C. Imaging was performed on a Zeiss
Axioplan 2 microscope using a 100x objective and an ORCA-AG camera.

Images were acquired with Openlab.

**Table 1: Yeast Strains**

- **2587** MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+
- **18591** MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, lte1::KanMX6
- **19900** MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, ura3::pRS306-mCherry-TUB1::URA3, KIN4-GFP::His3Mx6
- **20312** MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, rts1Δ::NatMx6
- **20918** MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, KIN4-GFP::His3Mx6, ura3::pRS306-mCherry-TUB1::URA3, rts1Δ::NatMx6
- **26156** MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, ura3::pRS306-mCherry-TUB1::URA3, KIN4-GFP::His3Mx6, lte1Δ::NatMx6
- **31122** MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, ura3::pRS306-mCherry-TUB1::URA3, KIN4-GFP::His3Mx6, lte1Δ::NatMx6, rts1Δ::NatMx6
- **32150** MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, lte1::KanMX6, rts1Δ::NatMx6,
References
