Regulation of the Localization of Lte1, a *S. cerevisiae* Mitotic Exit Activator

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Abstract

The regulation of eukaryotic cell division, which involves the faithful segregation of a complete DNA complement to each daughter cell, is a fundamental area of research in biology. Entry into mitosis is initiated by the action of mitotic cyclins complexed with the cyclin dependent kinase (CDK). Once the chromosomes have been successfully segregated, the exit from mitosis ensues. In order for cells to exit from mitosis, mitotic CDKs must be inactivated. The inactivation of mitotic CDKs, in turn, promotes cytokinesis. In *S. cerevisiae*, mitotic exit is controlled by the Mitotic Exit Network (MEN). In this simple eukaryote, the tight coupling of nuclear migration and mitotic exit is achieved in part by the spatial segregation of Lte1, a positive activator of the MEN, and Tem1, a GTPase that acts at the top of the MEN signaling cascade. The spatial segregation of Lte1 and Tem1 is particularly important in cells with mispositioned anaphase spindles, and plays a role in the prevention of aneuploidy. A model for the regulation of Lte1 localization across the cell cycle is proposed. Additionally, the role of Lte1 localization in mediating its ability to promote mitotic exit is examined. This work identifies novel connections between polarity determinants, Ras signaling, and mitotic exit.

Dedicated to my parents
for all their unconditional
love, support, and encouragement

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Chapter I: Introduction

Overview of the cell cycle

Cyclin-dependent kinases (CDKs) are the workhorses of the cell cycle. The CDK is the catalytic kinase subunit, which must associate with a regulatory cyclin subunit in order to be active (Morgan, 1997). The expression of each cyclin subunit is confined to a small window during the cell cycle via transcriptional regulation and regulated protein degradation. The association of different cyclin subunits with the CDK subunit (Cdc28 in budding yeast) allows the phosphorylation of distinct substrates at specific times in the cell cycle (Morgan, 1997).

In budding yeast, there are two types of cyclins: the Clns and the Clbs. The Cln family consists of Cln 1 – 3 and their essential function is confined to the G1 phase of the cell cycle. The Clbs can be separated into two groups. Clb5 and Clb6 first appear in late G1 and are denoted the S phase cyclins, while Clb 1, 2, 3, and 4 are expressed in G2 and mitosis and are known as the mitotic cyclins (Figure 1) (Andrews and Measday, 1998). Once cells have reached a critical size, the Cln/CDKs promote entry into the cell cycle. The commitment to the cell cycle is known as 'START' and includes spindle pole body (SPB) duplication, bud formation, and entry into S phase (Nasmyth, 1993). The critical role of the Cln/CDKs is to phosphorylate the mitotic cyclin-dependent kinase inhibitor (CKI) Sic1 (Schneider et al., 1996). Phosphorylated Sic1 is then targeted for degradation by a ubiquitin ligase called the SCF and by the 26S proteasome. The degradation of Sic1 allows the accumulation of Clb-

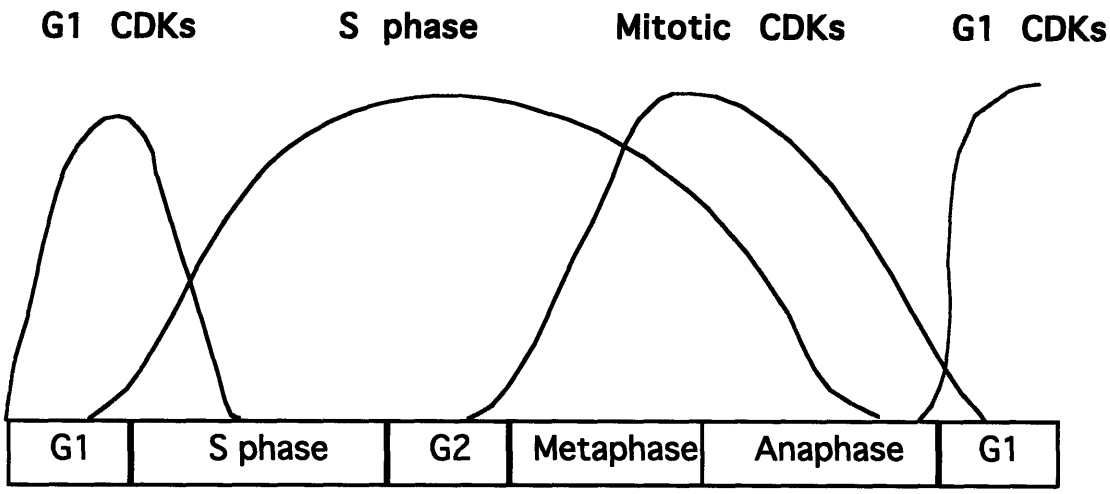

Figure 1: Cell cycle regulation of CDKs in *S. cerevisiae*
The cell cycle is regulated by the differential association of cyclins with the cyclin-dependent kinase Cdc28. During G1, the levels of G1 cycles rise and the activity of G1 CDKs promotes the passage of cells through START. The G1 cyclins in budding yeast are Cln1, Cln2, and Cln3. S-phase cyclins begin to increase at the end of G1, and are inactivated during mitosis. In budding yeast, Clb5 and Clb6-associated kinases are important for promoting DNA synthesis, but in their absence, the mitotic CDKs (Clb1, Clb2, Clb3, and Clb4) can substitute to initiate S phase. The mitotic CDKs are activated at the onset of mitosis and promote chromosome condensation and mitotic spindle formation. These functions can be promoted by Clb1-4, as well as by Clb5 and Clb6. The destruction of mitotic CDKs after chromosome segregation is completed allows cells to exit from mitosis, undergo cytokinesis, and enter into the next G1.

CDKs that promote DNA replication and chromosome segregation (M phase) (Feldman et al., 1997; Skowyra et al., 1997).

The key roles of the S phase cyclins are to activate DNA replication and spindle pole body duplication and to prevent the inappropriate re-initiation of DNA replication upon completion of S phase (Dahmann et al., 1995). Although the S phase and mitotic cyclins are expressed at different times, they are largely redundant as Clb1-4 can promote S phase in the absence of Clb5 and Clb6, and Clb5 can promote mitosis in strains lacking *CLB3* and *CLB4* (Schwob and Nasmyth, 1993). Cohesins (Scc1/Mcd1 in *S. cerevisiae*, Rad21 in *S. pombe* and *D. melanogaster* and Scc1 in human), which are the factors required to hold together the newly replicated chromosomes (sister-chromatids), are also laid down in S phase (reviewed in Hirano, 2000). The establishment of sister-chromatid cohesion allows the formation of a bipolar spindle in prophase, which is required for proper sister-chromatid segregation during mitosis.

The bipolar spindle is formed by microtubules (MT) emanating from the duplicated SPBs. The minus ends of these MTs are associated with the SPBs, while their plus ends are either attached to the sister-chromatids via kinetochores or overlap at the spindle midzone (reviewed in Kline-Smith and Walczak, 2004). In addition, astral MTs extend from the SPBs to the cell cortex and aid in the proper positioning of the mitotic spindle apparatus. In budding yeast, since the division site is predetermined, the mitotic spindle must be aligned such that each resulting daughter cell receives one complement of the replicated DNA. The spindle is aligned along the mother – bud axis by the action of microtubule motor proteins that subject the astral microtubules to forces at the cell cortex (reviewed in Schuyler and Pellman, 2001).

Once the bipolar metaphase spindle is established, the separation of sister-chromatids begins. Much of the mechanism whereby chromosome segregation is initiated at the metaphase – anaphase transition is conserved from yeast to man (reviewed in Nasmyth, 2002). A protease known as separase (Esp1 in budding yeast) cleaves the Scc1 component of the cohesin complex . Separase is held inactive by securin (Pds1 in budding yeast) until all of the chromosomes have been attached to the mitotic spindle in a bipolar manner. Once this occurs, an ubiquitin ligase known as the Anaphase Promoting Complex or Cyclosome (APC/C) complexed with its specificity factor Cdc20 mediates the destruction of Pds1, relieving the inhibition on Esp1 (Nasmyth, 2002). The cleavage of Scc1 by separase, which marks the metaphase – anaphase transition, is promoted by the phosphorylation of Scc1 by Polo kinase (Cdc5 in budding yeast) (Alexandru et al., 2001; Hauf et al., 2001; Sumara et al., 2002). Forces generated by the mitotic spindle apparatus then allow the separation of chromosomes to the poles of the dividing cell (reviewed in Kline-Smith and Walczak, 2004). Once the chromosomes have been successfully partitioned between the mother and daughter cells, the exit from mitosis ensues.

Mitotic exit is characterized by Clb-CDK inactivation, mitotic spindle disassembly, and chromosome decondensation. The removal of mitotic CDK activity establishes the conditions necessary for the completion of cytokinesis, the formation of pre-replicative complexes (preRCs) that are required for S phase initiation, and the establishment of the incipient bud site (reviewed in Stegmeier and Amon, 2004). Mitotic CDK inactivation in budding yeast is brought about by the ubiquitin-mediated destruction of Clb-CDKs. As in all eukaryotes, this process is initiated at the metaphase – anaphase

transition, but budding yeast is unique in that a pool of mitotic CDKs remains in the cell until late anaphase (Jaspersen et al., 1998; Visintin et al., 1998). The degradation of these persisting mitotic CDKs is achieved by the activation of the APC/C specificity factor Cdh1/Hct1, which associates with APC/C and targets Clb-CDKs for ubiquitination and degradation. In addition, the accumulation of the mitotic CDK inhibitor Sic1, which is promoted both by a transcriptional increase in protein levels and post-translational modifications of the protein, aids in the process of Clb-CDK destruction (reviewed in Stegmeier and Amon, 2004).

Regulation of mitotic exit by the MEN.

The M – G1 transition has recently been recognized as a cell cycle transition with multiple layers of regulation. Hartwell and colleagues first identified several essential genes involved in mitotic exit in their screen for budding yeast temperature-sensitive mutants that were defective in the cell cycle (Hartwell, 1971). Mitotic exit mutants are characterized by the presence of a long anaphase spindle, segregated DNA masses and high mitotic CDK activity. The mitotic exit regulators were characterized biochemically and ordered genetically into a core pathway known as the Mitotic Exit Network (MEN) (see Figure 2), which includes the SPB scaffold protein Nud1; the GTPase Tem1; the putative guanine-nucleotide exchange factor (GEF) Lte1; the two-component GTPase activating protein (GAP) Bub2-Bfa1; the protein kinases Cdc5, Cdc15, and Dbf2 (with its associated factor Mob1); the protein phosphatase Cdc14; and a scaffold protein Nud1 Figure 2; (Stegmeier and Amon, 2004). Tem1 localizes to the SPB and is negatively

regulated by the Bub2-Bfa1 GAP complex (Alexandru et al., 1999; Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Fesquet et al., 1999; Fraschini et al., 1999; Geymonat et al., 2002; Pereira et al., 2000; Wang et al., 2000). Tem1 is positively regulated by Lte1, although it is unclear what the biochemical function of Lte1 is (Jensen et al., 2002; Shirayama et al., 1994; Yoshida et al., 2003). The GTP-bound form of Tem1 is thought, though not proven, to recruit the Cdc15 kinase to both SPBs (Asakawa et al., 2001; Bardin et al., 2003; Lee et al., 2001a; Menssen et al., 2001; Visintin and Amon, 2001). This then promotes Dbf2-Mob1 localization to the SPB (Komarnitsky et al., 1998; Luca and Winey, 1998; Mah et al., 2001). The localization of Cdc15 and Dbf2-Mob1 to the SPB is thought to be important for the kinase activity of these proteins (Visintin and Amon, 2001). It is Cdc14 that ultimately dephosphorylates Clb-CDK substrates and allows cells to exit from mitosis (Jaspersen et al., 1999; Visintin et al., 1998). The critical function of the MEN is to promote the activation of Cdc14 by releasing the protein from its inhibitor in the nucleolus Cfi1/Net1(Shou et al., 1999; Straight et al., 1999; Traverso et al., 2001; Visintin et al., 1999). The dissociation of Cdc14 from Cfi1/Net1 causes Cdc14 to spread throughout the nucleus and cytoplasm where it can reach its targets, which include the APC/C specificity factor Cdh1/Hct1, the *SIC1* transcription factor Swi5, and Sic1 itself (Jaspersen et al., 1999; Knapp et al., 1996; Moll et al., 1991; Skowyra et al., 1997; Toyn et al., 1997; Verma et al., 1997; Visintin et al., 1998).

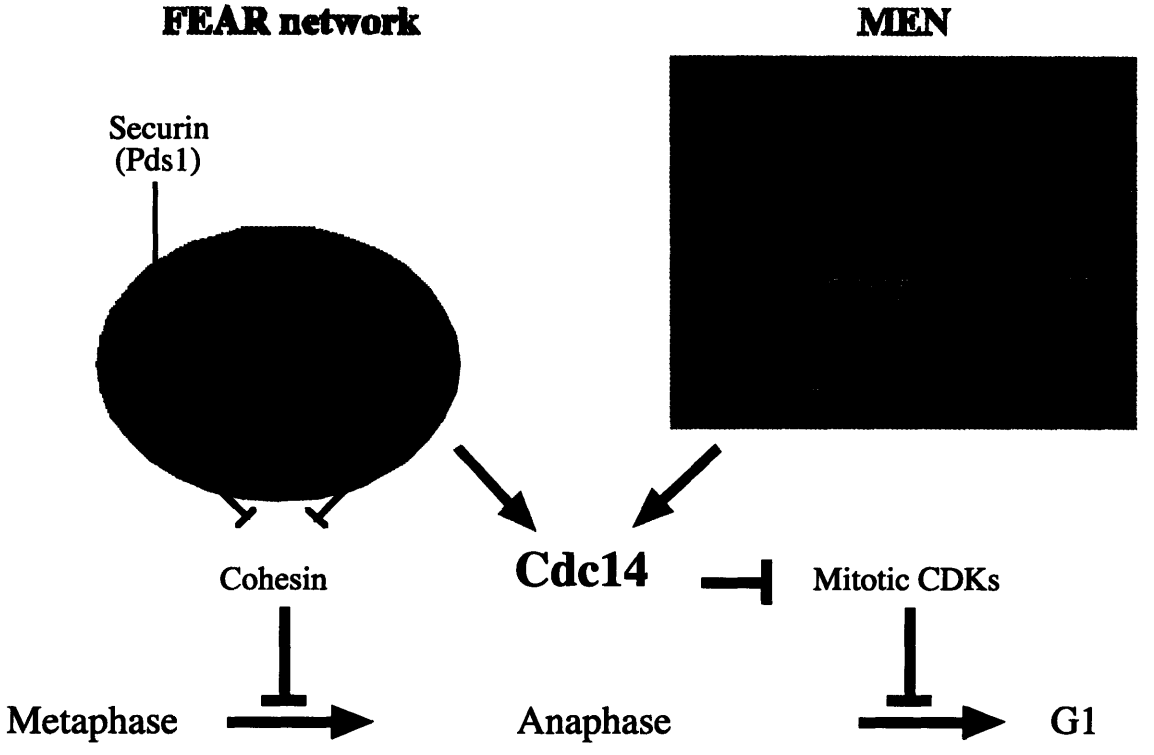

Figure 2: The regulation of anaphase onset and mitotic exit in budding yeast by the FEAR and the MEN.
The metaphase - anaphase transition is triggered by the destruction of cohesin, the glue that holds sister chromatids together. Securin inhibits Separase, which cleaves the cohesin subunit Scc1/Mcd1, thereby allowing sister chromatids to separate. Polo kinase aids in the dissolution of sister chromatid cohesion by phosphorylating Scc1/Mcd1. Separase and Polo also promote exit from mitosis by initiating the release of Cdc14 from the nucleolus during early anaphase as part of the Cdc14 Early Anaphase Release (FEAR) network. Additional components of the FEAR network are the kinetochore protein Slk19, and Spo12 and Bns1, two proteins of unknown function. A pathway known as the Mitotic Exit Network (MEN) promotes and maintains Cdc14 in the released state during late anaphase. The MEN components Tem1 (a GTPase), Bub2-Bfa1 (a two component GTPase activating complex), Cdc15 (a protein kinase), Dbf2 and Dbf20 (homologous protein kinases) and Mob1 (a Dbf2-associated factor) are anchored at the SPB by a scaffold protein Nud1. Components of the FEAR network are highlighted in blue, while MEN pathway components are in pink. Since some FEAR network components are known to regulate sister- chromatid separation, the FEAR network may ensure that exit from mitosis is not initiated prior to sister-chromatid separation.

Regulation of mitotic exit by the FEAR network.

Almost all of the components of the MEN are essential for cellular viability. However, both the putative GEF and the GAP for the Tem1 GTPase are non-essential in an unperturbed cell cycle at room temperature (Daum et al., 2000; Krishnan et al., 2000; Lee et al., 2001b; Shirayama et al., 1994; Yoshida et al., 2003). When cells are grown at low temperatures (14°C - 10°C), *LTE1* is required for cells to exit from mitosis (Shirayama et al., 1994). The dispensability of *LTE1* for normal cell cycle progression suggests that redundant activators of mitotic exit exist. In keeping with this idea, mutations in components of the FEAR (Cdc Fourteen Early Anaphase Release) network, a non-essential pathway that acts in early anaphase to promote Cdc14 activation, are lethal in combination with *lte1Δ* due to an inability to exit from mitosis (Stegmeier et al., 2002). That a MEN-independent release of Cdc14 exists was first discovered in MEN mutants. Several groups observed that Cdc14 is transiently released into the nucleus during early anaphase even in the absence of MEN activity (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). This transient release did not occur in cells lacking either *CDC14* or *CDC5* (Stegmeier et al., 2002; Yoshida et al., 2002). Other components of the FEAR network were subsequently identified and include Esp1, which is a proponent of chromosome segregation; the kinetochore protein Slk19, a nuclear protein of unknown function Spo12 and its homolog Bns1; and the replication fork block protein Fob1 (Pereira et al., 2002; Stegmeier et al., 2004; Stegmeier et al., 2002; Yoshida et al., 2002). These proteins collaborate to initiate the dissociation of Cdc14 from Cfi1. However, in

contrast to the MEN, the FEAR-mediated release of Cdc14 causes the protein to spread only into the nucleus, not into the cytoplasm. In addition, FEAR-induced Cdc14 release is transient, and does not allow the sustained release of Cdc14, which requires the MEN (Pereira et al., 2002; Stegmeier et al., 2004; Stegmeier et al., 2002; Yoshida et al., 2002). Although the order of function of the components of the FEAR network is not understood, genetic epistasis experiments indicate that this signaling cascade is made up of at least two branches. *ESP1* and *SLK19* function in parallel to *SPO12* and *BNS1* (Sullivan and Uhlmann, 2003; Visintin et al., 2003). *CDC5* seems to function as the ultimate effector of the pathway since the overexpression of *CDC5* can compensate for the loss of both the *ESP1* and *SPO12* branches (Visintin et al., 2003).

Temporal coordination of late mitotic events via MEN and FEAR components

The significance of the FEAR-activated release of Cdc14 is not well understood. Some studies indicate that the FEAR network primes the MEN for activation since FEAR network-released Cdc14 dephosphorylates and activates the MEN kinase Cdc15 (Jaspersen and Morgan, 2000; Stegmeier et al., 2002) and also may inactivate Bub2/Bfa1 GAP activity during early anaphase (Pereira et al., 2002; Yoshida et al., 2002). In addition, several lines of data suggest that Cdc14 released at the metaphase – anaphase transition acts to coordinate chromosome segregation with mitotic exit. First, both Esp1 and Cdc5 are key players in the process of sister-chromatid separation, in addition to promoting Cdc14 release and mediating mitotic exit (Nasmyth, 2002). The participation of Cdc5 and Esp1 in both these processes helps to ensure that mitotic exit does not occur

prior to chromosome segregation. Second, the FEAR network is required for the efficient segregation of telomeres and the rDNA array (D'Amours et al., 2004; Sullivan et al., 2004; Torres-Rosell et al., 2004)). The anaphase progression of cells lacking FEAR network activity leads to a loss in cell viability, which may be a result of genomic instability (D'Amours et al., 2004; Hartwell and Smith, 1985). Thus FEAR network-regulated Cdc14 release specifically helps to complete chromosome segregation, as mutations in proteins that only function in the MEN (such as *CDC15*) do not exhibit such chromosome loss phenotypes (D'Amours et al., 2004; Hartwell and Smith, 1985). Third, Cdc14 released by the FEAR network may regulate the localization of the chromosomal passenger proteins Sli15 and Ipl1 during early anaphase (Pereira and Schiebel, 2003). Chromosomal passenger proteins localize to kinetochores during metaphase, but translocate to the mitotic spindle apparatus during anaphase, which is thought to contribute to the stability of the mitotic spindle (Adams et al., 2001). Thus, FEAR network-released Cdc14 is likely to contribute to the temporal regulation between the partitioning of chromosomes and the exit from mitosis.

In addition to executing mitotic CDK inactivation, the MEN plays a direct and essential role in the separation of daughter cells following the completion of mitosis. Several groups previously reported that the MEN protein kinases Cdc5, Cdc15, Dbf2, Dbf20, and the Dbf2-associated factor Mob1 localize to the SPB during mitotic exit and to the bud neck during cytokinesis (Frenz et al., 2000; Song et al., 2000; Xu et al., 2000; Yoshida, 2001). Evidence for the functional importance of these proteins in cytokinesis came from the observation that *mob1-77* mutants that overexpress the CDK inhibitor *SIC1*, hence alleviating the need for *MOB1* in mitotic exit, are still impaired in

cytokinesis (Luca et al., 2001). These data suggest that the MEN collaborates with the cytokinetic machinery, which is made up of the acto-myosin ring, to ensure the execution of mitotic exit prior to cytokinesis. How the MEN and the cytokinetic machinery regulate each other in late mitosis is not understood.

A model for coupling nuclear migration with mitotic exit

In order to ensure that each daughter cell receives exactly one DNA complement during cell division, the division site must bisect the mitotic spindle. In fission yeast and higher eukaryotes, the division site is determined by the position of the mitotic spindle and signaling between the cell membrane and the mitotic spindle apparatus is likely to be important to coordinate chromosome segregation and cytokinesis in space. In *S. cerevisiae*, the division site is pre-determined and therefore the proper orientation of the spindle apparatus through the mother-bud neck prior to mitotic spindle disassembly and cytokinesis is crucial for the fidelity of chromosome segregation. It thus comes as no surprise that mechanisms exist that prevent exit from mitosis until the bud, the future daughter cell, receives a complete DNA complement. The activity of a surveillance mechanism termed the "the spindle orientation checkpoint" blocks exit from mitosis until the spindle is correctly oriented (Muhua et al., 1998; Yeh et al., 1995).

One mechanism that helps prevent exit from mitosis in cells with a mispositioned mitotic spindle is the spatial segregation of components of the MEN under these conditions. Lte1, a putative GEF for Tem1, becomes sequestered at the bud cortex concomitant with bud formation while Tem1 localizes specifically to the daughter-bound

SPB (Bardin et al., 2000; Pereira et al., 2000). Tem1 and Lte1 only come into contact when the daughter-bound SPB moves into the bud during anaphase (Bardin et al., 2000; Pereira et al., 2000). The spatial restriction of Tem1 and Lte1 is important for preventing exit from mitosis in cells with misaligned spindles. This idea is consistent with the finding that overexpression of *LTE1*, which causes the protein to be present in the mother cell as well as the daughter cell, allows cells with mis-oriented spindles to exit from mitosis (Bardin et al., 2000).

However, the spatial restriction of Lte1 and Tem1 is not the only mechanism that prevents cells with misaligned spindles from inactivating mitotic CDKs. Adames et al. first reported that certain mutants with spindle position defects inappropriately exited mitosis even when *LTE1* was deleted. The authors suggested that astral microtubules interacting with the bud neck act as a sensor for spindle position since inappropriate exit of cells with misaligned spindles correlated with loss of cytoplasmic microtubules from the bud neck (Adames et al., 2001). Castillon et al. corroborated this hypothesis with the finding that *arp1Δ* mutants, in which the mitotic spindle is frequently mis-positioned, inappropriately exit mitosis when septin ring function (described in detail below) at the mother – bud neck is compromised due to inactivation of the septin *CDC10*. In *arp1Δ cdc10Δ* cells, inappropriate mitotic exit was only partially prevented by deleting *LTE1*. Thus *CDC10* plays *LTE1*-dependent and independent roles in monitoring spindle position (Castillon et al., 2003). The nature of a septin-regulated signaling pathway remains elusive, but it may impinge on Tem1's GTPase activating protein complex Bub2/Bfa1 (Figure 3B). Deletion of *BUB2* allows cells with a misaligned mitotic spindle to exit from mitosis (Bardin et al., 2000; Pereira et al., 2000). Signals such as the SPB passing through

the bud neck may be required to inactivate Bub2/Bfa1, thus preventing activation of Tem1.

The regulation of spindle position in higher eukaryotes

The spindle position checkpoint also appears to function in fission yeast. Oliferenko and Balasubramanian (Oliferenko and Balasubramanian, 2002) found that a mutant defective in astral microtubule formation, *mia1Δ*, exhibits defects in mitotic spindle orientation and cells arrest in metaphase until the spindle orients correctly along the longitudinal axis of the cell. Evidence for a spindle position checkpoint also exists in mammalian cells. Rat epithelial cells, which were micro-manipulated so that the mitotic spindle is mis-positioned, delay anaphase onset until the spindle is reoriented along the long axis of the cell (O'Connell and Wang, 2000). In the absence of dynein, spindle repositioning does not occur and the cell cycle delay imposed by spindle mis-orientation also seems abolished (O'Connell and Wang, 2000). This suggests that in contrast to budding yeast dynein, which serves as a trigger of the spindle position checkpoint, dynein in mammalian cells not only functions to correct spindle position defects but is also required for checkpoint activation.

A spindle position checkpoint, however, does not appear to exist in the one-celled *C. elegans* embryo. The one-celled embryo divides asymmetrically to produce a larger anterior and a smaller posterior cell, which requires the asymmetric positioning of

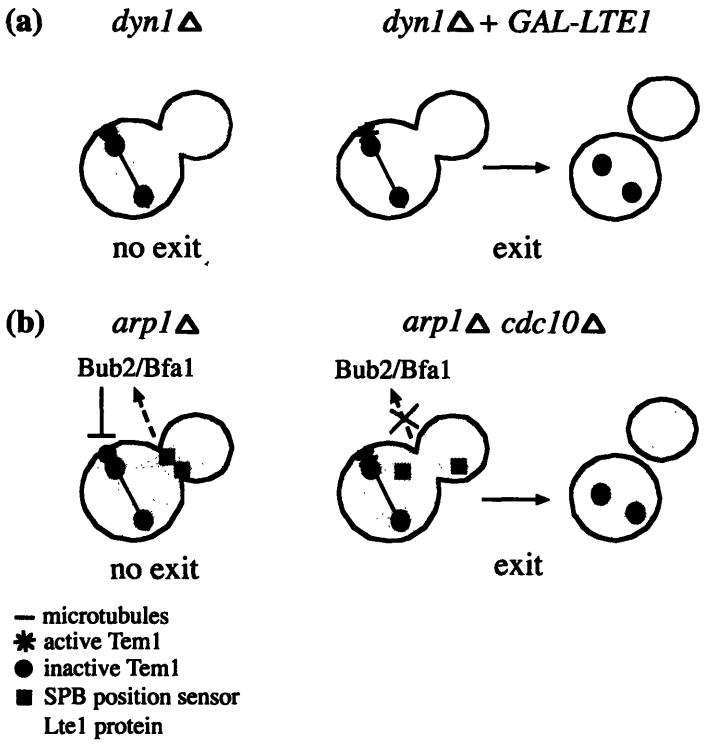

Figure 3: Mechanisms sensing nuclear position in *S. cerevisiae*.

(a) In the absence of dynein (*dyn1Δ*), 10% of cells have mispositioned spindles such that spindle elongation takes place entirely within the mother cell. These cells do not exit from mitosis until the spindle has been properly re-oriented due to the activity of the spindle position checkpoint. The spatial segregation of the mitotic exit activators Tem1 and Lte1 until the migration of the Tem1-bearing SPB into the bud, or daughter cell, is important for the fidelity of preventing inappropriate mitotic exit in cell with misaligned spindles. Cells with misoriented spindles that are defective for Lte1 localization (overexpression of *LTE1 [GAL-LTE1]*) exit from mitosis and accumulate anucleate and multi-nucleate cells.

(b) The passage of the SPB through the mother-bud neck may be required in order to inactivate a checkpoint that blocks inappropriate mitotic exit. This checkpoint, we speculate, could function by activating the Tem1 GAP Bub2/Bfa1 and thereby inhibit MEN activity. The septin *CDC10* may act as a scaffold for a sensor of the SPB passing through the mother-bud neck since in the absence of *CDC10*, such cells are able to exit from mitosis inappropriately in an *LTE1*-independent manner.

the mitotic spindle (Ahringer, 2003). The positioning of the spindle requires cytoplasmic dynein because RNAi-mediated loss of the *C. elegans* dynein homolog *dhc-1* causes spindle orientation defects (Gonczy et al., 1999). However, cell cycle progression does not seem to be delayed in cells with mis-positioned spindles, resulting in the rare occurrence of mis-segregated chromosomes (Gonczy et al., 1999). Perhaps a checkpoint in this instance is superfluous because the constraints of the embryo's eggshell force the spindle to re-position properly in most cases (Gonczy et al., 1999). It is also possible that the spindle position checkpoint, like other checkpoints, is not active during embryonic cell cycles (Dasso and Newport, 1990; Minshull et al., 1994).

How does Lte1 localization regulate mitotic exit?

Clearly, the sequestration of Lte1 to the bud compartment is important for maintaining genomic stability in cells with mispositioned spindles. Some data also suggested that a critical concentration of Lte1 could be required for Tem1 activation. For example, cells lacking the function of the actin motor Myo2 cannot form a bud and therefore grow to a very large size (Govindan et al., 1995; Johnston et al., 1991). In these cells, Lte1 is present throughout the cell and the release of Cdc14 from the nucleolus is delayed. The delay in Cdc14 release can be largely suppressed by the overexpression of *LTE1* in these cells (A.B and A.S. unpublished observations). This result can be interpreted in two ways. One conclusion is that if the sequestration of Lte1 in the bud compartment helps to concentrate the protein and this is needed for efficient Tem1 activation, then the dispersion of Lte1 throughout the enlarged *myo2-66* mutant cell could preclude efficient

mitotic exit. Another possibility is that Lte1 activation is impaired in cells lacking *MYO2*. These two scenarios are not mutually exclusive and imply that the localization of Lte1 to the bud affects its ability to promote mitotic exit.

Regulation of cell polarity throughout the cell cycle

Cell division in *S. cerevisiae* requires the formation of a bud by the actin-dependent vectorial secretion of cell wall constituents (Finger and Novick, 1998). The bud has two distinct growth phases: apical and isotropic. The apical growth phase spans from bud emergence to G2, and is characterized by polarized growth at the incipient bud site, which becomes the bud tip upon bud emergence. Shortly after bud emergence, bud growth switches to an isotropic mode of growth. In isotropically growing cells, the mother and bud are separated into distinct compartments that do not mix. This is achieved by the maintenance of exocyst and polarisome components at the bud cortex by the septin ring (reviewed in Faty et al., 2002).

The septin ring is a filamentous structure composed of Cdc3, Cdc10, Cdc11, Cdc12, and Shs1, which localizes to the incipient bud site just prior to bud emergence. The localized activation of the Rho GTPase Cdc42 at the incipient bud site by its GEF Cdc24 directs the formation of polarized actin filaments and the assembly of the septin ring. Cdc42 directs the initial formation of this scaffold through several effectors, including the PAK-like kinase Cla4 (Gladfelter et al., 2004). Throughout the cell cycle, the septins form a collar at the mother – bud neck. Septins act as a scaffold for the

localization of several proteins required for cytokinesis to the neck region, such as F-actin and myosin II (for review see Field and Kellogg, 1999). In addition, septins maintain cell polarity by acting as a diffusion barrier at the mother – bud neck.

The exocyst complex localizes to regions of polarized growth, and is required for vesicle docking and fusion events, which are an important part of bud growth. Vesicles containing components needed for cell wall formation travel to the bud along actin cables via myosin motor proteins (Karpova et al., 2000; Pruyne et al., 1998; Schott et al., 1999). The actin cables are polarized to the bud by the action of the polarizome complex, which includes the formin Bni1, as well as Spa2, Bud6, and Pea2. The polarisome localizes to the bud tip and later, to the bud cortex (reviewed in Pruyne et al., 2004). An intact septin ring is required to confine the exocyst and polarisome components to the bud cortex, thereby ensuring that polarized growth occurs only at the bud cortex (Barral et al., 2000). The mechanism by which septins compartmentalize the bud cortex is not known, but some data suggests that the interaction of septins with specific lipid moieties at the bud neck may act to concentrate certain lipids in the plasma membrane domain at this region of the cell cortex (Zhang et al., 1999). Presumably, this would affect the composition and fluidity of the membrane at the bud neck and thereby prevent the free diffusion of proteins anchored at the bud cortex.

How are morphological changes linked to cell cycle progression?

A checkpoint called the 'morphogenesis checkpoint' monitors cell morphology and links proper cytoskeletal changes to the cell cycle. Cells that are defective in switching from

apical to isotropic growth activate the morphogenesis checkpoint and exhibit a G_2 delay (Lew, 2003). Delaying nuclear division in response to morphological defects helps prevent the accumulation of aneuploid cells, since cytokinesis also requires a redirection of cell growth to the bud neck for septum formation. Therefore, the delay of morphologically defective cells in G_2 may have evolved to give cells time to make an attempt to repair polarity defects in time for the completion of cytokinesis.

The Wee1 kinase homolog *SWE1* produces the G_2 delay in response to morphological defects by phosphorylating a conserved inhibitory tyrosine on Cdc28 (Booher et al., 1993). Normally, Swe1 is degraded by the action of Hsl1 and Hsl7, a kinase and methyltransferase respectively, which localize specifically to the bud side of the mother bud neck and require proper function of the septin ring for their localization and activity. In the absence of Hsl1-Hsl7 activity, Swe1 is not degraded in G_2, producing a pronounced delay in cell cycle progression (reviewed in Lew, 2003). The mitotic cyclin Clb2 complexed with CDK as well as the Rho GTPase Cdc42 regulate the apical to isotropic switch in bud growth by coordinating to activate the Cla4 kinase, which results in re-organization of actin cytoskeletal polarity and the degradation of Swe1, allowing the progression of mitosis (Lew and Reed, 1993; Longtine et al., 2000; Tjandra et al., 1998). Thus Clb-CDKs link cell polarity to nuclear division and thereby act to preserve genomic stability.

Mitotic CDKs have also been shown to regulate cell polarity in the developing fly embryo. Asymmetric divisions of the neural progenitors in *D. melanogaster* embryos are required for the proper formation of the central nervous system. The asymmetric orientation of the mitotic spindle in neural progenital cells, called neuroblasts, is

necessary for the preferential segregation of cell-fate determinants into one of two sibling daughter cells (reviewed in Schaefer et al., 2001). The *Drosophila* Cdc2/B-type cyclin complexes are required for maintaining the asymmetric localization of these cell-fate determinants, thereby forming a link between nuclear division and cell polarity (Tio et al., 2001). Thus, the use of the mitotic machinery in the regulation of morphological events may be a common theme in eukaryotic cell division to ensure that the process of nuclear division is temporally and spatially connected to the site of cytokinesis.

Mitotic exit and cytokinesis in *S. pombe* and higher eukaryotes

Homologs of MEN components in S. pombe

A signaling cascade similar to the MEN also regulates late mitotic events in *S. pombe*. However, in contrast to the MEN, which is required for the inactivation of mitotic CDK activity, the Septation Initiation Network (SIN) primarily regulates the process of septum formation in fission yeast. Components of the SIN include the GTPase Spg1; the two-component GAP Cdc16-Byr4; the scaffold protein Cdc11; and the protein kinases Cdc7, Plo1, Sid1 (with its associated factor Cdc14), and Sid2 (with its associated factor Mob1) (see Figure 4 for a description of MEN homologs in the SIN). Mutations that disable SIN function cause an inability to constrict the actomyosin ring and complete cytokinesis. Conversely, mutations in Cdc16 or Byr4, which negatively regulate SIN, result in inappropriate septation in the absence of nuclear division (reviewed in Guertin et al., 2002).

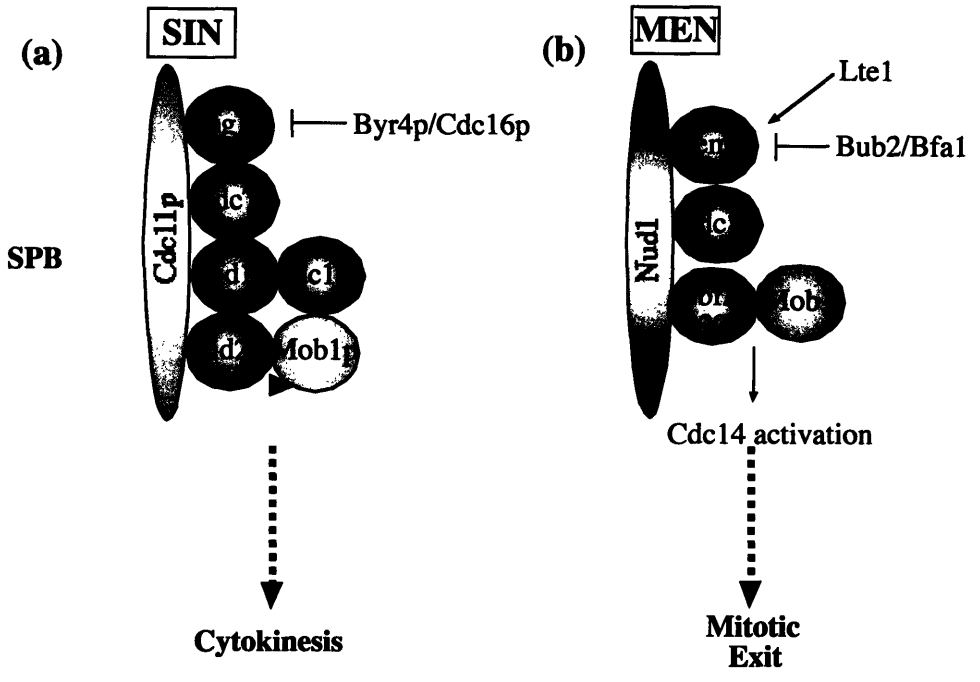

Figure 4: Architecture of the MEN and the SIN signaling pathways.

(a) The SIN in *S. pombe* regulates septum formation. It is composed of the scaffold protein Cdc11p, the GTPase Spg1p, the kinases Cdc7p, Sid1p, and Sid2p and the Sid1p and Sid2p-associated proteins Cdc14p and Mob1p respectively. SIN components localize to SPBs and later translocate to the septum.

(b) The MEN regulates mitotic exit in *S. cerevisiae*. Several components of the MEN localize to the SPB during mitotic exit.

Homologous MEN and SIN components are presented in similar colors.

Like the MEN, SIN activity is regulated by the subcellular localization of its components. All of the SIN components localize to the SPB. Cdc11 is constitutively present at the SPB and forms a scaffold that is necessary for the other members of the SIN to localize (Krapp et al., 2001). In interphase, Cdc16-Byr4 localize to the SPB, as does Spg1(Cerutti and Simanis, 1999; Li et al., 2000). Therefore, Spg1 is in its inactive GDP-bound state (Furge et al., 1998; Sohrmann et al., 1998). Upon entry into mitosis and formation of the metaphase spindle, Spg1 is activated at both SPBs by an unknown mechanism, and Cdc16-Byr4 dissociate from both SPBs (Cerutti and Simanis, 1999; Li et al., 2000). Spg1-GTP recruits the Cdc7 kinase, the Cdc15 homolog, to both SPBs, which persist at the SPB until the onset of anaphase B. At this point, Cdc16-Byr4 re-localizes to one SPB and this causes the inactivation of Spg1 and the dissociation of Cdc7 at this SPB (Cerutti and Simanis, 1999; Li et al., 2000). Although the mechanism that generates the asymmetric Spg1 activation state is not known, it is clear that this state is antagonized by high mitotic CDK activity (Chang et al., 2001). In addition, high mitotic CDK activity antagonizes the localization of Sid1-Cdc14 to the SPB (Chang et al., 2001; Guertin et al., 2000). The presence of non-destructible cyclin B in fission yeast causes cells to arrest with Cdc7 localized to both SPBs, prior to the asymmetric state. In such cells, Sid1-Cdc14 is not recruited to the SPB and downstream septation events do not occur, indicating that the inactivation of Spg1 at one SPB and the subsequent recruitment of Sid1-Cdc14 the single SPB containing Cdc7 is likely important for the regulation of septum formation (Chang et al., 2001; Guertin et al., 2000). Sid1-Cdc14 then recruits promotes the localization of Sid2-Mob1 to the SPB and then to the site of cytokinesis, resulting in actomyosing ring constriction (Guertin et al., 2000; Sparks et al., 1999).

The role of the SIN in mitotic CDK inactivation

In fission yeast, the destruction of mitotic CDKs is required for the onset of cytokinesis (Yamano et al., 1996). However, unlike the MEN, the SIN seems to directly antagonize CDKs only in cells in which cytokinesis is delayed. Like the MEN phosphatase Cdc14, the *S. pombe* Cdc14 homolog Clp1/Flp1 does plays a role in the inhibition of mitotic CDKs (Cueille et al., 2001; Trautmann et al., 2001). In fission yeast, the initiation of the next nuclear division cycle, accompanied by a rise in CDK activity, occurs around the time of septation. Because the SIN is inhibited in the presence of high CDKs, cells in which cytokinesis is delayed are in danger of being unable to complete cytokinesis due to the rise of CDKs in interphase (Martin-Castellanos et al., 1996). Therefore, a checkpoint termed the 'cytokinesis checkpoint' keeps CDKs low in interphase until the completion of cytokinesis (Le Goff et al., 1999; Liu et al., 2000; Liu et al., 1999; Trautmann et al., 2001). The key executor of this checkpoint is Clp1/Flp1, which is regulated by its subcellular localization (Cueille et al., 2001; Trautmann et al., 2001). In interphase, Clp1/Flp1 resides at the SPB and the nucleolus. Upon SIN activation during mitosis, Clp1/Flp1 becomes released from the nucleolus and spreads to the mitotic spindle and the actomyosin ring. Here, Clp1/Flp1 promotes the inhibitory phosphorylation of the CDK subunit Cdc2. The inactivation of the SIN causes Clp1/Flp1 to re-localize to the nucleolus, and allows the accumulation of activated CDK in interphase (Trautmann et al., 2001).

A comparison of the MEN and the SIN pathways

The similarities between the Cdc14 and Clp1/Flp1 highlight the conservation between the MEN and the SIN. Components of each localize to the SPB and the site of cell division, and both pathways seem capable of inhibiting CDKs as well as promoting cytokinesis. In addition, asymmetric SPB localization of proteins in the MEN as well as the SIN seems to be important for the integrity of these signaling pathways. The asymmetric localization of Tem1 in *S. cerevisiae* functions at least in part to couple the process of nuclear migration to mitotic exit as part of the 'spindle orientation checkpoint,' a checkpoint specifically important in budding yeast due to the pre-establishment of the division site (Bardin et al., 2000; Pereira et al., 2000). In fission yeast, however, no such challenges are presented, and spindle positioning is regulated by crosstalk between the cell membrane and the mitotic spindle apparatus during mitosis (Gachet et al., 2004). In addition, a similar requirement for Spg1 activation by a localized putative exchange factor does not seem to exist, since no exchange factor for Spg1 has yet been identified. Some studies suggest that Spg1 has an uncharacteristically high intrinsic GDP exchange rate, rendering an exchange factor unnecessary (Furge et al., 1998). Other studies indicate that the polo kinase Plo1 may promote the activation of Spg1. The overexpression of Plo1 promotes inappropriate septation in a Spg1-dependent manner, and promotes the recruitment of Cdc7 to both SPBs, indicating that Spg1 is in its GTP-bound state (Tanaka et al., 2001). It is also possible that an as yet unidentified exchange factor for Spg1 exists.

Mitotic exit in higher eukaryotes

The fact that cyclin B must be degraded in order for cells to exit from mitosis was first discovered using *Xenopus* oocytes (Murray et al., 1989). Thus a central feature of the molecular mechanism mediating this transition is conserved from budding yeast to multi-cellular organisms. However, whether pathways such as the MEN and FEAR network regulate mitotic exit in higher eukaryotes is not clear. The regulation of mitotic exit in budding yeast and mammalian cells is different in that a pool of cyclin B/ CDK persists until telophase in budding yeast, which is eliminated by APC/C^{Cdh1}. In contrast, in mammalian cells, all of the cyclin B is degraded at the metaphase to anaphase transition by APC/C^{Cdc20}. The role of APC/C^{Cdh1} in mammalian cells is to restrain mitotic CDK activity in late mitosis and thereby establish the G1 phase of the cell cycle (Peters, 2002). Whether the degradation of additional targets by APC/C^{Cdh1} in mammalian cells is required for mitotic exit remains to be seen. In mammals, two homologs of *CDC14*, hCdc14A and hCdc14B, have been identified. hCdc14A localizes predominantly to centromeres, and hCdc14B localizes to the nucleolus, at least in interphase cells (Kaiser et al., 2002; Mailand et al., 2002). Thus far, RNAi depletion studies have identified mainly a centrosomal regulation role for hCdc14A. The overexpression of hCdc14A leads to the premature splitting of centrioles in S phase, which results in the formation of several aberrant mitotic spindles (Kaiser et al., 2002; Mailand et al., 2002). The role of hCdc14B is even less well characterized (Kaiser et al., 2002). The study of how these and other MEN, FEAR network, and SIN homologs function to regulate late mitotic events is largely uncharted territory.

Thesis Summary

A great many strides have been made in elucidating the regulation of mitotic exit in the last five years. The work presented in this thesis has contributed to our understanding of the MEN in many ways. This work gives rise to a model for the regulation of Lte1 localization by Ras, the Rho GTPase Cdc42, its effector the PAK-like kinase Cla4, and the septin ring. Importantly, connections are established between Ras and Rho GTPase pathways in the regulation of mitotic exit. The findings presented here also suggest that the specific localization of Lte1 to the bud cortex may be linked to the protein's ability to promote mitotic exit. We can conclude from these studies that several different signaling pathways converge on the regulation of mitotic exit through Lte1. The mechanisms by which these pathways collaborate to monitor and execute mitotic exit remain to be challenges for the future.

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Chapter II: Control of Lte1 localization by cell polarity determinants and Cdc14

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Dr. Allison Bardin initially observed that Lte1 localization is cell cycle regulated.

Summary

The putative guanine nucleotide exchange factor Lte1 plays an essential role in promoting exit from mitosis at low temperatures. Lte1 is thought to activate a Ras-like signaling cascade, the Mitotic Exit Network (MEN). MEN promotes the release of the protein phosphatase Cdc14 from the nucleolus during anaphase, which is a prerequisite for exit from mitosis. Lte1 is present throughout the cell during G1, but is sequestered in the bud during S phase and mitosis by an unknown mechanism. We show that anchorage of Lte1 in the bud requires septins, the cell polarity determinants Cdc42 and Cla4, and Kel1. Lte1 physically associates with Kel1 and requires Kel1 for its localization in the bud, suggesting a role for Kel1 in anchoring Lte1 at the bud cortex. Our data further implicate the PAK-like protein kinase Cla4 in controlling Lte1 phosphorylation and localization. *CLA4* is required for Lte1 phosphorylation and bud localization. Furthermore, when overexpressed, *CLA4* induces Lte1 phosphorylation and localization to regions of polarized growth. Finally, we show that Cdc14, directly or indirectly, controls Lte1 dephosphorylation and delocalization from the bud during exit from mitosis. Restriction of Lte1 to the bud cortex depends on the cortical proteins Cdc42 and Kel1 and the septin ring. Cla4 and Cdc14, promote and demote Lte1 localization at and from the bud cortex, respectively, suggesting not only that the phosphorylation status of Lte1 controls its localization, but also indicating that Cla4 and Cdc14 are key regulators of the spatial asymmetry of Lte1.

Introduction

One of the underlying principles of development is the asymmetric distribution of cell fate determinants during cell division to create two cells of different fates. Cell division in the budding yeast *S. cerevisiae* is inherently asymmetric: the mother cell polarizes to form a bud, which then separates to become the daughter cell. Many proteins have been identified whose localization is restricted to the incipient bud site and the growing bud: proteins involved in bud site selection and bud formation (Casamayor and Snyder, 2002; Chant, 1999; Pruyne and Bretscher, 2000b), cell fate determinants (Beach et al., 1999; Bertrand et al., 1998; Bobola et al., 1996; Jansen et al., 1996; Sil and Herskowitz, 1996), and proteins involved in anchoring cytoplasmic microtubules in the bud (reviewed in Schuyler and Pellman, 2001).

Asymmetry of the dividing yeast cell is achieved both by the polarization of the actin cytoskeleton, and by the actin-dependent vectorial transport of polarity factors into the growing bud (reviewed in Finger and Novick, 1998; Pruyne and Bretscher, 2000a). The Rho-like GTPase *CDC42* is a key early determinant of actin polarization and bud formation. Local activation of Cdc42 by Cdc24 leads to actin polarization through the activation of multiple effectors (reviewed in Gulli and Peter, 2001; Pruyne and Bretscher, 2000a). Among the Cdc42 effectors are two PAK-like protein kinases Ste20 and Cla4 that perform partially overlapping functions in the establishment of cell polarity (reviewed in Gulli and Peter, 2001; Pruyne and Bretscher, 2000a). Cla4 kinase is not only activated by Cdc42 but also by mitotic cyclin-dependent kinases Clb-CDKs; (Benton et al., 1997; Tjandra et al., 1998), which causes Cla4 kinase activity to be cell cycle

regulated (Benton et al., 1997; Tjandra et al., 1998). Cla4 kinase is low during G1 rises during late S phase, peaks during mitosis and declines during exit from mitosis (Benton et al., 1997).

Polarized growth requires not only the establishment of polarity cues at the site of bud formation and growth and the transport of cargos thereto, but also maintenance of these cues and cargos in the bud. The septin ring has been shown to play a critical role in maintaining bud-localized proteins at the bud cortex (reviewed in Gladfelter et al., 2001). The septin ring is a complex of proteins that includes Cdc3, Cdc10, Cdc11, and Cdc12 (Byers and Goetsch, 1976; Haarer and Pringle, 1987; Kim et al., 1991), which localizes as a ring at the incipient bud site and encircles the mother-bud neck after bud formation. At the mother–bud neck, the septin scaffold functions as a diffusion barrier, blocking the passage of bud-cortex localized proteins to the mother cell cortex (Barral et al., 2000; Takizawa et al., 2000) (Gladfelter et al., 2001).

Spatial asymmetry also plays a role in cell cycle control. Lte1, a regulator of mitotic exit, is asymmetrically localized (Bardin et al., 2000; Pereira et al., 2000). Lte1 is distributed throughout the cell during G1 but becomes sequestered to the bud during S phase. Shortly before cells undergo cytokinesis, Lte1 asymmetry is lost and the protein is evenly distributed throughout the mother cell and the bud (Bardin et al., 2000; Pereira et al., 2000). Lte1 has homology to guanine nucleotide exchange factors (GEFs) for Ras-like GTPases and is required for exit from mitosis at low temperatures (Shirayama et al., 1994). Lte1 is thought to stimulate Tem1, a GTPase which functions near or at the top of a signaling cascade known as the mitotic exit network (MEN), and promotes the release

of the protein phosphatase Cdc14 from its inhibitor Cfi1/Net1 in the nucleolus (reviewed in Bardin and Amon, 2001). This phosphatase in turn promotes exit from mitosis by reversing phosphorylation on cyclin-dependent kinase (CDK) substrates.

The requirement for localization of Lte1 to the bud in the regulation of exit from mitosis appears to be two-fold. First, Lte1 localization to the bud helps to ensure that exit from mitosis does not occur prior to delivery of one chromosome complement into the bud. Tem1, Lte1's putative target, localizes to the spindle pole body destined to migrate into the bud. Thus, only after chromosome segregation has been initiated is Tem1 in the same compartment as Lte1 (Bardin et al., 2000; Pereira et al., 2000). Second, restricting Lte1 to the bud may also ensure that sufficient concentrations of Lte1 are present to activate Tem1 once the nucleus enters the bud. Cells lacking the actin motor *MYO2* fail to form a bud and the mother cell grows unusually large (Govindan et al., 1995; Johnston et al., 1991). In such cells Lte1 is present throughout the cell, but release of Cdc14 from the nucleolus is delayed. The delay in Cdc14 release is, to a large extent, abolished by overexpression of *LTE1*(Bardin et al., 2000).

In this study, we have begun to determine the mechanisms that control the localization of Lte1 at the bud cortex. We show that anchorage of Lte1 in the bud depends on a functional septin ring, Cdc42, Cla4, and Kel1. Kel1 physically interacts with Lte1 suggesting that Kel1 is a component of the machinery anchoring Lte1 at the bud cortex. The protein kinase Cla4 is required for Lte1 phosphorylation and when overexpressed during G1, is sufficient to cause Lte1 phosphorylation and localization to cortical regions, indicating that Cla4 regulates Lte1 phosphorylation and localization. Finally, we show that the protein phosphatase Cdc14, directly or indirectly, initiates Lte1

dephosphorylation and delocalization from the bud during exit from mitosis. We propose that Lte1 localization is controlled by phosphorylation and protein anchors at the cortex and that Cla4 and Cdc14 control the spatial asymmetry of Lte1.

Results

Lte1 is localized to the bud cortex and bud cytoplasm.

To gain insight into how the localization of Lte1 is regulated, we used deconvolution microscopy to examine in detail the localization of Lte1 fused to the green fluorescence protein (GFP). Lte1 staining appeared to be particulate in unbudded and budded cells (Figure 1A, B). The particulate nature of Lte1 staining was also observed in fixed cells or cells expressing Lte1 tagged with 13 MYC or 3 HA epitopes (data not shown), indicating that it was not an artifact of a particular epitope tag or fixation procedure. This result suggests that Lte1 associates with large structures, whose identity remains to be determined.

To examine Lte1 localization during the cell cycle, we determined the localization of a defined pool of Lte1-GFP. Cells expressing an *LTE1-GFP* fusion from the galactose inducible/glucose repressible *GAL1-10* promoter as the sole source of *LTE1* were arrested in G1 with α-factor pheromone. Subsequently cells were released into the cell cycle under conditions where Lte1 transcription was repressed. In G1 cells, Lte1 was localized throughout the cytoplasm of cells (Figure 1B, a). In small budded cells Lte1 was localized primarily at the bud cortex (Figure 1B, b, c). In cells with medium and large buds, which are bud sizes observed when cells are in late S phase and mitosis, Lte1

staining was also evident in the cytoplasm, being enriched in the bud cytoplasm (Figure 1B, d, e). This cytoplasmic localization of Lte1 is not likely to be an artifact of saturating anchors at the bud cortex due to *GAL1-10* promoter expression of Lte1, as in small budded cells, Lte1 is not seen in the bud cytoplasm. During exit from mitosis, Lte1 asymmetry was lost and the protein became uniformly distributed throughout mother cell and bud (Figure 1B, f).

The fact that Lte1 was not only cortical in medium and large budded cells but also present in the bud cytoplasm was particularly evident when Lte1 localization was compared to the localization pattern of the integral membrane protein Ist2 (Figure 1C;Takizawa et al., 2000). Whereas no cytoplasmic fluorescence was observed in cells expressing an *IST2-GFP* fusion, significant amounts of fluorescence in the cytoplasm were detected in cells expressing *LTE1-GFP*. Our findings suggest that Lte1 associates with the bud cortex in cells with small buds. In cells with medium and large buds, Lte1 appears to be released from cortical anchors in the bud, which leads to the uniform distribution of the protein during late stages of telophase.

Bud-specific accumulation of Lte1 is largely independent of the actin and microtubule cytoskeletons.

Both the actin and microtubule cytoskeletons have been shown to target proteins to regions within the cell (reviewed in Goode et al., 2000; Pruyne and Bretscher, 2000b). To determine whether actin filaments or microtubules were important for Lte1 delivery into the bud, we examined the consequences of disrupting these filaments using Latrunculin A (LAT-A) and nocodazole, respectively. Cells were treated with hydroxyurea (HU), which

arrests cells as budded cells in early S phase. Subsequently, cells were treated with either nocodazole or LAT-A, which caused depolymerization of microtubules and actin filaments, respectively (data not shown). Transcription of an *LTE1-HA* fusion was then induced from the *GAL1-10* promoter and the accumulation of Lte1-HA in the bud was examined over time. As shown in Figures 1D and supplemental Figure 1, disruption of microtubules did not affect the accumulation of Lte1 in the bud. Treatment of cells with Latrunculin A caused a minor delay in the bud-specific accumulation of Lte1 (Figure 1E) indicating that accumulation of Lte1 in the bud is largely independent of a functional actin cytoskeleton.

Anchorage of Lte1 in the bud depends on septins.

To determine how Lte1 is anchored in the bud, we analyzed Lte1 localization under conditions where structures important for establishing asymmetry, namely the actin or microtubule cytoskeletons, or the septin ring, were disrupted. We found that neither LAT A treatment nor nocodazole treatment caused loss of Lte1 from the bud in HU-arrested cells, in which Lte1 is already localized in the bud (Figure 2A, B). These results indicated that neither the actin nor the microtubule cytoskeleton was required for anchorage of Lte1 at the bud cortex.

Maintenance of Lte1 in the bud, however, required a functional septin ring. In temperature sensitive *cdc12-6* mutants, the septin ring is disrupted within 15 minutes of temperature shift to 37°C (Barral et al., 2000). The bud-specific localization of Lte1 was also lost in *cdc12-6* mutants within 15 minutes (Figure 2C). *CDC12* was, however, not required for maintenance of Lte1 phosphorylation as judged by the persistence of

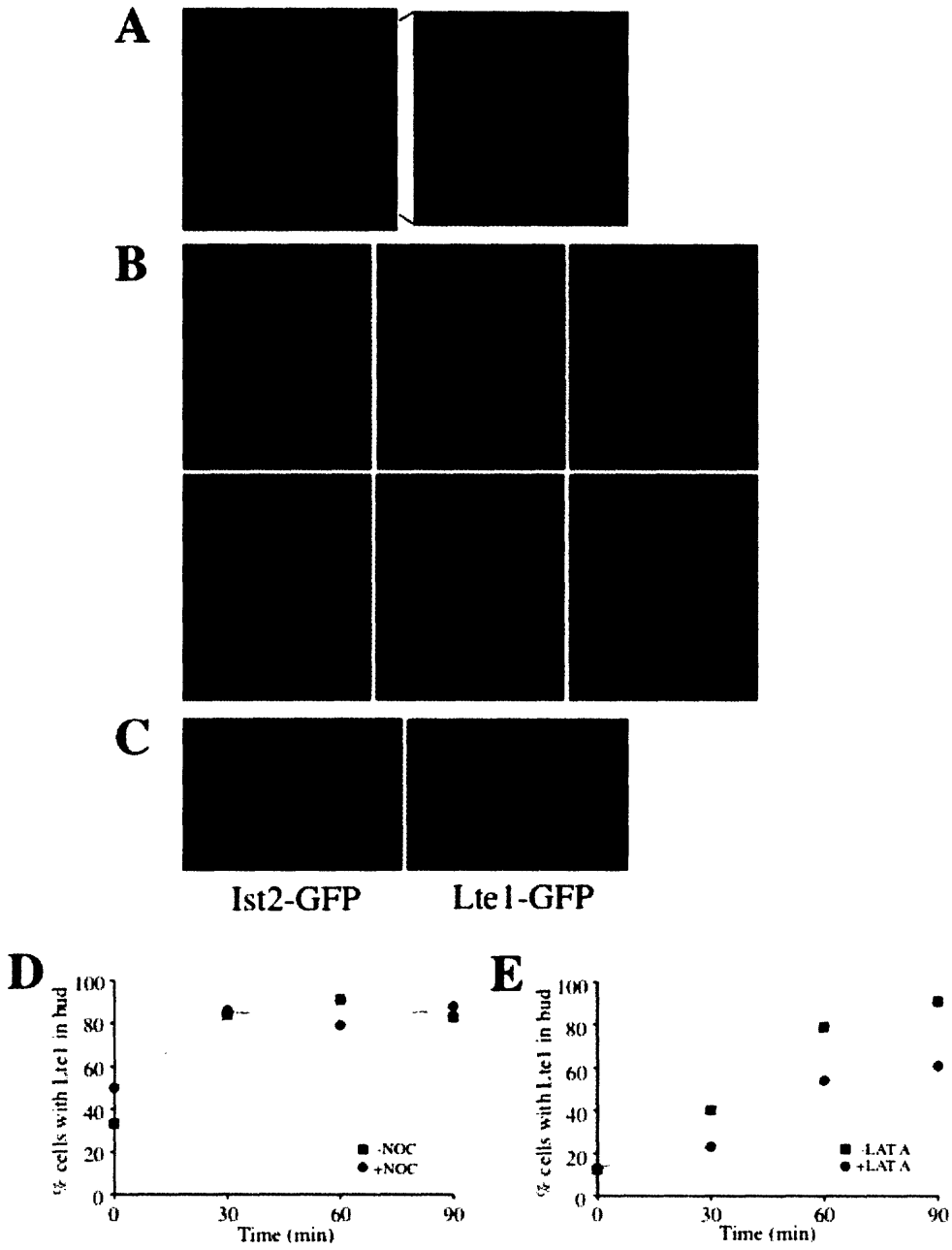
A
B
C
Ist2-GFP
Lte1-GFP
D
% cells with Lte1 in bud
100
80
60
40
20
0
0
30
60
90
Time (min)
-NOC
+NOC
E
% cells with Lte1 in bud
100
80
60
40
20
0
0
30
60
90
Time (min)
-LAT A
+LAT A

Figure 1

Figure 1: Characterization of Lte1 localization.

(A) *GAL-LTE1-GFP* (A3587) cells were grown in synthetic complete medium containing raffinose and galactose (SC+Raff+Gal) for 3 hours. Live cell images were taken and deconvolved using Deltavision software as described in Materials and Methods. The image shown is a single frame of a Volume Viewer movie generated from a Z-series consisting of twenty-five 200nm sections.

(B) *GAL-LTE1-GFP* cells (A3587) were arrested in SC+Raff medium with alpha-factor (3μg/ml) for 2.5 hours. Galactose was added for 1 hour 30 min to induce transcription of *GFP-LTE1*. Cells were then released into media lacking pheromone but containing glucose to repress production of the Lte1-GFP fusion. Cells were fixed and analyzed by deconvolution microscopy. The images shown represent a single section of a deconvolved image created from a Z-series of twenty-five 200nm sections. The cell shown in (a) is in the G1 stage of the cell cycle and was harvested 20 minutes after release from the pheromone arrest. Cells shown in (b) and (c) represent small budded cells and were harvested 40 and 60 minutes after release from the G1 block, respectively. The cell shown in (d) represents cells with a medium size bud and was harvested 80 minutes after release. The cell shown in (e) represents large budded cells and was harvested 120 minutes after release. In the cell shown in (f) bud-specific accumulation of Lte1-GFP is lost. This cell was harvested 140 minutes after release from the G1 block.

(C) *GAL-LTE1-GFP* (A3587) and *GAL-GFP-IST2* (A5085) cells were grown in SC + Raffinose + Galactose for 3 hours and fixed. The images represent a single section of a deconvolved image created from a Z-series of twenty-five 200nm sections.

(D) A *GAL-LTE1-3HA* strain (A2067) was arrested with 10 mg/ml hydroxyurea (HU) for 2 hours. The culture was then split and nocodazole (15 μg/ml Noc) was added to one of the cultures. After 2 hours galactose was added to induce expression of the *LTE1-HA* fusion. After 60 minutes (t=0) glucose was added to repress *LTE1-3HA* transcription. Time points were taken at indicated times to determine the percentage of cells with Lte1-HA concentrated in the bud.

(E) *GAL-LTE1-3HA* (A2067) cells were grown as described in (D) except 200 μM Latrunculin A (LAT-A) was added instead of nocodazole. 30 minutes after LAT-A addition, transcription of *LTE1-3HA* was induced for 60 minutes followed by repression of transcription by glucose addition (t=30). The percentage of cells with Lte1 in the bud was analyzed at the indicated times.

slower migrating forms of Lte1 throughout the course experiment (Figure 2D; Bardin et al., 2000; Lee et al., 2001).

KEL1 is required for Lte1 localization at the bud cortex.

While septins are required to restrict Lte1 to the bud, it is not likely that septins themselves anchor Lte1 in the bud, as their localization is restricted to the mother–bud neck (reviewed in Gladfelter et al., 2001). *KEL1* has recently been shown to physically interact with Lte1 (Gavin et al., 2002; Ho et al., 2002) raising the possibility that this protein plays a role in Lte1 localization. *KEL1* is required for cell fusion and wild-type cell morphology and localizes to the tip of the mating projection, to the presumptive bud site in G1 cells, and to the bud cortex during bud growth (Philips and Herskowitz, 1998).

To investigate the role of *KEL1* in localization of Lte1, we first determined whether the two proteins associated with each other. Kel1-HA was detected in Lte1-GFP immunoprecipitates (Figure 3A) indicating that the two proteins form a complex. Furthermore, we found that overexpression of *LTE1* caused a change in the localization of Kel1. In α-factor arrested cells Lte1 is present throughout the cell, whereas Kel1 is concentrated at the tip of the mating projection (shmoo tip; Bardin et al., 2000; Pereira et al., 2000; Philips and Herskowitz, 1998). Overexpression of *LTE1* caused a decline in cells with Kel1 concentrated at the shmoo tip (Figure 3E), indicating that Lte1, when present at high levels, can titrate Kel1 away from the shmoo tip.

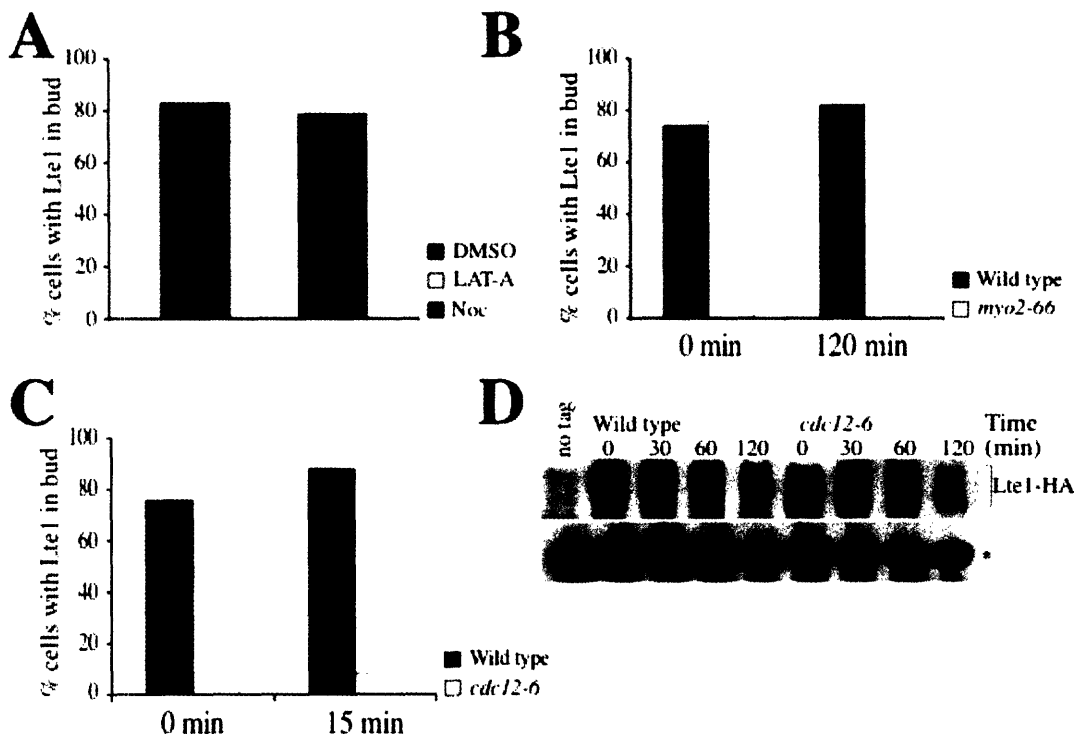
A
% cells with Lte1 in bud
100
80
60
40
20
0
DMSO
LAT-A
Noc
B
% cells with Lte1 in bud
Wild type
myo2-66
0 min
120 min
C
% cells with Lte1 in bud
Wild type
cdc12-6
0 min
15 min
D
no tag
Wild type
cdc12-6
Time (min)
0 30 60 120 0 30 60 120
Lte1-HA

Figure 2

Figure 2: Maintenance of Lte1 in the bud requires the septins.

(A) Cells carrying an *LTE1-13MYC* fusion (A3717) were arrested with 10 mg/ml HU for 2 hours. Subsequently cells were treated with 15 μg/ml nocodazole (Noc) or 200 μM latrunculin A (LAT-A) for 2 hours and the percentage of cells with Lte1 localized to the bud was determined.

(B) Wild type (A3717) and *myo2-66* mutant (A3941) cells carrying an *LTE1-13MYC* fusion were arrested with 10 mg/ml HU for 2 hours at 23°C. Subsequently cells were shifted to 37°C for 2 hours and the percentage of cells with Lte1 localized to the bud was determined.

(C) Wild type (A1949) and *cdc12-6* (A3210) strains were arrested at 23°C with 10 mg/ml HU for 2 hours. Both strains were then shifted to 37°C (t=0) and samples were taken at the indicated times to determine the percentage of cells with Lte1 localized to the bud.

(D) Wild type (A1949) and *cdc12-6* (A3210) cells were arrested in HU for 2 hours and subsequently shifted to 37°C to depolymerize the septin ring. Samples were taken at indicated times and Lte1-HA phosphorylation status was examined by Western blot analysis. The asterisk denotes a cross-reacting band that serves as a loading control.

Kel1 not only physically interacted with Lte1, but was also essential for Lte1 localization at the bud cortex. Lte1 was not localized in the majority of HU-arrested *kel1Δ* cells, (Figure 4C) or in *kel1Δ* cells progressing though the cell cycle in a synchronous manner (Figure 3C). However, deletion of *KEL1* had little effect on Lte1 phosphorylation, as judged by western blot analysis (Figure 3B). Cells lacking *KEL2,* a protein that shares 44% homology with Kel1 and forms a complex with it (Philips and Herskowitz, 1998) did not exhibit defects in Lte1 localization (Figure 4C). We conclude that Kel1 is found in a complex with Lte1 and is required for Lte1 localization to the bud.

CDC42 and its effector _CLA4_ are required for Lte1 localization and phosphorylation.

CDC42 is required for the bud cortex association of many proteins (reviewed in Gulli and Peter, 2001; Pruyne and Bretscher, 2000a). To determine whether *CDC42* was required for the maintenance of Lte1 in the bud, we arrested temperature sensitive *cdc42-17* cells with HU at the permissive temperature (20°C) and examined Lte1 localization upon shift of cells to 37°C, the restrictive temperature of *cdc42-17* mutant. Figure 4A shows that localization of Lte1 at the bud cortex was already compromised at the permissive temperature and was lost even further upon temperature shift. Gladfelter, et al. (Gladfelter et al., 2002) have previously shown that the *cdc42-17* allele does not affect the septin ring structure in this type of experiment, suggesting that *CDC42* does not regulate Lte1 localization solely through its role in establishing a stable septin ring. Interestingly, inactivation of *CDC42* also led to the disappearance of phosphorylated Lte1

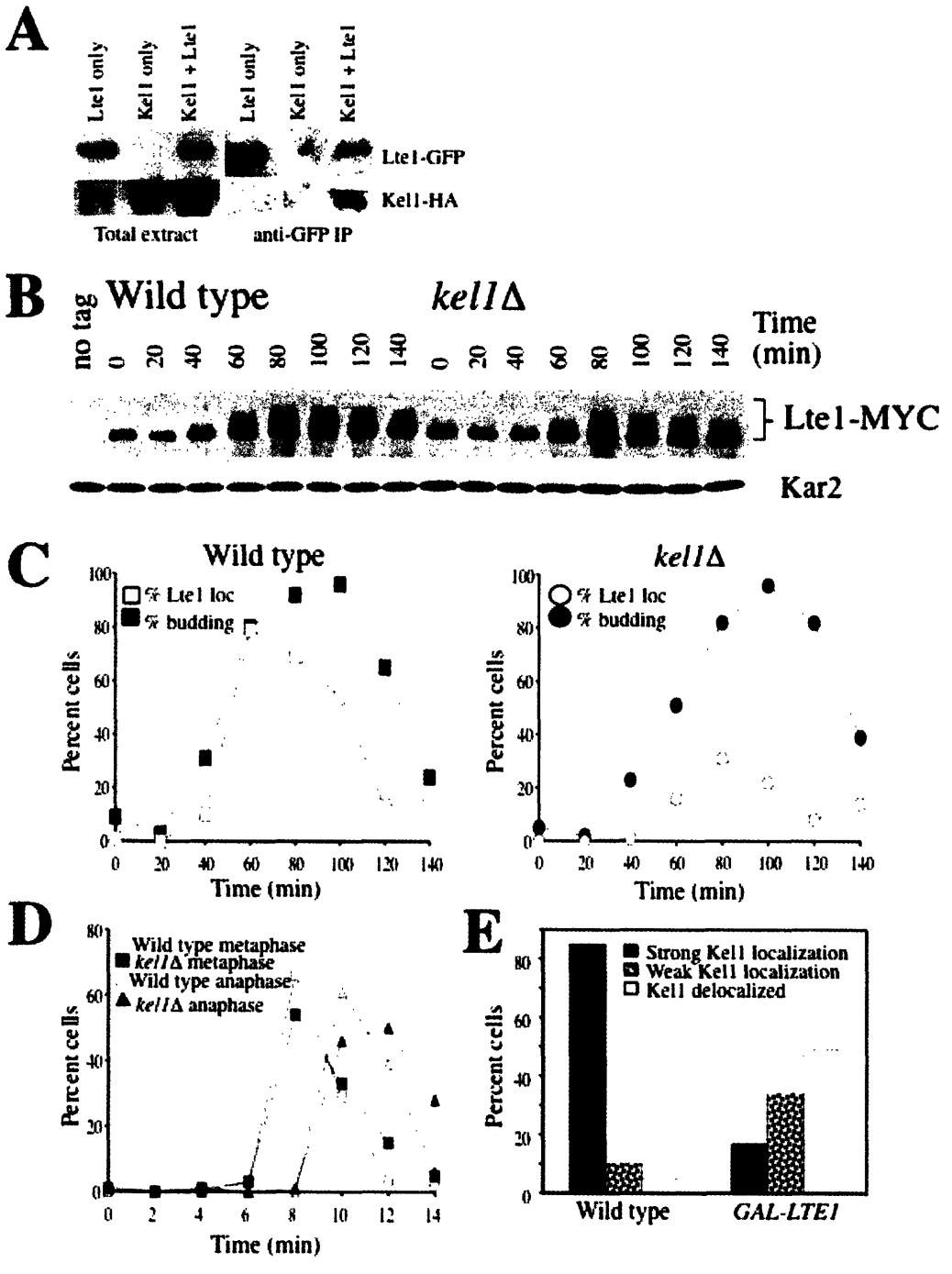
A
Lte1 only
Kel1 only
Kel1 + Lte1
Lte1 only
Kel1 only
Kel1 + Lte1
Lte1-GFP
Kel1-HA
Total extract
anti-GFP IP
B
no tag
Wild type
kel1Δ
0 20 40 60 80 100 120 140 0 20 40 60 80 100 120 140
Time (min)
Lte1-MYC
Kar2
C
Wild type
kel1Δ
% Lte1 loc
% budding
Percent cells
Time (min)
D
Wild type metaphase
kel1Δ metaphase
Wild type anaphase
kel1Δ anaphase
Percent cells
Time (min)
E
Strong Kel1 localization
Weak Kel1 localization
Kel1 delocalized
Percent cells
Wild type
GAL-LTE1

Figure 3

Figure 3: Kel1 forms a complex with Lte1 and is required for its localization to the bud cortex.
(A) Cells containing a *GAL-LTE1-GFP* fusion (A3587), a *KEL1-3HA* fusion (A6524), or both *GAL-LTE1-GFP* and *KEL1-3HA* (A6526) were grown in YEP+ Raffinose and induced with 2% Galactose for 3 hours. Cells were then harvested and Lte1-GFP was immunoprecipitated using anti-GFP antibodies as described in Materials and Methods. The presence of Kel1-HA in the immunoprecipitates was subsequently detected by western blot analysis.

(B - D) Wild type cells (A3717) and cells deleted for *KEL1* (A5479) both carrying *LTE1-13MYC* were arrested in G1 by addition of alpha-factor pheromone (5 μg/ml) for 2 hours. Cells were then released into media lacking pheromone and samples were taken at the indicated times to analyze Lte1-MYC protein levels (B), the percentage of budded cells and the percentage of cells with Lte1 localized in the bud (C) and the percentage of cells with metaphase and anaphase spindles (D).

(E) Wild-type (A6524) and *GAL-LTE1* (A6525) cells, carrying a *KEL-3HA* fusion were arrested with alpha-factor pheromone (5 μg/ml) for 2 hours. Overexpression of *LTE1* was induced for 3 hours by the addition of galactose and the percentage of cells with Kel1-HA localized to the shmoo tip was determined.

(Figure 4B). This decrease of phosphorylated Lte1 is not a consequence of loss of Lte1. from the bud, as Lte1 remains phosphorylated in *cdc12-6* mutants, despite the protein being delocalized (Figure 2C, D). The reason why Lte1 protein levels also decline at 37°C in the HU arrest is at present unclear, but may be due to enhanced protein turnover at this temperature. Lte1 was also not phosphorylated in *cdc42-17* cells progressing through the cell cycle in a synchronous manner (Figure 4E, H) indicating that Lte1 phosphorylation and localization depend on *CDC42* throughout the cell cycle.

Next we determined whether known effectors of Cdc42 were required for Lte1 localization in the bud. Bni1 is an effector of Cdc42 (reviewed in Gulli and Peter, 2001; Pruyne and Bretscher, 2000a) and has recently been shown to nucleate actin filaments (Pruyne et al., 2002; Sagot et al., 2002). Deletion of neither *BNI1* nor *STE20,* another downstream effector of *CDC42,* affected Lte1 localization or phosphorylation (Figure 4C, D; data not shown). Deletion of *CLA4*, in contrast, resulted in complete delocalization of Lte1 from the bud (Figure 4C). Furthermore, Lte1 was not significantly phosphorylated in exponentially growing *cla4Δ* cells (Figure 4D) or in *cla4Δ* cells progressing through the cell cycle in a synchronous manner (Figure 4F, G). Interestingly, the protein levels of the mitotic cyclin Clb2, which were examined to monitored cell cycle progression, were elevated, in cells lacking *CLA4* suggesting that this mutant has difficulties in inactivating mitotic CDKs, a key aspect of exit from mitosis (see below). Our results show that *CDC42* and *CLA4* but not *STE20* and *BNI1* are required for Lte1 phosphorylation and localization to the bud.

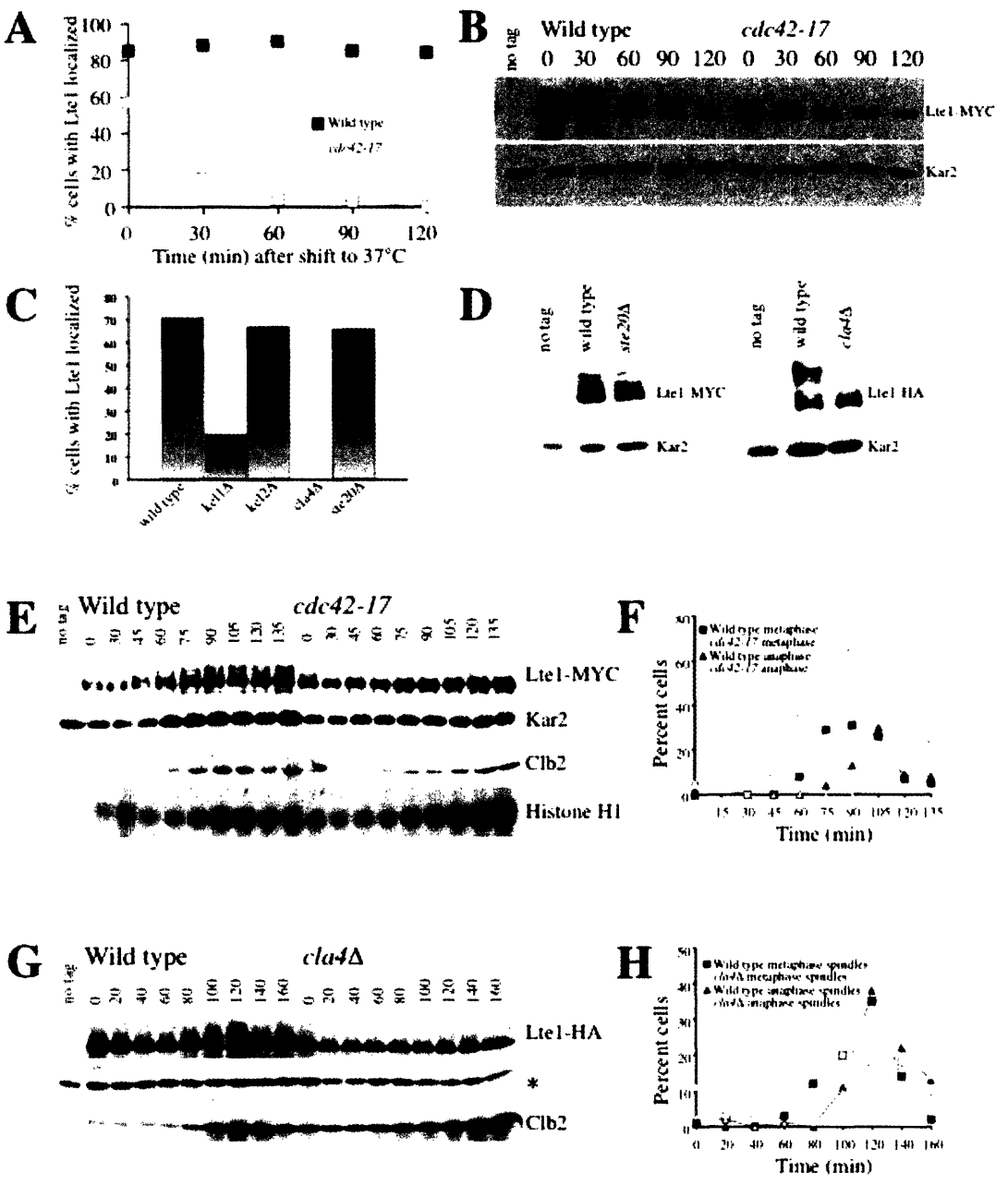
A
% cells with Lte1 localized
Wild type
cdc42-17
Time (min) after shift to 37°C
B
no tag
Wild type
cdc42-17
0 30 60 90 120 0 30 60 90 120
Lte1-MYC
Kar2
C
% cells with Lte1 localized
wild type
kel1Δ
kel2Δ
cla4Δ
ste20Δ
D
no tag
wild type
ste20Δ
Lte1-MYC
Kar2
no tag
wild type
cla4Δ
Lte1-HA
Kar2
E
Wild type
cdc42-17
Lte1-MYC
Kar2
Clb2
Histone H1
F
Percent cells
Time (min)
G
Wild type
cla4Δ
Lte1-HA
*
Clb2
H
Percent cells
Time (min)

Figure 4

Figure 4: *CDC42* and *CLA4* are required for Lte1 localization to the bud cortex and Lte1 phosphorylation.

(A, B) Wild type (A3717) and *cdc42-17* (A5556) cells carrying a *LTE1-13MYC* fusion were arrested for 2 hours with HU (10mg/ml) at 20°C. The cells were then shifted to 37°C while maintaining the HU arrest and the percentage of cells with Lte1 asymmetrically localized to the bud was quantitated by indirect immunofluorescence for each time point indicated (A). The phosphorylation status of Lte1 was also monitored by western blot analysis (B). Kar2 is shown as a loading control.

(C) Wild type (A2587), *kel1Δ* (A5479), *kel2Δ* (A5481), *cla4Δ* (A5718) and *ste20Δ* (A6125) cells carrying an *LTE1-13MYC* fusion were grown to exponential phase and the percentage of cells in which Lte1 was localized at the bud cortex was determined.

(D) Wild type (A1949) and *cla4Δ* (A5718) cells with an *LTE1-3HA* fusion and wild type and *ste20Δ* (A6125) cells carrying an *LTE1-13MYC* fusion were grown to exponential phase and the phosphorylation status of Lte1 was determined by Western blot analysis.

(E, H) Wild type (A3717) and *cdc42-17* (A5556) cells with an *LTE1-13MYC* fusion were arrested with 5μg/ml alpha-factor pheromone at 20°C for 2 hours. Cells were then released into media lacking pheromone at 37°C to inactivate *cdc42-17* and samples were taken at the indicated times. (E) Lte1-MYC phosphorylation status, Clb2 protein levels, and Clb2 kinase activity using histone H1 as a substrate. The percentage of metaphase and anaphase spindles is graphically represented in (H). Kar2 serves as a loading control.

(F, G) Wild type (A1949) and *cla4Δ* (A5718) cells with an *LTE1-3HA* fusion were arrested with 5μg/ml alpha-factor pheromone at 25°C for 2 hours. Cells were then released into media lacking pheromone and samples were taken at the indicated times to examine Lte1-HA protein phosphorylation status (F), Clb2 protein levels (F), and the percentage of cells with metaphase and anaphase spindles (G). The asterisk (*) indicates a cross-reacting band that serves as a loading control.

Overexpression of *CLA4* is sufficient to induce Lte1 localization and phosphorylation.

As *CLA4* is required for septin ring function (Holly and Blumer, 1999), which we have shown to be required for maintenance of Lte1 in the bud, the effect of *CLA4* on Lte1 localization could be indirect. To determine whether *CLA4* plays a more direct role, we asked whether overexpression of *CLA4* could induce Lte1 phosphorylation and localization. In cells arrested in G1 with α-factor, Lte1 is neither highly phosphorylated nor localized to a distinct region within the cell (Bardin et al., 2000; Lee et al., 2001). α-factor arrested cells are, however, polarized as many polarity determinants localize to the shmoo tip (reviewed in Casamayor and Snyder, 2002; Chant, 1999; Pruyne and Bretscher, 2000b). Overexpression of *CLA4* from the galactose-inducible *GAL1-10* promoter in α-factor arrested cells led to partial phosphorylation of Lte1 (Figure 5C) and caused the protein to localize to the shmoo tip in 30 percent of cells (Figure 5A). In HU-arrested cells, expression of *CLA4* from the *GAL1-10* promoter caused a higher degree of Lte1 phosphorylation and bud localization (Figure 5B). (Note that *GAL-CLA4* is the only copy of *CLA4* present in the cell, and thus no localization or phosphorylation of Lte1 is observed prior to galactose addition). Association of Lte1 with the shmoo tip, but not Lte1 phosphorylation caused by overexpressed *CLA4*, was significantly reduced when *KEL1*was deleted (Figure 5) indicating that localization of Lte1 induced by *CLA4* required *KEL1*.

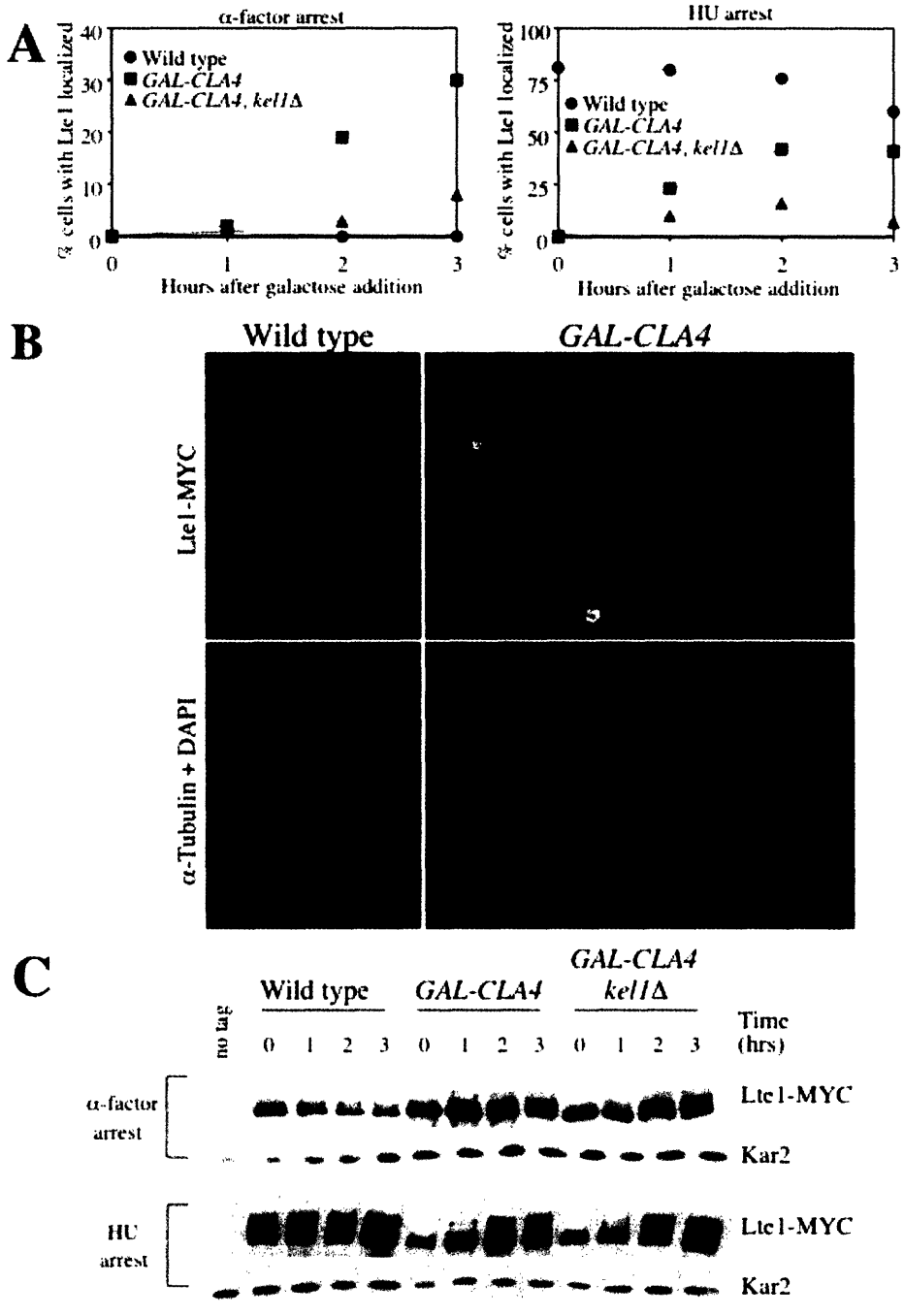
A
α-factor arrest
HU arrest
% cells with Lte1 localized
Wild type
GAL-CLA4
GAL-CLA4, kel1Δ
Hours after galactose addition
B
Wild type
GAL-CLA4
Lte1-MYC
α-Tubulin + DAPI
C
GAL-CLA4
Wild type
GAL-CLA4
kel1Δ
no tag
Time (hrs)
α-factor arrest
HU arrest
Lte1-MYC
Kar2

Figure 5

Figure 5: Overexpression of *CLA4* is sufficient to induce Lte1 phosphorylation and localization.

(A - C) Wild type cells (A3717) *GAL-CLA4::cla4* (A6023) and *GAL-CLA4::cla4 kel1Δ* (A6507) cells containing an *LTE1-13MYC* fusion were arrested with either 5μg/ml alpha-factor pheromone or 10mg/ml HU for two hours. Two-percent galactose was then added to induce the overexpression of *CLA4* while the arrests were maintained for the duration of the experiment. Samples were taken at the indicated times and the percentage of cells containing Lte1 in the bud (A, B) and the phosphorylation status of Lte1 (C) were examined. (B) shows the localization of Lte1 as detected by indirect immunofluorescence of both wild type and *GAL-CLA4* cells arrested with α-factor pheromone after a 3 hour galactose induction. Spindle morphology and DNA masses were visualized with an antibody against α-tubulin and 4'6-diamidino-2-phenylindole (DAPI) respectively.

Lte1 is dephosphorylated and delocalized from the bud during exit from mitosis.

Having identified some of the proteins that are required for Lte1 phosphorylation and localization to the bud cortex, we next asked how Lte1 phosphorylation and localization are lost during exit from mitosis. We first established the exact time during mitosis when Lte1 phosphorylation and localization from the bud were lost. Cells were arrested in late anaphase using a *cdc15-2* mutation (Surana et al., 1993). Upon release from the *cdc15-2* block, kinase activity associated with the mitotic cyclin Clb2 declined within 21 minutes (Figure 6C). Lte1 was dephosphorylated, as judged by a decrease in Lte1 mobility, 35 minutes after release from the *cdc15-2* block (Figure 6B). Loss of Lte1 from the bud occurred at the same time (Figure 6A).

Overexpression of *CDC14* is sufficient to induce Lte1 dephosphorylation and loss of Lte1 from the bud cortex.

Lte1 is dephosphorylated during exit from mitosis when the protein phosphatase Cdc14 is activated. This observation prompted us to investigate whether Lte1 dephosphorylation depended on *CDC14*. Indeed Lte1 was highly phosphorylated in *cdc14-3* mutants and *cdc15-2* mutants when Cdc14 is inactive, and was little, if at all phosphorylated when Cdc14 was hyperactive due to deletion of its inhibitory subunit *CFI1/NET1* (Figure 6D). Furthermore, overexpressing *CDC14* in S phase-arrested cells, when Lte1 is phosphorylated, caused dephosphorylation of Lte1 (Figure 6E), showing that

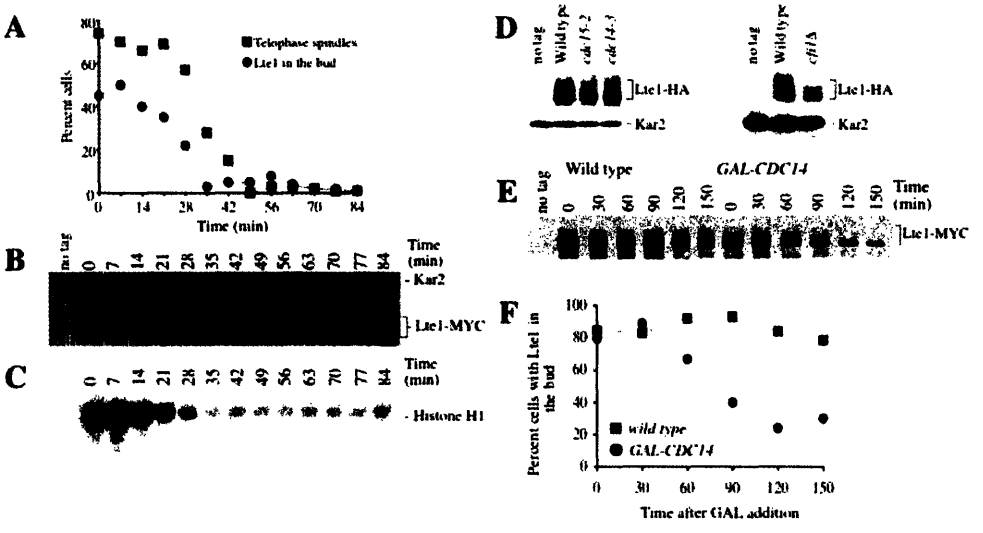

Figure 6: Loss of Lte1 localization from the bud cortex is controlled by *CDC14*. (A - C) *cdc15-2* (A3789) carrying a Lte1-13MYC fusion were incubated for 2 hours at 37°C to arrest cells in telophase. Subsequently cells were transferred into 23°C medium and the percentage of cells with telophase spindles and Lte1 localized to the bud (A) was analyzed at the indicated times. The phosphorylation status of Lte1 was monitored by Western blot analysis (B). Inactivation of Clb2 –associated kinase activity was assayed using Histone H1 as a substrate (C).

(D) Wild type (A1949), *cdc15-2* (A1960), and *cdc14-3* (A2252) cells carrying an *LTE1-HA* fusion were incubated at 37°C for 2 hours and Lte1 phosphorylation was examined by Western blot analysis (blot on the left). Wild type (A3717) and *cfi1Δ* (A3958) cells carrying a *LTE1-13MYC* fusion were harvested for Western blot analysis and Lte1 migration was analyzed (blot on the right). Kar2 was used as a loading control.

(E-F) Wild type (A3717) and *GAL-CDC14* (A3848) cells containing an *LTE1-13MYC* fusion were arrested in HU for 2 hours. *GAL-CDC14* transcription was then induced by addition of galactose while the HU arrest was maintained. Samples were taken to analyze Lte1 phosphorylation by Western blotting (E) and the percentage of cells with Lte1 localized to the bud (F).

overexpression of *CDC14* is sufficient to induce Lte1 dephosphorylation. The kinetics of Lte1 dephosphorylation upon overexpression of *CDC14* was similar to that observed for the Cdc14 substrate Bfa1 (Pereira et al., 2002). As soon as Lte1 dephosphorylation occurred due to overexpression of *CDC14*, bud-specific localization of Lte1 was lost (Figure 6F). Lte1 was, however, not dephosphorylated in *cdc12-6* or *kel1Δ* mutants (Figure 2D; 3B) indicating that loss of phosphorylation is not a consequence of loss of Lte1 from the bud cortex. The finding that the localization of another bud-specific protein, She3 (Bobola et al., 1996; Jansen et al., 1996), whose localization also depends on septins was not affected (Figure 7A, B), excluded the possibility that overexpression of *CDC14* caused a general loss of bud-specific proteins. Furthermore, the septin ring structure was not grossly affected and septin ring splitting was not induced by high levels of Cdc14 as judged by the localization pattern of a Cdc12-GFP (Figure 7C, data not shown). Our results show that high levels of Cdc14 are sufficient to cause the loss of bud-specific localization of Lte1. However, we have not been able to test whether Cdc14 can dephosphorylate Lte1 *in vitro*. Thus, we cannot exclude the possibility that *CDC14* is required for activation of a yet to be identified protein phosphatase that dephosphorylates Lte1.

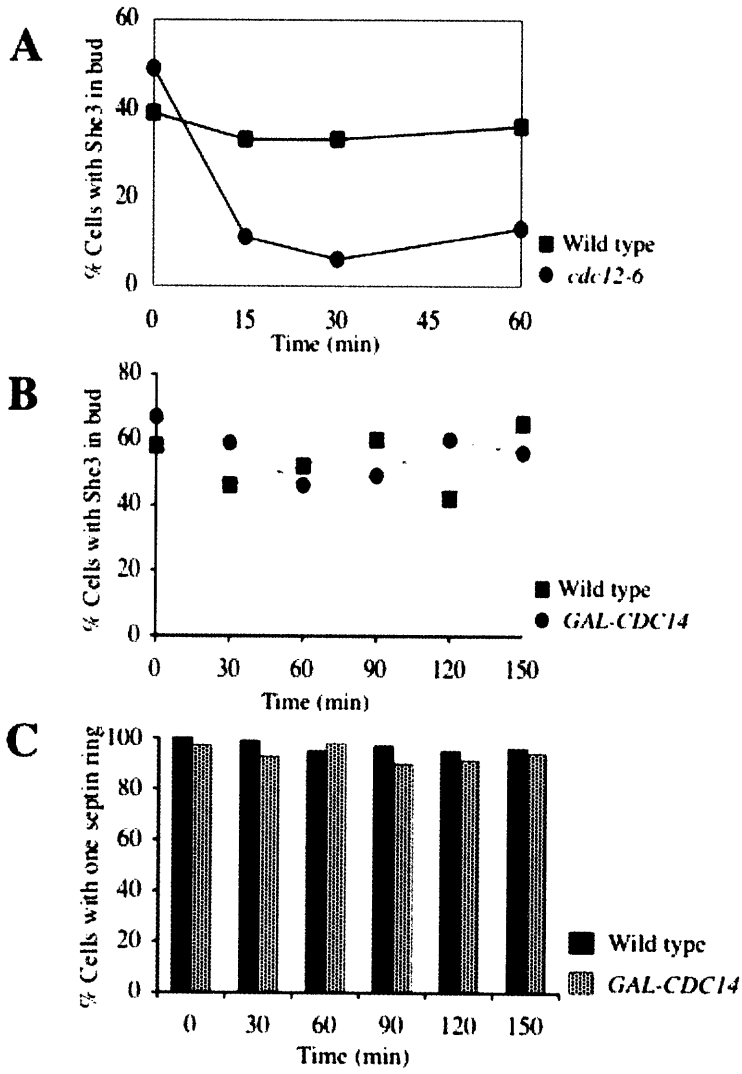
A
% Cells with She3 in bud
60
40
20
0
0
15
30
45
60
Time (min)
Wild type
cdc12-6
B
% Cells with She3 in bud
80
60
40
20
0
0
30
60
90
120
150
Time (min)
Wild type
GAL-CDC14
C
% Cells with one septin ring
100
80
60
40
20
0
0
30
60
90
120
150
Time (min)
Wild type
GAL-CDC14

Figure 7

Figure 7: Overexpression of *CDC14* does not affect She3 localization or septin ring structure.

(A) Wild type (A3987) and *cdc12-6* (A4469) cells containing a *SHE3-3MYC* fusion were arrested with HU for 2 hours. The cells were then shifted to 37°C and the percentage of cells with She3 confined to the bud was determined at the indicated times.

(B) Wild type (A3987) and *GAL-CDC14* (A4468) cells were arrested for 2 hours in the presence of HU. Galactose was then added to induce overexpression of *CDC14* (t=0). The percentage of cells with She3 localized to the bud was then determined at the indicated times.

(C) Wild type (A5336) and *GAL-CDC14* (A5505) cells containing a *CDC12-GFP* fusion on a centromeric plasmid were grown in SC-Leu+Raff medium and arrested and induced as described in (B). Samples were taken and Cdc12-GFP localization was visualized to examine septin ring morphology.

Figure 8: *KEL1* and *CLA4* are required for efficient exit from mitosis.
(A) Wild type (A1411) and *kel1Δ* (A6108) cells carrying a *CDC14-HA* fusion were arrested with 5μg/ml alpha-factor pheromone in YEP+ Raffinose medium at 25°C. Cells were then released into media lacking pheromone at 14°C and the percentage of cells with Cdc14 released from the nucleolus (circles), metaphase (squares) and anaphase (triangles) spindles was determined.

(B) Wild type (A3298) and *cla4Δ* (A6556) cells carrying a *CDC14-13MYC* fusion were arrested with 5μg/ml alpha-factor pheromone at 30°C. Cells were then released into media lacking pheromone at 14°C and the percentage of cells with Cdc14 released from the nucleolus (circles), metaphase (squares) and anaphase (triangles) spindles was determined.

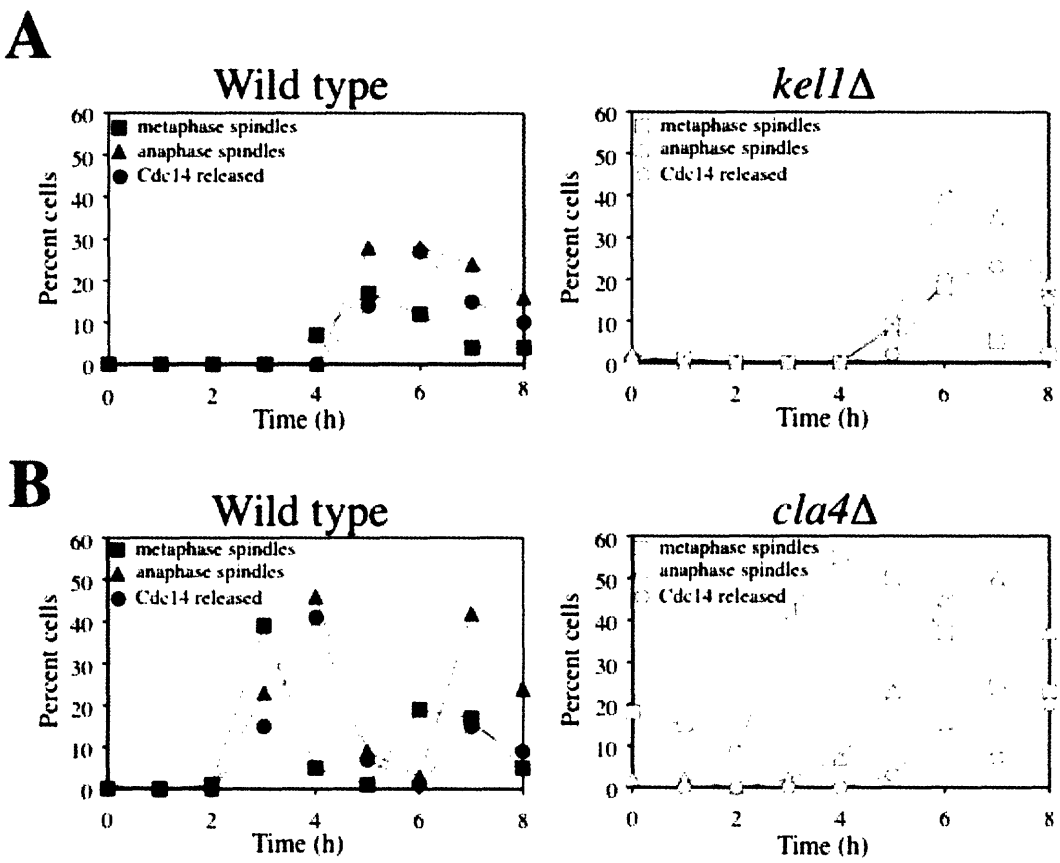
A
Wild type
kel1Δ
metaphase spindles
anaphase spindles
Cdc14 released
Percent cells
Time (h)
B
Wild type
cla4Δ
metaphase spindles
anaphase spindles
Cdc14 released
Percent cells
Time (h)

Figure 8

The roles of *KEL1* and *CLA4* in exit from mitosis.

The observation that *KEL1* and *CLA4* regulate the localization of Lte1 prompted us to investigate the consequence of inactivating these genes on exit from mitosis at low temperatures (14°C). Cdc14 release from the nucleolus and mitotic spindle disassembly were to some extent impaired in the mutant progressing through the cell cycle at 14°C (Figure 8A). This finding indicates that either a critical concentration of Lte1 in the bud was necessary for efficient exit from mitosis or that *KEL1* was also required for the function of Lte1 or other MEN components. Inactivation of *CLA4* had a much more dramatic effect on cell cycle progression than deletion of *KEL1*. At 14°C *cla4Δ* cells were severely delayed in release of Cdc14 from the nucleolus and mitotic spindle disassembly (Figure 8B; Hofken and Schiebel, 2002). The finding that exit from mitosis was moderately delayed in *kel1Δ* cells, but severely delayed in cells lacking *CLA4* indicates that *CLA4* not only regulates Lte1 localization but also other aspects of mitotic exit. We conclude that Kel1 and Cla4 are important for exit from mitosis at low temperatures.

Discussion

The localization of Lte1 is thought to play a role in regulating MEN. The restriction of Lte1 to the bud and the asymmetric localization of Tem1 to the daughter-bound SPB helps to couple nuclear migration to mitotic exit: Tem1 can only be activated by Lte1

when the daughter-bound SPB enters the bud (Bardin et al., 2000; Pereira et al., 2000). Bud-specific accumulation of Lte1 is also important to concentrate the protein therein to ensure efficient activation of Tem1 (Bardin et al., 2000). Here we investigate how Lte1 localization is regulated. We show that Lte1 anchorage at the bud cortex depends on septins, *CDC42, CLA4* and *KEL1*. We further show that *CLA4* is required for Lte1 phosphorylation and that the phosphorylation status of Lte1 affects its ability to localize to regions of polarized growth. The protein phosphatase Cdc14 appears to antagonize Lte1 phosphorylation and its association with the bud cortex, suggesting that *CDC14* controls the spatial asymmetry of Lte1 localization. Finally we show that Kel1 and Cla4, likely through the regulation of Lte1, control exit from mitosis.

Initial capture of Lte1 at the bud cortex.

Lte1 accumulation in the bud was largely unaffected when actin filaments were disrupted by Latrunculin A treatment. This finding indicates that active transport plays little if any role in creating Lte1 asymmetry. What mechanisms could be responsible for accumulation of Lte1 in the bud? Selective degradation of Lte1 in the mother cell is not likely to be responsible. In *cdc12-6* or *kel1 Δ* mutants, where Lte1 is no longer concentrated in the bud, the protein is found to be evenly distributed between mother cell and bud. Perhaps a diffusion – capture mechanism, whereby Lte1 diffuses throughout the cytoplasm and is captured by anchors at the bud cortex, promotes Lte1 localization.

Anchorage of Lte1 in the bud.

The septins, which function as a diffusion barrier at the bud neck (Barral et al., 2000; Takizawa et al., 2000), are required to maintain Lte1 at the bud cortex. However, it is not likely that septins themselves anchor Lte1 therein, as septins localize solely to the mother – bud neck. Could Kel1 be an Lte1 anchor in the bud? We believe so. Kel1 can be coimmunoprecipitated with Lte1 Figure 3A; (Gavin et al., 2002; Ho et al., 2002). Furthermore, deletion of *KEL1* significantly reduced the accumulation of Lte1 in the bud. It is also clear, however, that an association of Lte1 with Kel1 cannot be the sole determinant of Lte1 localization. First, Lte1 localization is not completely abolished in *kel1Δ* cells. Second, Lte1 does not localize to the presumptive bud site during G1 nor is it enriched at the shmoo tip. It is, thus, possible that Kel1 regulates Lte1 localization indirectly, by controlling anchors of Lte1 in the bud. Alternatively, owing to the relationship we observe between bud localization of Lte1 and Lte1 phosphorylation, we speculate that Lte1 phosphorylation could contribute to the localization of the protein.

Whether *KEL1* also regulates the exchange activity of Lte1 is an important question that remains to be addressed. Clearly, *kel1Δ* cells retain some Lte1 activity as this mutant does not arrest in telophase at 14°C, as *lte1Δ* cells do. However, Cdc14 release from the nucleolus and exit from mitosis were delayed in the mutant raising the possibility that either *LTE1* is not fully functional or that *KEL1* regulates some other aspect of mitotic exit.

Cdc42 and Cla4 regulate Lte1 phosphorylation and localization.

The experimental conditions chosen to determine *CDC42*'s role in Lte1 localization did not affect septin structure (Gladfelter et al., 2002) indicating that *CDC42* is directly regulating Lte1 anchorage at the bud cortex. The finding that Lte1 is not localized in cells lacking *CLA4* , however, could be explained by *CLA4's* role in septin ring formation, which, in turn, is required for confining Lte1 to the bud (Holly and Blumer, 1999). The observation that overexpression of *CLA4* is sufficient to target Lte1 to the shmoo tip in α-factor arrested cells where Lte1 is normally diffuse suggests otherwise. It is possible that ectopic overexpression of *CLA4* enables the septin ring to behave as a diffusion barrier around the shmoo tip. We favor the idea that *CLA4* is involved in anchoring Lte1 at the cortex independently of the septins and that *CDC42* exerts its function in Lte1 localization through *CLA4*.

How does Cla4 regulate Lte1 localization? Cla4 could directly anchor Lte1 at cortical regions or could play a regulatory role in this process. Cla4 protein is present and localized to shmoo tips in pheromone-arrested cells, but its kinase activity is low during this stage of the cell cycle (Benton et al., 1997; Holly and Blumer, 1999). If Cla4 were simply an anchor for Lte1 at the bud cortex, Lte1 should be localized to shmoo tips, but Lte1 is diffuse in α-factor arrested cells. Furthermore, overexpression of *CLA4* in stages of the cell cycle when its kinase activity is normally low, is sufficient to induce Lte1 phosphorylation and localization. We therefore, believe that it is Cla4's catalytic activity rather than presence of the protein *per se* that is important for Lte1 anchorage in the bud.

It is of course also possible that Cla4 acts both as an anchor and a regulator of Lte1 localization. Once the kinase function of Cla4 is activated, Cla4 could phosphorylate Lte1 as well as tether it at the bud cortex.

Several lines of evidence suggest that *CLA4* is not only required for Lte1 localization at the bud cortex but is also required for Lte1 phosphorylation. First, mirroring Lte1 phosphorylation, Cla4 kinase activity is low during G1 and early stages of S phase, accumulates as cells complete S phase, is high during mitosis and declines as cells exit from mitosis (Bardin et al., 2000; Benton et al., 1997; Lee et al., 2001); Figure 6B. Second, Lte1 phosphorylation is low in cells lacking *CLA4*. Finally, overexpression of *CLA4* is sufficient to induce Lte1 phosphorylation in stages of the cell cycle when the protein is normally not phosphorylated. The phosphorylation of Lte1 induced by overexpression of *CLA4* was less pronounced in α-factor than in HU-arrested cells. This could mean that overexpression of *CLA4* only partially overcomes the mechanisms that keep its kinase activity low during G1. Alternatively, other protein kinases could contribute to Lte1 phosphorylation. Lte1 phosphorylation has been shown to be decreased in mutants defective for the polo kinase *CDC5* (Lee et al., 2001). As Lte1 phosphorylation is higher in states of high Clb-CDK activity, Clb-CDKs could also contribute to Lte1 phosphorylation.

Cells lacking *CLA4* were severely delayed in telophase, indicating that *CLA4* was required for exit from mitosis at low temperatures. The defect in exit from mitosis in *cla4Δ* mutant is not solely due to Lte1 being mis-localized. In *kel1Δ* cells, where Lte1 is delocalized, Cdc14 release from the nucleolus and exit from mitosis are modestly delayed

compared to *cla4Δ* cells. This indicates that *CLA4* has roles in exit from mitosis other than anchoring Lte1 in the bud. *CLA4* could regulate proteins other than Lte1 during exit from mitosis. Alternatively, *CLA4* could not only regulate Lte1 localization and phosphorylation, but also exchange activity.

A correlation between Lte1 phosphorylation and localization.

We find that when Lte1 is phosphorylated, it localizes to the bud, and when it is dephosphorylated, it is present uniformly throughout the cell. When Lte1 dephosphorylation is induced by overexpressing *CDC14,* the bud-specific localization of the protein is lost. Conversely, when *CLA4* is overexpressed in α-factor arrested cells, Lte1 becomes partially phosphorylated and localized to the shmoo tip. One possibility is that phosphorylation/dephosphorylation induces changes in the structures responsible for anchoring Lte1 in the bud. However, overexpression of *CDC14* in HU arrested cells did not lead to a general loss in bud-specific protein accumulation as She3 localization was not perturbed by high levels of Cdc14. Another possibility that we were able to exclude was that loss of Lte1 from the bud causes dephosphorylation of the protein as Lte1 was not dephosphorylated in *cdc12-6* or *kel1Δ* cells, where Lte1 localization is lost. Based on these observations, we favor the idea that the phosphorylation status of Lte1 itself determines the protein's localization. The key test of this hypothesis would be to examine the consequences of mutating the phosphorylated residues in Lte1 to amino acids that can no longer be phosphorylated, and to then examine the effects of these mutations on Lte1 localization. This analysis is complicated by the fact that Lte1 is a very large protein (170

kD) and we were thus far unable to eliminate all phosphorylated residues in Lte1 (A. S., unpublished observations).

A model for the regulation of Lte1 localization.

We would like to propose a model where Lte1 localization is regulated by at least two determinants: proteins that anchor Lte1 at the bud cortex and phosphorylation of Lte1 itself. Anchorage of Lte1 at the bud cortex requires septins, Kel1 and phosphorylation of Lte1 mediated by the kinase Cla4. Once in the bud, Lte1 appears to be maintained in a strictly cortical manner during early stages of the cell cycle, but becomes increasingly cytoplasmic during later cell cycle stages. It appears that the protein is being "released " from its cortical anchors. We propose that Cdc14 induces dephosphorylation of Lte1, which triggers the release of Lte1 from cortical anchors in the bud. Dephosphorylated cytoplasmic Lte1, which is not restricted by the cortical septin barrier, then diffuses throughout mother and daughter cell cytoplasm leading to equal distribution of Lte1 prior to cytokinesis.

We further suggest that Cdc14, whose activity oscillates throughout the cell cycle, is employed to create changes in the spatial distribution of Lte1. Another temporal oscillator, CDK activity, has previously been implicated in creating asymmetry. In *Drosophila melanogaster*, the neural progenitor determinant Inscuteable localizes to the apical cortex of the cell and the maintenance of this localization during mitosis requires *CDC2* activity (Tio et al., 2001). Thus it may well be that employing a temporal oscillator in creating spatial asymmetry is a common theme in the regulation of cell division.

Materials and Methods

Yeast Strains and Growth Conditions

All strains were derivatives of strain W303 (K699). The *GAL-LTE1-GFP*, *LTE1-13MYC*, *kel1::HIS3*, *kel2::HIS3*, *cla4::kanMX*, *ste20::HIS3* and *GAL-CLA4* strains were constructed using the PCR-based method described in (Longtine et al., 1998). Growth conditions for individual experiments are described in the figure legends. In cases where growth media is unspecified, cells were grown in YEP + 2% glucose.

Immunoblot Analysis and Clb2 Kinase Assays

For immunoblot analysis of the total amount of Clb2, Lte1-3HA, Lte1-13MYC, and Kar2, 10 ml samples of cells were harvested and pellets were resuspended in lysis buffer (50mM Tris-HCl pH 7.5, 1mM EDTA, 60mM β-glycerophosphate, 0.1mM $NaVO_3$, 15mM para-Nitro-phenyl phosphate, 40μg/ml Aprotinin, 50mM DTT, 1mM Pefablock (Boehringer Manheim), 10μg/ml TLCK (Sigma), 10μg/ml TPCK (Sigma), 10μg/ml Pepstatin A (Sigma), 10μg/ml Leupeptin (Sigma)). TCA extracts were prepared by the addition of 100μl 20%TCA to cell pellets instead of lysis buffer. Glass beads (Sigma) were added and samples were vortexed for 10 minutes on a Vibrax vortexer. Samples were run on either a 6% SDS-PAGE gel (for Lte1) or 10% SDS-PAGE gel (for Clb2). Clb2 kinase activity was assayed as described in (Surana et al., 1993).

Co-immunoprecipitation analysis

Cells were harvested, washed twice with 10mM Tris, pH7.5, and resuspended in CO-IP buffer (1% NP40 (weight/ volume), 150mM NaCl, 50mM Tris, pH7.5, 60mM β-glycerophosphate, 0.1mM $NaVO_3$, 15mM para-Nitro-phenyl phosphate, comeplete EDTA-free protease inhibitor cocktail (Roche)). Anti-GFP antibody (Chemicon) was added to each sample and incubated for 1 hour at 4°C. Protein-G coupled beads were then added and samples were incubated with rotation at 4°C for 2 hours. Samples were washed 6 times with CO-IP buffer, boiled in SDS and run on a 6% SDS-PAGE gel for subsequent western blot analysis.

Fluorescence microscopy

Indirect in situ immunofluorescence methods were as described in (Bardin et al., 2000). Primary anti-HA mouse antibody (Covance) was used at a1:400 dilution. Primary anti-MYC antibody (Covance) was used at a 1:1000 dilution. Secondary anti-mouse antibodies (Jackson Laboratories) were used at a concentration of 1:800 or 1:1500, respectively. Phalloidin staining was performed on cells without methanol/acetone fixation using anti-phalloidin-FITC conjugated antibodies (Molecular Probes) for 30 minutes at a 1:5 dilution. GFP-tagged proteins were visualized after 10 minutes of fixation performed as described below. Cells were analyzed on a Zeiss Axioplan 2 microscope and images were captured using a Hamamatsu camera controller. Openlab 3.0.2 software was used to process immunofluorescence images. At least 100 cells were analyzed per time point.

Deconvolution microscopy

Cells were grown and induced as indicated in the legend to Figure 1. For live cell imaging, cells were spun down and resuspended in 50μl synthetic complete (SC) media containing 2% glucose. Cells were then mounted on a glass slide and observed on a DeltaVision microscope system with a 100X objective at room temperature. DeltaVision software was used to deconvolve each Z-series. To create three-dimensional movie frames, VolumeViewer was used after deconvolution. For fixed cell imaging, cells were fixed for 10 minutes in 2% formaldehyde. Cells were then washed in 0.1M potassium phosphate buffer, pH 6.6 and resuspended in the same. Microscopy was performed as for live cells.

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Chapter III: Ras and the Rho effector Cla4 collaborate to target and anchor Lte1 at the bud cortex

A version of this manuscript has been submitted for publication by Seshan, A.

and Amon A.

Summary

Lte1, a protein important for exit from mitosis, localizes to the bud cortex as soon as the bud forms and remains there until cells exit from mitosis. Ras, the Rho GTPase Cdc42 and its effector the protein kinase Cla4 are required for Lte1's association with the bud cortex. Here we investigate how Ras, and the Cdc42 effector Cla4 regulate the localization of Lte1. We find that Ras2 and Lte1 associate in stages of the cell cycle when Lte1 is phosphorylated and associated with the bud cortex and that this association requires *CLA4*. Additionally, *RAS1* and *RAS2* are required for *CLA4*-dependent Lte1 phosphorylation. Our findings suggest that Cla4-dependent phosphorylation promotes the initial association of Lte1 with Ras at the bud cortex and that Ras is required to stabilize phosphorylated forms of Lte1 at the bud cortex. Our results also raise the interesting possibility that the localization of Lte1 affects the protein's ability to promote mitotic exit.

Introduction

In its GTP-bound state, Ras activates a multitude of effectors thereby promoting cell growth and proliferation. Budding yeast contains two Ras proteins, Ras1 and Ras2. In the presence of ample nutrients, these two proteins redundantly promote the production of cAMP by activation of adenylate cyclase. cAMP in turn activates protein kinase A (PKA), an important regulator of the G1 – S phase transition in budding yeast, by binding to its inhibitory subunit Bcy1 and promoting its dissociation from PKA [reviewed in reference (Thevelein and de Winde, 1999)]. In addition to controlling the G1 – S phase transition in response to nutrient availability, Ras also regulates exit from mitosis in budding yeast. When the essential role of Ras in promoting the G1 – S phase transition is eliminated (by deleting *BCY1)*, cells lacking both *RAS1* and *RAS2* are able to proliferate at room temperature (Yoshida et al., 2003). However, at low temperatures, cells lacking both Ras homologs arrest in late anaphase prior to exit from mitosis (Morishita et al., 1995; Yoshida et al., 2003), indicating that Ras is also required for this cell cycle transition.

Exit from mitosis is characterized by mitotic spindle disassembly and requires the inactivation of mitotic cyclin-dependent kinases (Clb-CDKs), brought about by the protein phosphatase Cdc14 [reviewed in reference (Stegmeier and Amon, 2004)]. A GTPase signaling cascade known as the Mitotic Exit Network (MEN) regulates the activity of Cdc14. It promotes the release of the protein phosphatase from its inhibitor Cfi1/Net1 in the nucleolus during anaphase. The GTPase Tem1acts at or near the top of the MEN pathway and is negatively regulated by a GTPase activating protein (GAP)

complex composed of Bub2 and Bfa1 and positively regulated by a putative exchange factor Lte1 (Stegmeier and Amon, 2004). Activation of Tem1 is thought to stimulate the protein kinase Cdc15, which in turn activates the protein kinase Dbf2 and its associated factor Mob1. Activation of Dbf2 eventually contributes to the release of Cdc14 from its inhibitor in the nucleolus, but the mechanism(s) whereby the protein kinase accomplishes this are not known (Stegmeier and Amon, 2004).

The activation of the GTPase Tem1 by the MEN activator Lte1 is thought to contribute to the stimulation of MEN signaling. The effects of Lte1 stimulation are seen in cells grown at low temperatures, when Lte1 is essential for exit from mitosis (Shirayama et al., 1994) and in cells in which the anaphase nucleus is mispositioned (Bardin et al., 2000; Castillon et al., 2003; Pereira et al., 2000). Cells defective in aligning the mitotic spindle along the mother – bud axis undergo anaphase entirely within the mother cell (Carminati and Stearns, 1997; Yeh et al., 1995). Such mutants are prevented from exiting from mitosis until the mitotic spindle aligns properly by the dual action of the GAP complex Bub2/Bfa1 and the spatial segregation of Lte1 and Tem1 in these cells (Bardin et al., 2000; Castillon et al., 2003; Pereira et al., 2000). Tem1 and Lte1 are spatially segregated until the nucleus moves into the bud during anaphase. Lte1 is distributed throughout the cell in G1, but becomes sequestered in the bud during S phase. Tem1 localizes to the spindle pole body (SPB) destined to migrate into the bud. Thus, only after nuclear division is initiated and the nucleus moves into the bud are Tem1 and Lte1 in the same cellular compartment (Bardin et al., 2000; Pereira et al., 2000).

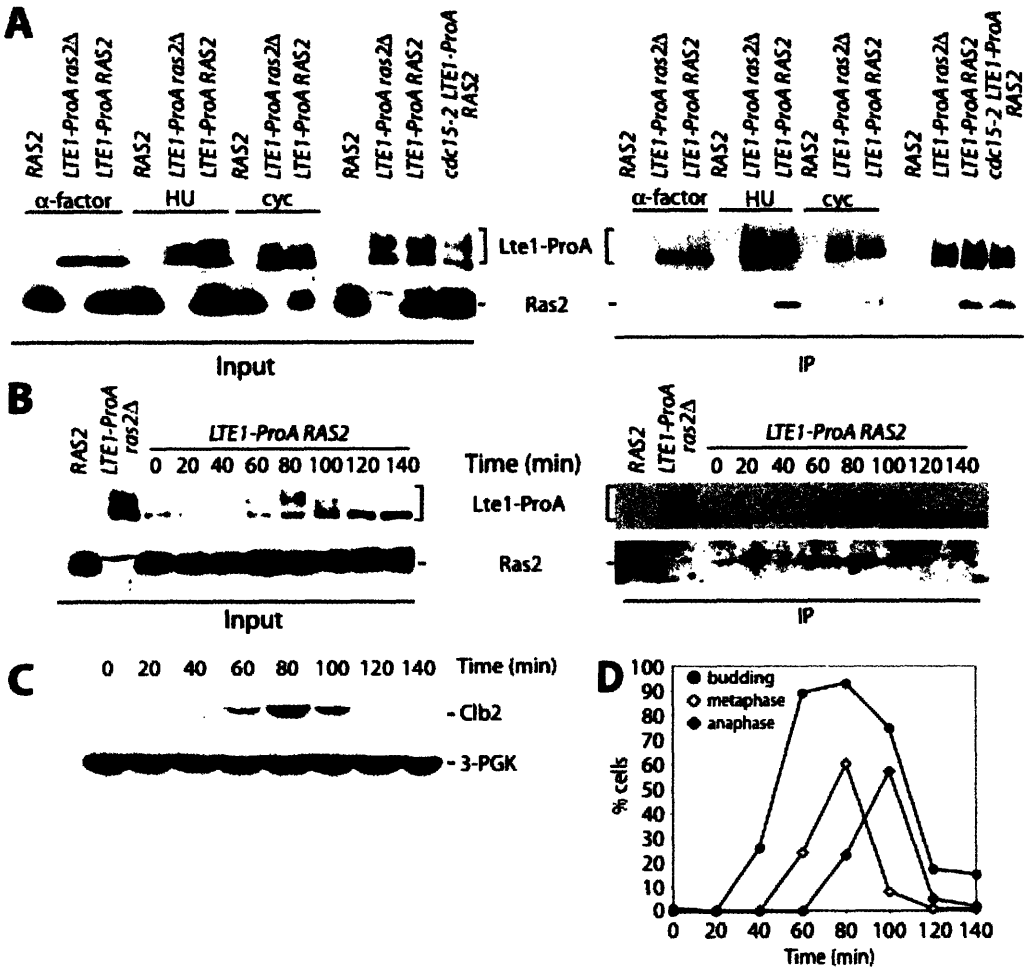
The nature of Lte1's biochemical activity and the mechanism by which this protein promotes Tem1 function have both remained unclear. However, a recent study by Yoshida et al. showed that Lte1 is regulated by Ras, hence providing insight into how Ras controls exit from mitosis (Yoshida et al., 2003). *RAS1* and *RAS2*, like *CDC42* and its effector *CLA4*, are required for localizing Lte1 to the bud cortex and for exit from mitosis at low temperatures. Here we investigate how Ras and the Cdc42 effector Cla4 regulate the localization of Lte1. We find that Lte1 preferentially associates with the GTP-bound form of Ras *in vivo* and requires residues within Ras necessary for the GTPase's interaction with adenylate cyclase. Importantly, we find that Ras2 and Lte1 only form a complex in stages of the cell cycle when Lte1 is phosphorylated and associated with the bud cortex. This association between Ras2 and Lte1 requires *CLA4*. Likewise, *RAS1* and *RAS2* are required for *CLA4*-dependent Lte1 phosphorylation. We conclude that Cla4-dependent phosphorylation promotes the initial association of Lte1 with Ras at the bud cortex and that Ras is required to stabilize phosphorylated forms of Lte1 at the bud cortex. Our data further suggest that the cortical association of Lte1 with Ras2 is important for Lte1 function.

Results

Lte1 and Ras2 form a complex from S phase until anaphase.

Lte1 associates with the bud cortex from the time of bud formation until anaphase. During exit from mitosis, the protein dissociates from the bud cortex into the cytoplasm, where it remains throughout G1 (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan

et al., 2002). How Lte1 is anchored at the bud cortex is poorly understood, but requires *CDC42*, its effector *CLA4* as well as the redundant Ha-Ras homologs *RAS1* and *RAS2* (Chiroli et al., 2003; Hofken and Schiebel, 2002; Seshan et al., 2002; Yoshida et al., 2003). *RAS1* and *RAS2* are not only required for Lte1 localization to the bud cortex, but also form a complex with Lte1 *in vitro* and *in vivo* (Yoshida et al., 2003). These data suggest that Ras either directly anchors Lte1 at the bud cortex or is required for Lte1 bud anchors to capture the protein. In either case, Lte1 should be found in a complex with Ras only in stages of the cell cycle when Lte1 associates with the bud cortex, that is from S phase until anaphase but not when Lte1 is cytoplasmic during G1. We found that Ras2 co-immunoprecipitates with Lte1 in S-phase arrested cells (using hydroxyurea to arrest cells; HU; Figure 1A); and anaphase-arrested cells (using a temperature sensitive *cdc15-2* mutant to arrest cells), but not in G1-arrested cells (using α-factor pheromone to arrest cells), implying that the proteins form a complex only from S phase to anaphase. Similar results were obtained when we analyzed Lte1 - Ras2 complex formation in cells progressing through the cell cycle in a synchronous manner. Concomitant with bud formation, 60 minutes upon release from a α-factor-induced G1 arrest, Ras2 and Lte1 were found in a complex (Figure 1B, D). The association between the two proteins initiated well before the metaphase peak and persisted until anaphase. As cells exited mitosis, as judged by the decline in Clb2 protein levels and anaphase spindle disassembly, complex formation between Ras2 and Lte1 was lost (Figure 1B-D). Our results indicate that Lte1 only forms a complex with Ras2 when the protein is present at the bud cortex, indicating that Lte1 – Ras2 complex formation is spatially and temporally regulated during the cell cycle.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Input
IP
B
LTE1-ProA RAS2
0 20 40 60 80 100 120 140
Time (min)
C
- Clb2
- 3-PGK
D
budding
metaphase
anaphase
% cells
Time (min)

Figure 1

Figure 1: The association between Lte1 and Ras2 is cell cycle regulated.

(A) *ras2Δ* cells carrying a *LTE1-ProA* fusion (A11370), wild type cells carrying a *LTE1-ProA* (A7750), and wild type untagged cells (A2587) were arrested with either 10 mg/ml hydroxyurea (HU) for 2 hours 30 min, 5μg/ml alpha-factor for 2 hours, or allowed to cycle through the cell cycle. *cdc15-2* cells containing a *LTE1-ProA* fusion (A9746) were incubated for 2 hours at 37°C to arrest cells in telophase prior to mitotic exit. HU-arrested samples of wild type (A2587), *ras2Δ LTE1-ProA* (A11370) and *LTE1-ProA* (7750) were included as controls for the *cdc15-2* arrest experiment. Lte1 immunoprecipitates were examined by Western blot analysis for the presence of Lte1 and Ras2.

(B-D) Wild type cells carrying a *LTE1-ProA* fusion (A7750) were arrested in G1 by addition of alpha-factor pheromone (5μg/ml) for 2 hours. Cells were then released into medium lacking pheromone and samples were taken at the indicated times to analyze (B) the co-immunoprecipitation of Ras2 with Lte1, (C) protein levels of the mitotic cyclin Clb2 and (D) the percentage of cells with metaphase and anaphase spindles and buds. Wild type (A2587) and *LTE1-ProA ras2Δ* (A11370) cells were included as negative controls for the co-immunoprecipitation experiments. 3-PGK is included as a loading control in the Western blot analysis.

***CDC14* regulates the association between Lte1 and Ras2.**

Our findings revealed a correlation between the phosphorylation status of Lte1 and its ability to associate with Ras2 (Figure 1). In G1 cells, Lte1 is not highly phosphorylated, and it cannot associate with Ras2. In contrast, highly phosphorylated forms of Lte1 predominate from S phase to anaphase, which is when an association between Lte1 and Ras2 is detected [Figure 1; (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et al., 2002)]. We therefore investigated the role of Lte1 phosphorylation in Lte1 – Ras2 complex formation. Cdc14 is the ultimate effector of the Mitotic Exit Network (MEN) and, when overproduced, is sufficient to induce Lte1 dephosphorylation and the dissociation of Lte1 from the bud cortex(Jensen et al., 2002; Seshan et al., 2002). In order to examine the effects of Cdc14 on the Lte1-Ras2 complex, we examined whether overexpression of *CDC14* was able to induce the dissociation of the Lte1-Ras2 complex. Cells containing *CDC14* under the control of the galactose-inducible *GAL1-10* promoter were arrested in S phase with HU. Galactose was then added to induce the overexpression of *CDC14* while maintaining the HU arrest. Sixty minutes after galactose addition, Ras2 was undetectable in Lte1 immunoprecipitates from *GAL-CDC14* cells (Figure 2). Loss of the interaction between Lte1 and Ras2 occurred concomitantly with the dephosphorylation and delocalization of Lte1 from the cell cortex that is observed in *GAL-CDC14* induced cells [Figure 3; (Jensen et al., 2002; Seshan et al., 2002)] Our results suggest that phosphorylation of Lte1 is important for the protein's ability to associate with Ras2.

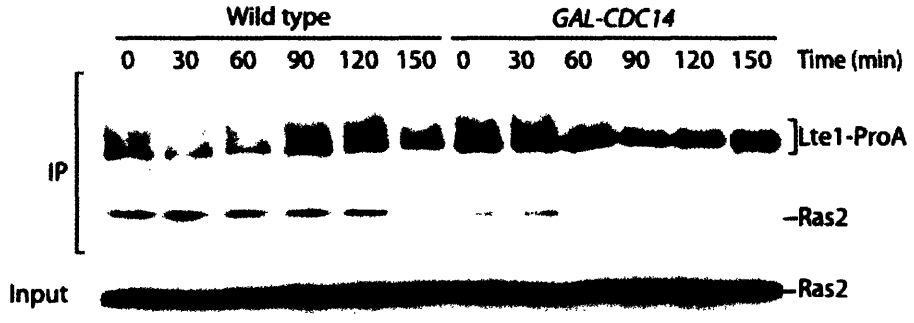

Figure 2: Cdc14 promotes the dissociation of the Ras2 – Lte1 complex.
Wild type (A7750) and *GAL-CDC14* (A10936) cells containing a *LTE1-ProA* fusion were arrested in 10 mg/ml HU for 2 hours 30 min. *GAL-CDC14* transcription was then induced by addition of galactose while the HU arrest was maintained throughout the course of the experiment. Samples were taken at the times indicated to examine the association of Ras2 with Lte1 by co-immunopreciptiation analysis.

Lte1 associates with activated forms of Ras2 and requires residues in Ras that are important for binding to adenylate cyclase.

Lte1 has been shown to associate with GTP-bound Ras, but not with GDP-bound Ras *in vitro* (Yoshida et al., 2003). To determine whether Lte1 exhibits an increased affinity for Ras-GTP *in vivo*, we examined the ability of Lte1 to associate with a mutant form of Ras in which glycine 19 was mutated to valine (*RAS2*^{V19}). This mutant Ras protein exhibits decreased GTPase activity, which is associated with increased stability of the GTP-Ras complex (Crechet et al., 1990). Ras2^{V19} co-immunoprecipitated with Lte1 in HU-arrested cells more efficiently than wild type Ras2 (Figure 3, lanes 2 and 5) suggesting that Lte1 binds preferably to Ras-GTP *in vivo*.

LTE1 was first identified as a multicopy suppressor of the *RAS2*^{V19} heat shock sensitivity (Shirayama et al., 1994), a phenotype characteristic of mutants in which the PKA pathway is overstimulated (Nikawa et al., 1987; Sass et al., 1986). It has been suggested that Lte1 performs this function by binding directly to Ras^{V19} and preventing its association with adenylate cyclase, thereby quenching the hyper-activation of the PKA pathway (Yoshida et al., 2003). To further investigate whether the regions within Ras necessary for the interaction with adenylate cyclase are also important for the interaction with Lte1, we examined two alleles of *RAS2* (*RAS2*^{V19G41} and *RAS2*^{V19N45}) that are defective in associating with adenylate cyclase but are capable of activating the Ste20-MAPK pathway (Akasaka et al., 1996; Mosch et al., 1999). Both mutations reduced the interaction between Ras2^{V19} and Lte1 (Figure 3, lanes 3 and 4) indicating that residues within Ras2 that are important for binding to adenylate cyclase are also important for the interaction between Lte1 and Ras2. These results indicate that Lte1 could function as a

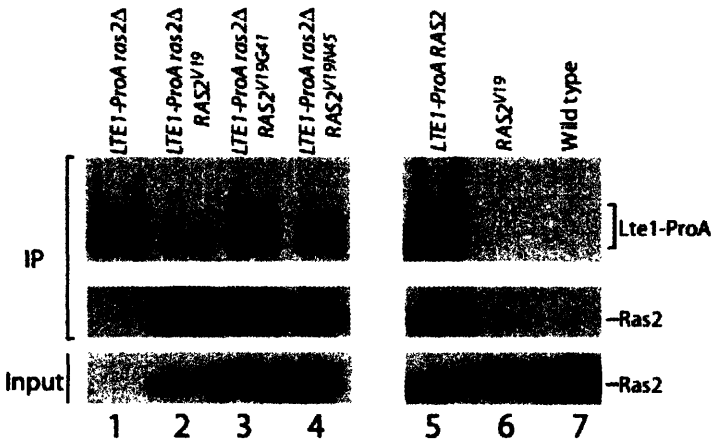

Figure 3: Lte1 associates with activated forms of Ras2 and requires residues within Ras2 necessary for adenylate cyclase binding.

ras2Δ cells carrying an *LTE1-ProA* fusion and transformed with YCplac22 (A12867), YCplac22-*RAS2*^{V19} (A12868), YCplac22-*RAS2*^{V19G41} (A12869) or YCplac22-*RAS2*^{V19N45} (A12870) were arrested in SC-Trp medium with 10 mg/ml HU for 2 hours 15 min at 30°C. The association of Ras2 mutants with Lte1 was analyzed by co-immunopreciptiation analysis. Wild type cells carrying an *LTE1-ProA* fusion were also arrested in SC medium as above and the co-immunoprecipitation of Ras2 with Lte1 was examined. Wild type untagged cells transformed with YCplac22 (A12871) and YCplac22-*RAS2*^{V19} (A12872) were included as negative controls.

competitive inhibitor of Cyr1 binding to $Ras2^{V19}$ *in vivo*. Additionally, since *STE20* has previously been shown to be dispensable for Lte1 localization (Hofken and Schiebel, 2002; Seshan et al., 2002), these data lend support to the idea that Ras2-GTP directly recruits Lte1 to the bud cortex.

CDC42 and _CLA4_ but not septins are required for the association between Lte1 and Ras2.

The septin ring, which encircles the mother – bud neck, plays a key role in restricting Lte1 to the bud cortex. Cells carrying mutations in its components fail to restrict Lte1 and many other proteins to the bud cortex (Barral et al., 2000; Hofken and Schiebel, 2002; Seshan et al., 2002; Takizawa et al., 2000). The Rho-like GTPase Cdc42 and its effector Cla4 are also required for Lte1's association with the bud cortex (Chiroli et al., 2003; Hofken and Schiebel, 2002; Seshan et al., 2002). To determine whether any of these genes were required for the association between Lte1 and Ras2, we examined the ability of Lte1 to co-immunoprecipitate Ras2 in cells defective in the function of the septin ring, *CDC42* or *CLA4*. Ras2 was efficiently co-immunoprecipitated with Lte1 in cells carrying a deletion in the septin ring component *SHS1/ SEP7 (*Figure 4A) and in cells carrying a temperature- sensitive allele of the septin *CDC12* (Figure 4B). Lte1 was also found in a complex with Ras2 in cells lacking the Kelch-repeat protein *KEL1*, which has been implicated in being required for Lte1-HA localization to the bud cortex but not for that of an Lte1-GFP fusion [Figure 4C; data not shown; (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et al., 2002)]. These results indicate that neither *KEL1* nor an intact

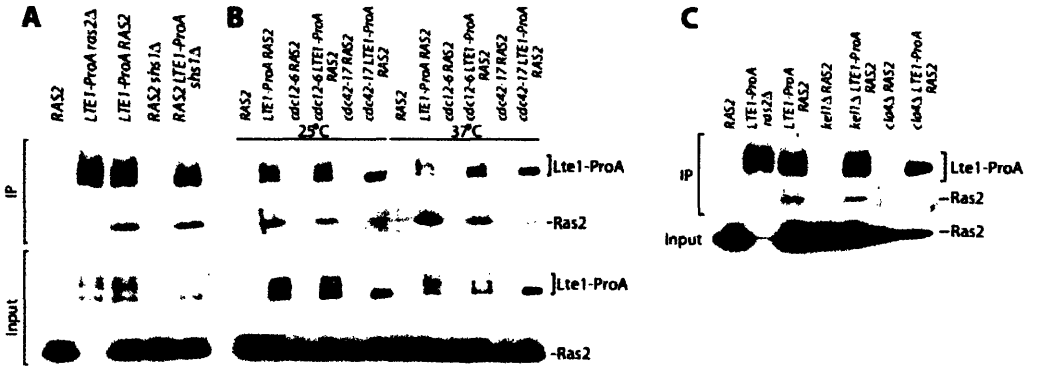

Figure 4: The association of Ras2 and Lte1 depends upon *CLA4*, but not the septin ring.

(A) Untagged wild type (A2587) and *shs1Δ* (A11708) cells in addition to wild type (A7750), *ras2Δ* (A11370) and *shs1Δ* (A11701) cells carrying an *LTE1-ProA* fusion were arrested in HU for 2 hours 20 minutes. Cells were collected and subjected to immunoprecipitation analysis. The presence of Ras2 in Lte1 immunoprecipitates was determined.

(B) Wild type (A2587), *cdc12-6* (A4270), and *cdc42-17* (A5395) untagged cells and wild type (A7750), *cdc12-6* (11753) and *cdc42-17* (A11257) cells carrying an *LTE1-ProA* fusion were arrested at 20°C with 10 mg/ml HU for 2 hours 30 min and half of the cultures were collected. The remainder of each culture was then shifted to 37°C for 45 minutes and an additional 5 mg/ml HU was added. Samples were collected to analyze the association between Lte1 and Ras2.

(C) Wild type (A2587), *kel1Δ* (A5313), and *cla4Δ* (A5816) cells and wild type (A7750), *kel1Δ* (A9748), *cla4Δ* (A11130) and *ras2Δ* cells carrying an *LTE1-ProA* fusion were arrested with 10 mg/ml HU for 2 hours 30 min and collected. Each sample was then analyzed for the co-immunoprecipitation of Ras2 with Lte1.

septin ring is necessary for Lte1 and Ras2 to form a complex. In contrast, Lte1 failed to associate with Ras2 in *cdc42-17* mutants (Figure 4B) or cells lacking *CLA4* (Figure 4C), indicating that the Rho GTPase and its effector *CLA4* are required for the association of Lte1 with Ras2. These findings, together with the observation that Lte1 is phosphorylated and capable of promoting inappropriate mitotic exit in *shs1Δ/sep7Δ* mutants (Castillon et al., 2003), but not phosphorylated and impaired for mitotic exit in *cdc42-17* mutants and *cla4Δ* mutants, raise the interesting possibility that the association of Lte1 with Ras at the bud cortex is regulated by phosphorylation and that bud cortex association and/or Lte1 phosphorylation is important for Lte1's mitotic exit promoting activity (see Discussion).

Ras is required for Lte1 phosphorylation but fails to target Lte1 to the bud cortex. The protein kinase Cla4 is not only required for the association of Lte1 with Ras2, but also for Lte1 phosphorylation (Hofken and Schiebel, 2002; Seshan et al., 2002). Interestingly, Ras also appears to be important for Lte1 phosphorylation. In cells lacking both *RAS1* and *RAS2*, slower migrating forms of Lte1, which are due to phosphorylation (Bardin et al., 2000), did not accumulate, whereas the absence of only *RAS2* did not affect Lte1 phosphorylation [Figure 5A, B; *BCY1* was also deleted in these cells to retain viability; (Toda et al., 1987)]. Furthermore, we found that *CLA4*-induced targeting to the shmoo tip and phosphorylation of Lte1 in α-factor arrested cells depended on Ras. Overexpression of *CLA4* in alpha-factor arrested wild-type cells caused both the accumulation of Lte1 at the shmoo tip and ectopic Lte1 phosphorylation [Figure 5C, D; (Hofken and Schiebel, 2002; Seshan et al., 2002)]. In contrast, overexpression of *CLA4*

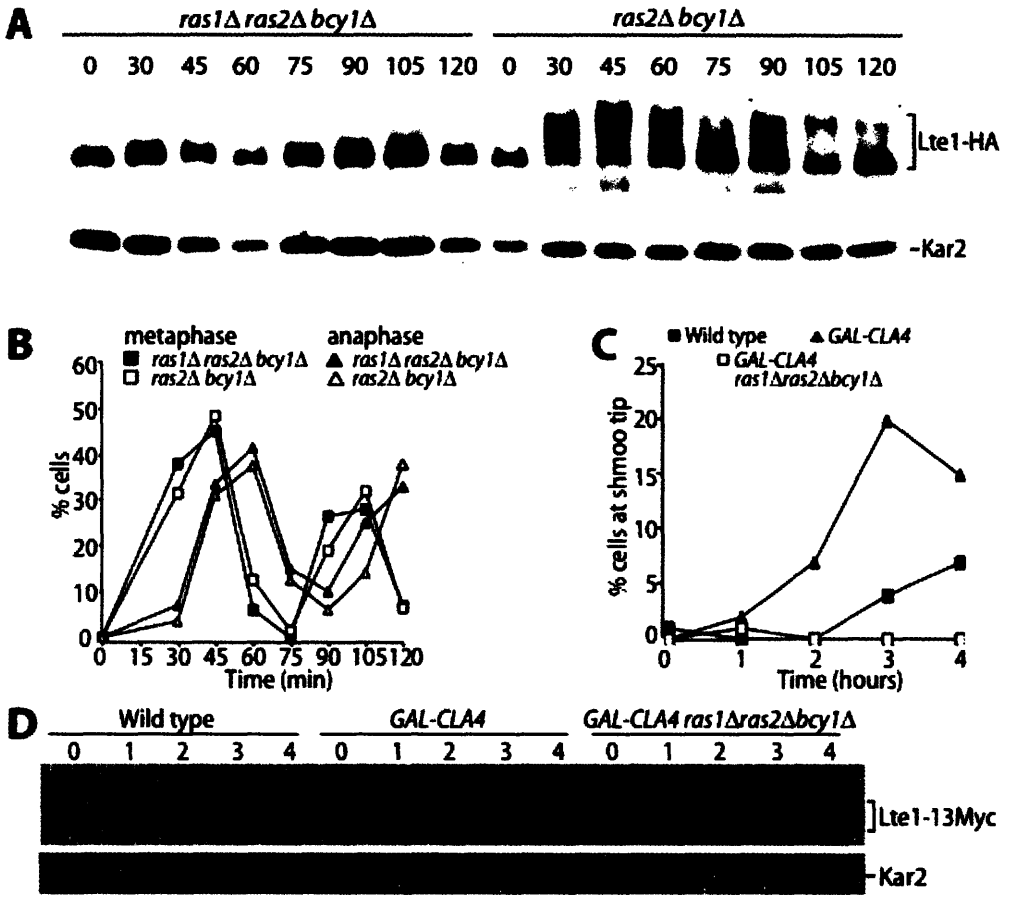

Figure 5: Ras activity is required for Lte1 phosphorylation across the cell cycle and ectopic targeting to regions of polarized growth by *CLA4*.
(A-B) *ras2Δ bcy1Δ* (A12572) and *ras1Δ ras2Δ bcy1Δ* (A12570) cells carrying an *LTE1-HA* fusion were arrested in G1 at 30°C with 5 μg/ml alpha-factor pheromone for 2 hours. Cells were then released into medium lacking pheromone and samples were taken at the indicated times to analyze (A) the phosphorylation status of Lte1 by Western blot analysis and (B) the percentage of cells with metaphase and anaphase spindles.

(C-D) Wild type cells (A3717), *GAL-CLA4* cells (A6023) and *GAL-CLA4 ras1Δ ras2Δ bcy1Δ* cells (A12936) all carrying an *LTE1-13MYC* fusion were arrested with 5 μg/ml alpha-factor pheromone for 2 hours 30 min. Two-percent galactose was then added to induce the overexpression of *CLA4* while the G1 arrest was maintained. Samples were taken at the indicated times and the percentage of cells with Lte1 at the shmoo tip (C) and the phosphorylation status of Lte1 (D) were examined. Kar2 was included as a loading control.

failed to phosphorylate and target Lte1 to the shmoo tip in *ras1Δ ras2Δ bcy1Δ* cells (Figure 5C, D). The lack of Lte1 phosphorylation was not due to a requirement of Ras for Cla4 activity since the phenotypes characteristic of cells lacking *CLA4*, such as a prolonged G2 delay due to activation of the morphogenesis checkpoint (Lew, 2003; Tjandra et al., 1998) were not observed in *ras1Δ ras2Δ bcy1Δ* triple mutants (Figure 5B). In addition, the introduction of a dominant active allele of *CDC42* to hyper-stimulate Cla4 activity did not allow localization of Lte1 in budded cells lacking *RAS1* and *RAS2* (Yoshida et al., 2003).

The observation that Ras was required for Lte1 phosphorylation and localization to the bud cortex raised the possibility that the binding of Ras to Lte1 was the event that initiated the localization of Lte1 at the bud cortex. If this were the case, expression of a form of Ras that exhibits high-binding affinity to Lte1 should be able to target Lte1 to a polarized cell cortex. To test this hypothesis, we examined whether Lte1 could associate with RAS2^{V19} or localize to the shmoo tip of alpha-factor arrested cells expressing *RAS2^{V19}*. In contrast to the overexpression of *CLA4,* expression of *RAS2^{V19}* was neither sufficient to promote hyper-phosphorylation of Lte1 (Figure 6A), nor association of Lte1 with the activated -Ras allele (Figure 6C) nor association of Lte1 with the shmoo-tip (Figure 6B). Together, our findings support the idea that *CLA4*-dependent phosphorylation is required for the initial association of Lte1 with Ras at the bud cortex and that Ras is required to stabilize phosphorylated forms of Lte1 and to anchor Lte1 at the bud cortex.

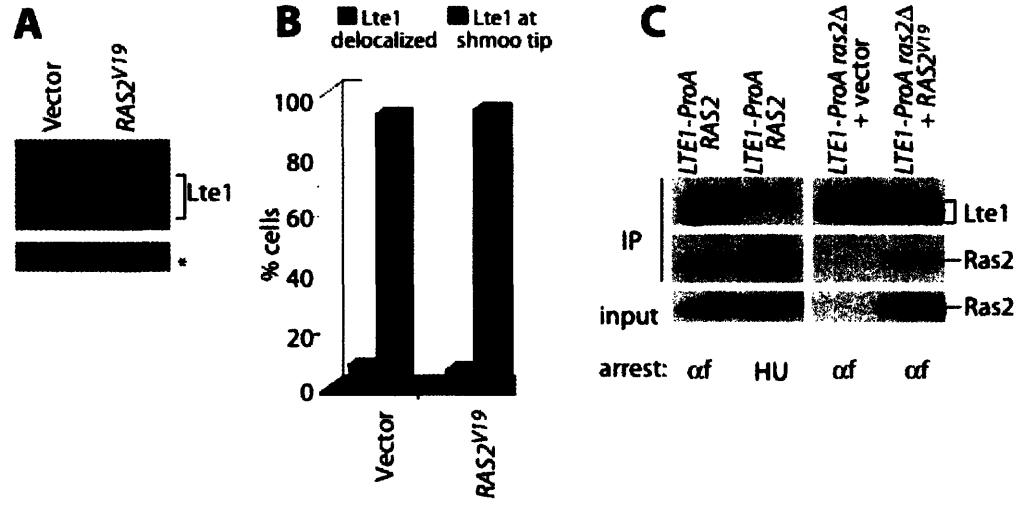

Figure 6: Activated Ras fails to target lte1 to the shmoo tip.
(A-B) Wild type cells carrying an *LTE1-13MYC* fusion and transformed with YCplac22 (A13207) or YCplac22-*RAS2*^{V19} (A13208) were arrested with 5 μg/ml of the alpha-factor pheromone in SC-Trp medium for 2 hours 20 min. Cells were either fixed for indirect immunofluorescence or collected for western blot analysis. The phosphorylation status of Lte1 (A) and the percentage of cells with Lte1 at the shmoo tip (B) were determined (N = 200 cells).

(C) *ras2Δ* cells carrying an *LTE1-ProA* fusion and transformed with YCplac22 (A12867) or YCplac22-*RAS2*^{V19} (A12868) were arrested in SC-Trp medium with 5 μg/ml α–factor for 2 hours at 30°C. The association of Ras2 mutants with Lte1 was analyzed by co-immunopreciptiation analysis. Wild type cells carrying an *LTE1-ProA* fusion were also arrested in SC medium with either 10 mg/ml HU for 2 hours (as a positive control) or with α–factor as above and the co-immunoprecipitation of Ras2 with Lte1 was examined.

Discussion

Regulation of Lte1 localization.

The localization of Lte1 is thought to be important for regulating the MEN. Restriction of Lte1 to the bud, coupled with the asymmetric localization of Tem1 to the daughter-bound SPB, allows the coordination of nuclear position and mitotic exit (Bardin et al., 2000; Pereira et al., 2000). The importance of this regulation is revealed in cells where the mitotic spindle is not aligned along the mother – bud axis. In such cells, nuclear division occurs within the mother cell and exit from mitosis is prevented. Loss of the restriction of Lte1 to the bud leads to untimely exit from mitosis and generates aploid and multinucleate cells (Bardin et al., 2000; Castillon et al., 2003). Owing to this necessity to confine Lte1 to the bud, it is important to understand how Lte1 localization is regulated.

Several proteins have been implicated in controlling Lte1 localization, but how the proteins function together to direct and anchor Lte1 at the bud cortex is poorly understood. Our data suggest that Lte1 associates with Ras2 concomitant with bud formation. This is also the point in the cell cycle at which the protein becomes localized to the bud cortex, which suggests that Ras could capture Lte1 at the bud cortex. However, mechanisms in addition to Ras binding must exist to direct Lte1 to the bud cortex. Ras is present at the cell cortex of mother and daughter cell throughout the cell cycle (Yoshida et al., 2003), yet Lte1 is restricted to the bud cortex and present there only during S phase, G2 and mitosis. What other factors could be necessary for proper localization of Lte1? Several lines of evidence suggest that the *CLA4*-mediated phosphorylation of Lte1

could be contributing to this event. First, Lte1 phosphorylation occurs concomitantly with Ras2 – Lte1 complex formation. Second, overexpression of *CDC14* induces not only dephosphorylation of Lte1 but also the dissociation of the Lte1 - Ras2 complex and delocalization of Lte1 from the bud [this study; (Jensen et al., 2002; Seshan et al., 2002)]. Third, the protein kinase Cla4, which is required for Lte1 phosphorylation (Chiroli et al., 2003; Hofken and Schiebel, 2002; Seshan et al., 2002), is also required for Lte1 – Ras2 complex formation and is, when overexpressed, sufficient to target Lte1 to the shmoo tip in G1-arrested cells in a *RAS1* and *RAS2*-dependent manner.

Interestingly, not only are Cdc42 and Cla4 required for Lte1 association with Ras at the bud cortex, but Ras also appears to be necessary for phosphorylated forms of Lte1 to accumulate. Based on this observation, we propose that phosphorylation of Lte1 by Cla4 specifically targets Lte1 to the bud cortex where it directly binds to Ras or to other proteins whose anchoring activity depends upon Ras function. Binding to bud cortex anchors stabilizes phospho-Lte1 and further promotes phosphorylation of the protein by Cla4 and perhaps other protein kinases. Phosphorylated Lte1 is then maintained at the bud cortex by the septin ring. A key question concerning this model is whether Ras itself serves a structural role in anchoring Lte1 at the bud cortex, or whether other known Ras signaling pathways are important for anchorage to occur. Three observations suggest the former to be true. First, Lte1 and Ras2 associate with each other *in vivo* and *in vitro* (Yoshida et al., 2003), Second, the PAK-like kinase Ste20, an upstream regulator of the Ras-activated MAPK signaling pathway, is not required for Lte1 localization or phosphorylation (Hofken and Schiebel, 2002; Seshan et al., 2002). Third, in *bcy1Δ* cells, in which cAMP-dependent protein kinase A (PKA) signaling is independent of Ras

activity, the mutation of Ras residues important for binding to adenylate cyclase decreased the Ras2 – Lte1 interaction. These findings argue for a structural role of the Ras adenylate cyclase binding domain in Lte1 anchorage, rather than a signaling role. Since the MAPK pathway and the PKA pathway are the only known Ras-activated signaling cascades in budding yeast, it is likely that Ras-GTP mediated recruitment of Lte1 to the bud cortex constitutes a new Ras-signaling pathway that promotes mitotic exit.

Is the association with Ras necessary for Lte1's mitotic exit function?

Mutants that affect Lte1 localization fall into two classes. In one class of mutants, Lte1 is still phosphorylated and is able to bind to Ras2, indicating that the protein is present at the cell cortex, though no longer restricted to the bud. This group consists of mutants that affect septin ring function. In *cdc12-6* and *shs1Δ* cells, Lte1 is phosphorylated and found in a complex with Ras2 [this study; (Jensen et al., 2002; Seshan et al., 2002)]. In the other class of mutants, which includes *cdc42-17, cla4Δ* and *ras1Δ ras2Δ*, Lte1 phosphorylation, the association of Lte1 with Ras2, and Lte1's association with the cell cortex are abolished. Interestingly, in septin ring mutants, such as in *shs1Δ* cells, Lte1 retains its ability to promote exit from mitosis (Castillon et al., 2003), whereas in *cdc42-17, cla4Δ* and *ras1Δ ras2Δ* mutants, Lte1 is no longer able to promote exit from mitosis (Hofken and Schiebel, 2002; Seshan et al., 2002; Yoshida et al., 2003). These findings raise the possibility that Lte1 needs to be associated with the bud cortex, or perhaps with Ras itself, in order to promote exit from mitosis. In this scenario, Cla4 phosphorylation would allow Lte1 to associate with Ras2 at the cell cortex, where the protein would

become active to promote mitotic exit. The dissociation of Lte1 and Ras2 upon Cdc14 activation would provide a mechanism for the inactivation of Lte1 and the termination of MEN signaling upon mitotic exit initiation. Such a negative feedback loop would help to restrict MEN activity to exit from mitosis.

How could Ras promote the mitotic exit function of Lte1? Both the N- and C-termini of Lte1 are homologous to the Cdc25 Ras GEF. The N-terminus contains a poorly defined GEFN domain, whereas the C-terminus contains the Cdc25 homology domain (CHD). Yoshida et al. suggested that Lte1 is not a GEF for Tem1 since an internal domain of Lte1 termed the mini-domain (aa 659-926), that does not include the CHD or the GEFN domains and does not associate with the bud cortex, was capable of rescuing the mitotic exit phenotype of *ras1Δ ras2Δ bcy1Δ* mutants when overproduced (Yoshida et al., 2003). Instead Yoshida et al. proposed that the GEF domain was necessary for binding of Lte1 to Ras-GTP. *LTE1* function is required for the survival of cells lacking components of the Cdc Fourteen early anaphase release (FEAR) network, such as *SPO12*, which is normally non-essential for mitotic exit (Stegmeier et al., 2002). We find that overexpression of neither the mini-domain nor a truncation of Lte1 lacking its CHD were sufficient to suppress the synthetic lethality of *spo12Δ lte1Δ* cells, suggesting that both GEF homology domains are important for Lte1 function as a MEN activator (Figure 7). Based on this observation and the physical interaction between Ras and Lte1 we propose that Ras could function as an activator of Lte1's GEF domain. Recently, it was reported that the Ras-specific nucleotide exchange factor Son of sevenless (SOS) is activated by the binding of Ras-GTP at an allosteric binding site in Sos known as the Ras exchanger

motif (Rem) domain, which interacts with the Sos catalytic domain. Ras-GTP binding at the allosteric site results in increased affinity for Ras-GDP at the catalytic site of Sos

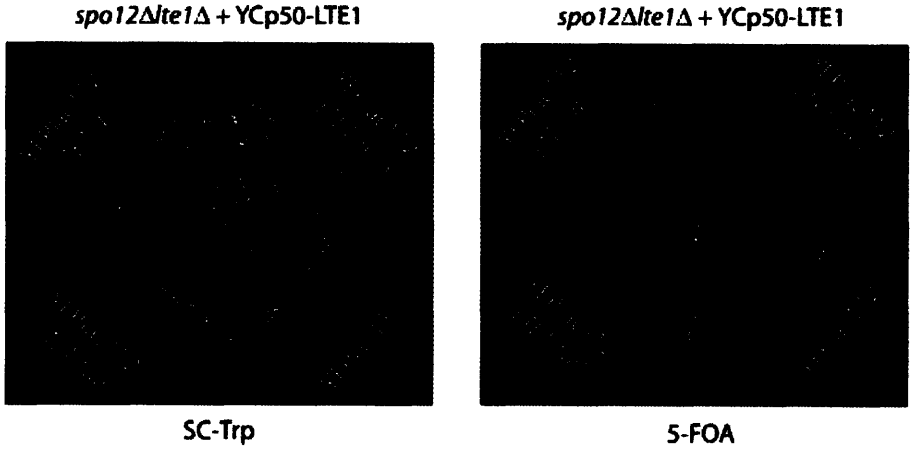

Figure 7: The N- and C- termini of Lte1 are required for *LTE1* function.
spo12Δ lte1Δ cells carrying a *URA3*-marked YCp50-*LTE1* plasmid were transformed with the *TRP*-marked YEplac112 vector and also with YEplac112 containing full-length *LTE1* (LTE1-full), *LTE1* lacking its C-terminus (amino acids 1-1307;LTE1-dC), or a middle region of *LTE1* (amino acids 659-926; LTE1-mini). Strains were replica-plated from SC-Trp plates onto 5-FOA plates and incubated at 30°C for 2 days to test for the ability of the YEplac112 plasmids to rescue the *spo12Δ lte1Δ* synthetic lethality.

(Sondermann et al., 2004). A careful examination of the GEFN domain of Lte1 (aa 24-157) and the Rem domain of Sos (aa 596-741) revealed the potential for significant structural homology between these regions (Figure 8). Sos contains six alpha-helices in its Rem domain, the first three of which are widely conserved among other Ras-specific exchange factors such as Cdc25, Sdc25, and Ras guanine-nucleotide-release factor [RasGRF; reviewed in (Boguski and McCormick, 1993)]. The region of the Sos Rem domain that regulates Ras-GTP binding is situated in helices 4 and 6, which are conserved in DmSos and in the predicted secondary structure of Lte1 (Rost and Sander, 1993), but not in Cdc25, Scd25, or RasGRF [Figure 8; (Boriack-Sjodin et al., 1998)]. The significant sequence homology between the Rem domain of Sos and the N-terminus of Lte1, coupled with the fact that the N-and C-termini of Lte1 have been shown to interact *in vitro* (Jensen et al., 2002) suggests that Lte1 is indeed a GEF for Tem1 that may be regulated directly by another GTPase, Ras. We hypothesize that Cla4-mediated binding of Ras-GTP to Lte1 allows the catalytic domain of Lte1 to catalyze GDP-exchange on Tem1, thereby promoting mitotic exit. In closing we note that such a mechanism would provide a novel mechanism by which Ras-GTPase pathways could regulate one another.

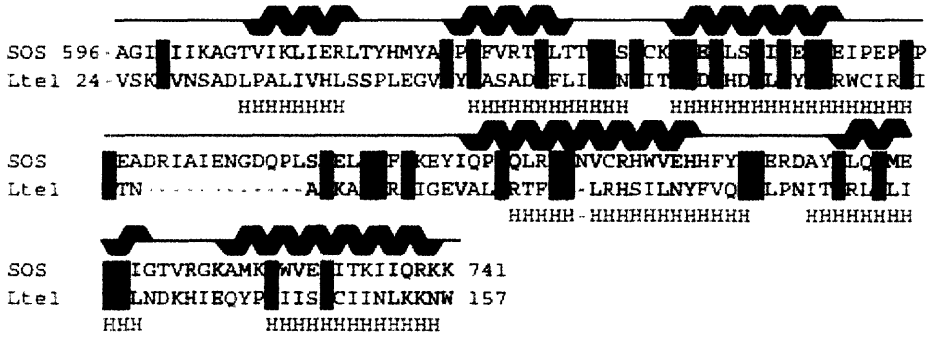

Figure 8: Sequence alignment of mammalian Sos REM domain and the N-terminus of Lte1 indicates structural homology.

The alignment was generated using ClustalW (Henikoff and Henikoff, 1993) to identify homology between the REM domain of Sos (amino acids 596-741) and the N-terminus of Lte1 (amino acids 24-157). Secondary structure predictions for Lte1 were obtained using the PHD prediction program, (Rost and Sander, 1993) while Sos secondary structure is presented as reported from the Sos crystal structure.(Boriack-Sjodin et al., 1998) Strictly conserved residues are marked by red boxes, similar residues by yellow boxes. Alpha-helices in the Sos structure are represented by green helices above the Sos protein sequence. The predicted helices in the Lte1 N-terminus are indicated by the presence of "H" underneath the Lte1 protein sequence.

Materials and Methods

Yeast Strains and Growth Conditions

All strains were derivatives of strain W303 (A2587). The *ras1::HIS3, ras2::HIS3, bcy1::kanMX, LTE1-ProA, shs1::TRP1,* and *cla4::HIS3* strains were constructed by using the PCR-based method described in reference (Longtine et al., 1998). Growth conditions for individual experiments are described in the figure legends. In cases in which growth media is unspecified, cells were grown in YEP + 2% glucose.

Co-immunoprecipitation analysis

Cells were harvested, washed once with 10mM Tris, pH7.5, and resuspended in CO-IP buffer (1% NP40 (weight/ volume), 150mM NaCl, 50mM Tris, pH7.5, 1mM DTT, 40μg/ml Aprotinin, 60mM β-glycerophosphate, 0.1mM $NaVO_3$, 15mM para-Nitro-phenyl phosphate, 5mM $MgCl_2$, complete EDTA-free protease inhibitor cocktail (Roche)). Rabbit IgG-coupled Dynabeads were added to each sample and incubated with rotation for 1-2 hours at 4°C. Samples were washed 6 times with CO-IP buffer and separated on a 6%-10% step SDS-PAGE gel for subsequent western blot analysis.

Immunoblot Analysis

For immunoblot analysis of the total amount of Clb2, Lte1-3HA, Lte1-13MYC, Lte1-ProA, Kar2, and 3-PGK, 10-25 ml samples of cells were harvested and pellets were resuspended in lysis buffer (50mM Tris-HCl pH 7.5, 1mM EDTA, 60mM β-glycerophosphate, 0.1mM

$NaVO_3$, 15mM para-Nitro-phenyl phosphate, 40μg/ml Aprotinin, 50mM DTT, complete EDTA-free protease inhibitor cocktail (Roche)). TCA extracts were prepared by fixation in 1 volume 5%TCA to cell pellets instead of lysis buffer. Glass beads (Sigma) were added and samples were vortexed for 3 times 45 seconds on a BioRad Biopulverizer. Samples were run on either a 6% SDS-PAGE gel (for Lte1), 10% SDS-PAGE gel (for Clb2), or a 6%-10% step gel (for simultaneous detection of Lte1 and Ras2). Primary mouse anti-MYC antibody (Covance) was used at a concentration of 1:500, with a secondary anti-mouse antibody concentration of 1:5000. Primary mouse anti-HA antibody (Covance) was used at a concentration of 1:750 with a secondary anti-mouse concentration of 1:2000. Peroxidase anti-peroxidase antibody (Sigma) was used at a dilution of 1:500 to detect Lte1-ProA. Primary goat anti-Ras2 antibody (Santa Cruz) was used at a concentration of 1:1000. For co-IP experiments, blots were incubated in primary antibody at 4° C overnight, and in secondary antibody for 4-5 hours. Otherwise, blots were incubated in primary antibody for 2 hours, and in secondary antibody for 1 hour. Secondary anti-goat antibody was used at a concentration of 1:5000. Mouse and rabbit antibodies were prepared in 1% milk, 1% BSA, while goat antibodies were prepared in 1% ovalbumin.

Fluorescence microscopy

Indirect *in situ* immunofluorescence methods were as described in reference (Bardin et al., 2000). Primary anti-MYC antibody (Covance) was used at a 1:1000 dilution. Secondary anti-mouse antibodies (Jackson Laboratories) were used at a concentration of 1:1500. Cells were analyzed on a Zeiss Axioplan 2 microscope and Openlab 3.1.5 software was used to process immunofluorescence images. At least 100 cells were analyzed per time point.

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Chapter IV: Discussion and Future Directions

Summary and Conclusions

The regulated localization of Lte1 to the bud cortex is important mainly in cells in which the mitotic spindle apparatus is not aligned along the mother-bud axis. In such cells, the spatial segregation of Lte1 and Tem1 ensures that mitotic exit does not occur until the spindle re-aligns itself properly, allowing the accurate partitioning of the genetic material. The work presented in this thesis has shown that Lte1 localizes to the bud cortex upon bud formation. The ability of Lte1 to localize to the bud is independent of the actin and microtubules cytoskeletons, suggesting that a diffusion – capture mechanism regulates the association of the protein with the bud cortex. This cortical localization is promoted by Ras, the Rho GTPase Cdc42 and its effector the PAK-like kinase Cla4, as well as the septin ring, which acts as a diffusion barrier for cortical Lte1. The phosphorylation status of Lte1 is likely to play a causal role in the protein's ability to associate with the cell cortex. The role of Cla4 is most probably both catalytic and structural, since Lte1 associates only with the bud cortex, while Ras localizes to both the mother and daughter cortices. Once Cla4 is directed to the bud tip of the apically growing bud by Cdc42, Lte1 is phosphorylated and associates with Cdc42, Cla4 and Ras at the developing bud cortex. The Lte1-Ras-Cla4-Cdc42 complexes are maintained specifically at the bud cortex by the septin ring, which inhibits passage of these cortical complexes through the mother – bud neck. The association of Lte1 with Ras or other proteins at the cortex may also positively regulate Lte1's mitotic exit promoting ability. The dephosphorylation and dissociation of Lte1 from the bud cortex can be initiated by the MEN phosphatase Cdc14, indicating

that, once activated, Cdc14 may promote its own inactivation by terminating the activation of Lte1 (Figure 1).

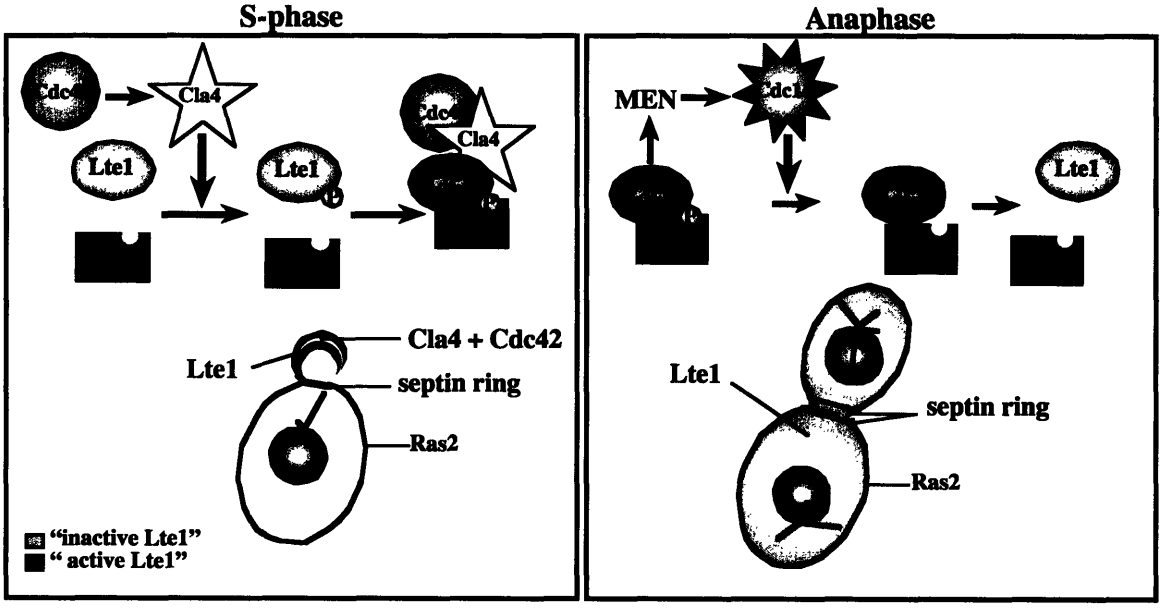

Figure 1: A model for Lte1 localization
The GTPase Cdc42 activates the Cla4 kinase, which both localize to the bud tip at the beginning of S phase. Cla4 then promotes the phosphorylation of Lte1, which directs Lte1 to Ras2 at the cortex. The association of Lte1 with the cortex also induces the protein to be in an 'active' form. The Lte1-Ras2-Cla4-Cdc42 complex is retained at the bud cortex by the septin ring at the mother – bud neck. In anaphase, 'active' Lte1 promotes MEN activation and Cdc14 is released from its inhibitor. Cdc14 then promotes the dephosphorylation of Lte1, which causes the protein to dissociate from Ras2 and the bud cortex and once again revert to its 'inactive' conformation.

The Ha-Ras oncogene promotes cell growth and proliferation in mammalian cells. Unregulated activation of this GTPase is linked to human cancers of all types (Coleman et al., 2004). The two *S. cerevisiae* Ras homologs, *RAS1* and *RAS2*, also play a role in cell cycle regulation. The cAMP-activated protein kinase A (PKA) is a key activator of the G1 – S phase transition in budding yeast, and Ras proteins initiate the production of cAMP by activating adenylate cyclase (Toda et al., 1985; Wigler et al., 1988). In addition, a study of *S. cereviae* cells lacking Ras activity revealed a late mitotic role for Ras that was independent of its requirement for adenylate cyclase activation (Morishita et al., 1995). Cells lacking *RAS1*, *RAS2,* and *BCY1* (the deletion of *BCY1* alleviates the PKA activation requirement of Ras mutants) cannot exit from mitosis at low temperatures (10°C; (Morishita et al., 1995). These cells are unable to efficiently release the Cdc14 phosphatase from its nucleolar inhibitor at 10°C, a phenotype that can be suppressed by the overexpression of *LTE1*, *TEM1*, and several other downstream MEN components (Morishita et al., 1995; Yoshida et al., 2003). Ras proteins are needed for Lte1 localization to the bud cortex, and directly associate with Lte1 in a cell-cycle regulated manner (Seshan and Amon, 2005; Yoshida et al., 2003). The fact that the accumulation of phosphorylated forms of Lte1 requires both Ras and Cla4 function implies that the phosphorylation of Lte1 allows the protein to associate with Ras. Interestingly, the association of Lte1 and Ras2 is abolished in mutants such as *cdc42-17* and *cla4Δ* that are unable to exit from mitosis at low temperatures (Seshan and Amon, 2005). This is a phenotype that is typical of *lte1Δ* cells, pointing to the possibility that the association of Lte1 with Ras is required for the ability of Lte1 to function as a MEN activator. Since

Lte1 binds preferably to GTP-bound forms of Ras,(Seshan and Amon, 2005; Yoshida et al., 2003) we hypothesize that Ras-GTP binds to Lte1 and activates the protein's GEF activity.

Our data supports the idea that both the C-terminal Cdc25 homology GEF (CHD) domain and the N-terminal GEFN domains of Lte1 are important for the protein's ability to promote mitotic exit (Seshan and Amon, 2005). A contradictory hypothesis has been proposed in which an internal region of the protein termed the mini-domain (aa 659-926) that does not include either the GEFN or the CHD domain complements an *lte1Δ* phenotype when overexpressed (Yoshida et al., 2003). However, we did not find that the mini-domain was sufficient to substitute for the essential function of *LTE1* in a *spo12Δ* background. We are unable to explain the discrepancy between these results.

Recent advances in our understanding of the structural interaction between the canonical guanine-nucleotide exchange factor, Sos, and its substrate Ras revealed that Sos is not only an activator of Ras, but also its substrate. Sondermann and colleagues discovered that the binding of Ras-GTP at an allosteric site in Sos known as the Ras exchanger motif (Rem) domain increases the affinity of Sos for Ras-GDP at the catalytic site (Sondermann et al., 2004). The predicted structure of the GEFN domain of Lte1 (aa 24-157) indicates that this region of the protein forms six alpha-helices (Rost and Sander, 1993). A thorough alignment of the GEFN domain of Lte1 with the Rem domain of Sos (aa 596-741) revealed that there is significant sequence homology between these two proteins. In addition, 3D-crystal structure analysis of the Sos Rem domain reveals six alpha helices (Boriack-Sjodin et al., 1998). The first three of these helices are widely-conserved among Ras GEFs while helices four, five and six are conserved only in a

subset of Ras GEFs. Since the interface of the Rem domain in Sos that binds Ras-GTP is formed by residues located in helices four and six, the conservation of these helices in the predicted Lte1 structure as well as sequence homology suggest that Ras-GTP may bind Lte1 at its GEFN domain. *In vitro* studies of Lte1 and Ras2 suggested that the CHD of Lte1 was sufficient for Lte1 – Ras2 binding. However, this region of Lte1 was not sufficient to mediate *in vivo* association of the two proteins (Yoshida et al., 2003). In addition, the N- and C-termini have been shown to be required for the proper localization of Lte1 and have been shown to interact *in vitro* (Jensen et al., 2002) supporting the idea that these domains of the protein are important for its function.

The finding that Ras and Cla4 act together to initiate mitotic exit through Lte1 raises many questions about how this regulation might be occurring. In addition, though this work suggests that Lte1's GEF domains are important for its function, the nature of this protein's biochemical activity remains to be determined. Proper control of mitotic exit is important for cellular viability, as is evidenced by the multiple layers of regulation imposed on this process. It will be of interest to continue to characterize how regulators of mitotic exit in *S. cerevisiae* aid in the preservation of the temporal and spatial order of mitotic events, and to determine whether similar mechanisms exist in multi-cellular organisms.

Unanswered Questions and Future Directions

The work presented in this thesis provides describes some of the mechanisms by which Lte1 is regulated, and by which this protein may initiate mitotic exit. In the next sections, I will discuss in detail the mechanisms by which Lte1 may be regulated by both elements within Lte1 and trans-acting factors. In addition, I will consider the possible reasons that *LTE1* is not required for mitotic exit at normal temperatures, despite the fact that most GTPases are not efficient in catalyzing their own GDP-exchange. Next, I will discuss how the regulation of Lte1 and other mitotic exit components by the polarity determinant *CDC42* provides a novel temporal and spatial link between mitotic exit and cytokinesis. I will also consider a possible mechanism by which cells ensure that the execution of mitotic exit precedes cytokinesis. Finally, I will highlight the functional conservation of MEN components in *S. pombe* and higher eukaryotes.

Role of elements within Lte1 in the regulation of Lte1 localization and function

Much work has been accomplished in the past five years in elucidating the mechanisms by which Lte1 is confined to the bud. We now know that phosphorylation by the PAK-like kinase Cla4 likely contributes to the sequestering of Lte1 to the bud compartment. Cla4-mediated phosphorylation is both necessary and sufficient for targeting Lte1 to areas of polarized growth (Hofken and Schiebel, 2002; Seshan et al., 2002). Since the kinase activity of Cla4 is required for its function in regulating Lte1 localization (Hofken and Schiebel, 2002), Lte1 may be a direct target of Cla4 kinase activity. Alternatively, other kinases that depend upon Cla4 kinase activity may contribute to Lte1 phosphorylation. For instance, Jensen et al. showed that mitotic Clb-CDK complexes immunoprecipitated from nocodazole-arrested cells can phosphorylate a bacterially produced Lte1 500 amino acid fragment *in vitro* (Hofken and Schiebel, 2002). In addition, we have found that Clb-CDK complexes are necessary for the maintenance of Lte1 phosphorylation in S-phase arrested cells (unpublished data). The requirement for Clb-CDKs may be due to the regulation of Cla4 *in vivo* kinase activity by Clb-CDKs (Tjandra et al., 1998). The phosphorylation of Cla4 by Clb2-CDK, together with the GTPase Cdc42, activates the kinase in the initiation of the apical – isotropic bud growth transition (Tjandra et al., 1998). However, it is also clear that Cln-CDK complexes can phosphorylate Lte1 *in vitro* (Jensen et al., 2002). The consequences of Cln-CDK phosphorylation on Lte1 localization and activity are unclear, although one group has reported that Lte1-GFP localizes to the incipient bud site and hypothesizes that Cln-CDK complexes prime Lte1 for recruitment to the bud cortex in a Cdc42-dependent manner

(Jensen et al., 2002). The mutation of consensus serine/threonine CDK-phosphorylation sites (S/T-P-X-K/R, S/T-P-K/R, and K/R-S/T-P) in Lte1 to alanine residues leads to a visible diminishment in Lte1 phosphorylation, suggesting that these sites are phosphorylated *in vivo* [unpublished data; (Jensen et al., 2002)]. However, these phospho-mutants were still phosphorylated in a cell-cycle regulated manner and also were able to localize to the bud cortex like wild type Lte1 [unpublished data(Jensen et al., 2002)].

Because the sites that are phosphorylated on Lte1 are not known, it is unclear whether phosphorylation directly mediates the ability of Lte1 to localize to the bud cortex, or whether the association of Lte1 with the cortex allows phosphorylated forms of the protein to accumulate. Correlative evidence suggests that the ectopic dephosphorylation of Lte1 by the Cdc14 phosphatase initiates its delocalization (Jensen et al., 2002; Seshan et al., 2002) and Lte1 is an *in vitro* substrate of Cdc14 (Seshan et al., 2002). In addition, Lte1 associates with Ras, which is required for its proper localization, only in stages when the protein is phosphorylated and localized to the bud cortex. Lte1 does not complex with Ras in G1 α-factor arrested cells unless it is ectopically phosphorylated by Cla4 (Seshan and Amon, 2005). The Lte1-Ras association also appears to be regulated by the phosphorylation status of Lte1, since ectopic dephosphorylation of Lte1 by *CDC14* induces Lte1 and Ras2 to dissociate (Seshan and Amon, 2005). It will be important, however, to determine which residues on Lte1 are phosphorylated. Determining whether any of these phosphorylated sites are required for the protein to localize to the cell cortex and to associate with Ras2 will allow us to distinguish whether the phosphorylation of Lte1 precedes its ability to associate with the

cell cortex, or whether the transient association of Lte1 with the cortex is needed for phosphorylated forms of the protein to accumulate. Additionally, since the consequences of Lte1 localization to the bud cortex on mitotic exit are still poorly understood, the generation of unphosphorylatable alleles of Lte1, as well as phospho-mimic alleles of Lte1, will aid in elucidating the role that Lte1 localization plays in promoting mitotic exit.

Role of trans-acting factors in the regulation of Lte1 localization and function

We hypothesize that the binding of Ras-GTP to Lte1 might allow the tighter association of Lte1 with its substrate Tem1 (see Figure 2). Owing to the labile nature of the association between Tem1 and Lte1 (Hofken and Schiebel, 2002), this could best be tested using an *in vivo* approach such as FRET to examine the association of Lte1 and Tem1 under various conditions. Sondermann et al. were able to demonstrate the *in vivo* relevance of Ras-GTP binding to Sos at the allosteric site by generating Sos mutants that could not bind Ras at the Rem domain, but retained the ability to bind Ras-GDP at the catalytic site. These mutants were considerably less efficient in activating Ras-signaling when expressed in a mammalian cell line (Sondermann et al., 2004). It would be interesting to generate homologous mutants in the GEFN domain of Lte1 and test whether the association with Ras is lost. One could then perform a more careful examination of the consequences of such an *LTE1* mutant on the execution of mitotic exit and determine whether the Lte1 – Ras association is of functional importance.

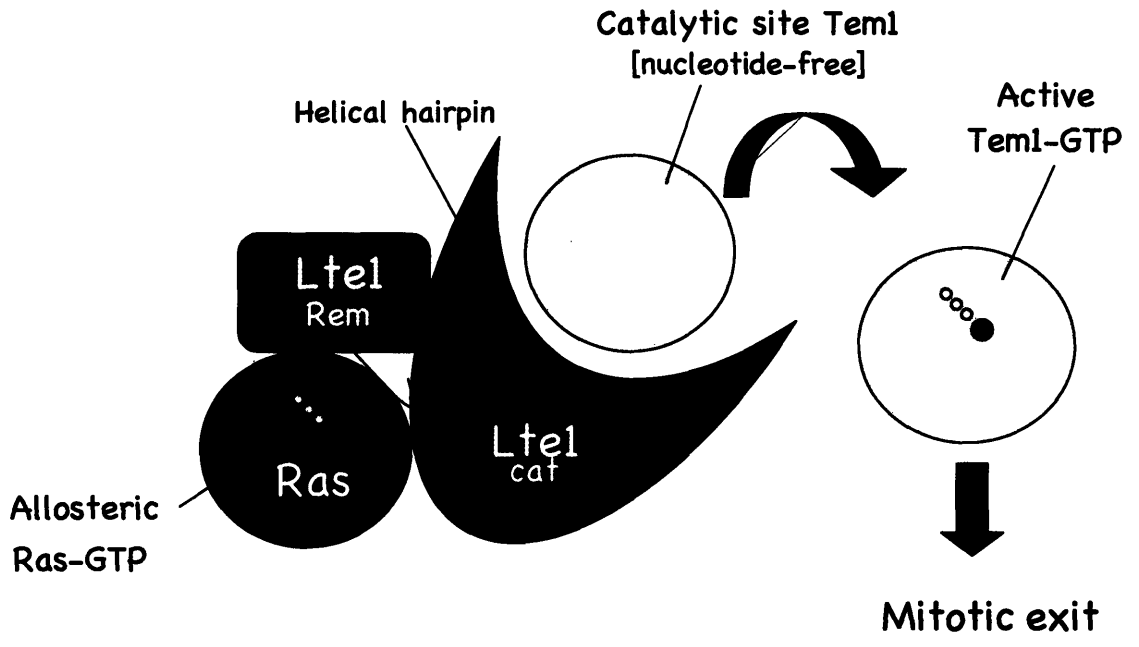

Figure 2 A model for the activation of Lte1's catalytic exchange activity by the binding of Ras-GTP to the Rem domain of Lte1. (see text for details)

CDC42 and *CLA4* are important not only for the regulated association of Lte1 and Ras, but also for the mitotic exit function of Lte1 (Hofken and Schiebel, 2002; Seshan and Amon, 2005; Seshan et al., 2002; Yoshida et al., 2003). The role of *CDC42* in Lte1 regulation seems to be linked to its essential function in activating the Cla4 kinase (Hofken and Schiebel, 2002). The fact that the accumulation of phosphorylated forms of Lte1 requires both Ras and Cla4 function implies that the phosphorylation of Lte1 allows the protein to associate with Ras. Cla4 and Ras could therefore function together to activate Lte1. It would be interesting to test whether a constitutively-GTP-bound version of Ras, together with increased amounts of *CLA4*, activates inappropriate mitotic exit in an *LTE1*-dependent manner. This could be examined using the *dyn1Δ* mutant, in which a proportion of cells undergo anaphase within the mother cell due to mitotic spindle

misalignment (Muhua et al., 1998; Yeh et al., 1995). In such cells, mitotic exit does not occur until the spindle re-aligns itself along the mother – bud axis, in part due to the spatial segregation of Lte1 and its putative substrate Tem1 (Bardin et al., 2000). However, if *LTE1* is overexpressed, some of the protein is not contained in the bud and leaks into the mother cell, causing a proportion of these cells to exit from mitosis inappropriately (Bardin et al., 2000). The overexpression of *LTE1* and *CLA4* as well as the activated $RAS2^{VI19}$ allele should increase the inappropriate mitotic exit of cells with mispositioned spindles, if Ras-GTP and Cla4 do indeed coordinate to increase Lte1's MEN activating ability.

Another protein that may have a role in the regulation of Lte1 is Kel1. The Kel1 kelch-repeat containing protein was found to interact with Lte1 in two high-throughput screens (Gavin et al., 2002; Ho et al., 2002). In addition, *KEL1* was identified as a negative regulator of the MEN since *kel1Δ* was found to suppress the cold-sensitivity of *lte1Δ* mutants (Hofken and Schiebel, 2002; Jensen et al., 2002). Strangely, the localization of Lte1 to the bud cortex appeared to be impaired when the localization of an Lte1-HA fusion protein was examined by indirect immunofluorescence (Seshan et al., 2002), but not when the localization of a Lte1-GFP fusion was observed (Hofken and Schiebel, 2002). The reasons for this discrepancy are unclear at present, but may be a reflection of the methods used in live-cell imaging as opposed to indirect immunofluorescence. For example, in order to examine the localization of a protein using an indirect antibody staining approach, it is necessary to first digest the yeast cell wall. In mutants in which the integrity of the yeast cell membrane is perturbed, the digestion of the cell wall may cause a disruption of the membrane and result in the displacement of

proteins that are confined to this compartment. It is possible that *KEL1* contributes to the fidelity of the cell membrane, since its gene product has been shown to regulate cell fusion events during the process of mating (Philips and Herskowitz, 1998). Thus, perhaps in *kel1Δ* cells, the cell membrane is more sensitive to perturbations and becomes compromised upon subjection to zymolyase treatment. This could result in the displacement of proteins localized to the cell membrane, such as Lte1, in indirect immunofluorescence analysis. *KEL1* is also not required for the association of Lte1 with Ras2 at the cell cortex (Seshan and Amon, 2005). Thus, the localization of Lte1 may not depend upon *KEL1*, but it is clear that these two proteins can interact throughout the cell cycle by co-immunoprecipitation analysis (unpublished results). The functional significance of this interaction remains to be seen.

How does Lte1 activate Tem1?

In the absence of *in vitro* exchange assays for Lte1, the biochemical identity of this protein and the mechanism by which it functions are unclear. Genetically, *LTE1* acts positively on MEN signaling upstream of Tem1, but whether the protein acts directly or indirectly to promote Tem1 activation is an important unresolved issue. An interaction between Tem1 and Lte1 by co-immunoprecipitation assays has not been detected (A.S. unpublished data(Hofken and Schiebel, 2002), suggesting that if the proteins do associate *in vivo*, they exhibit a weak and/or transient interaction. This could be either because Lte1 acts indirectly to activate Tem1, or because Tem1 is an enzymatic substrate of Lte1, and therefore their interaction is characterized by weak binding. If Lte1 does not promote GDP exchange on Tem1, several other possible mechanisms of Tem1 activation can be

envisioned. For example, Lte1 could enhance the ability of Tem1 and Cdc15 to interact at the dSPB (daughter SPB) during anaphase. Another possibility is that Lte1 increases the ability of Tem1 to bind to the SPB in an indirect way. Still another possibility is that Lte1 prevents the two-component GAP Bub2/Bfa1 from binding to and inhibiting Tem1 upon dSPB entry into the daughter cell.

Although there is conflicting evidence for the requirement of Lte1's GEF domain for its function in mitotic exit, it remains a possibility that Lte1 does, indeed, stimulate GDP exchange on Tem1. In this case, one important question is whether Lte1 is associated with Ras at the cell cortex, or becomes released from the cortex in an activated form when it promotes GDP-exchange on Tem1. Previous studies using quantitative time-lapse fluorescence microscopy suggest that cortical contact of the SPB bearing Tem1 does not correlate with the onset of mitotic exit (Molk et al., 2004). In order to rationalize these findings with a model in which Lte1 is activated by Ras-GTP at the cell cortex, one possibility is that Lte1, once activated by Ras, would be released from the cortex in a stabilized active form. This activated Lte1 would then come into contact with Tem1 on the SPB and activate the GTPase.

However, detailed FRAP experiments on Tem1 in anaphase indicate that Lte1 may not have to dissociate from the cortex in order to activate Tem1. Tem1 on the dSPB increases in intensity upon penetration of the dSPB into the bud in an *LTE1* dependent manner. In addition, it appears that Tem1 localized to the dSPB is dynamic and exchanges with the cytoplasmic pool of Tem1 in anaphase (Molk et al., 2004). We have found that Lte1 and Ras2 can associate robustly in cells arrested in late anaphase using the *cdc15-2* mutation (Seshan and Amon, 2005). Genetic studies have placed Tem1 and

Lte1 upstream of Cdc15 in the MEN signaling cascade, indicating that Lte1 could be confined to the bud cortex until after mitotic exit has been initiated. In this scenario, cytoplasmic Tem1 would be activated by contacting Lte1 at the cortex transiently. Although Tem1 has not been observed at the bud cortex to any appreciable level (Molk et al., 2004), it is possible that the interaction with Tem1 and Lte1 at the cortex is too transient to be observed or that cytoplasmic Tem1 is too low in abundance to visualize.

In order to distinguish between these possibilities, it will be useful to generate Lte1 mutants that cannot be released from the cortex, but that are still capable of associating with Ras normally. If such mutants can initiate mitotic exit at low temperatures, or can complement the synthetic lethality of *spo12Δ lte1Δ* cells, this would support the hypothesis that a cytoplasmic pool of Tem1 is activated by cortically-confined Lte1. By the same token, alleles of Tem1 that are anchored to the SPB, but can still recruit Cdc15 to the SPB demonstrating functionality, should not be able to exit from mitosis efficiently since a cytoplasmic pool of Tem1 would not be present. The feasibility of generating a functional allele of Tem1 that is anchored to the SPB is of concern. Still, it will be interesting to examine the dynamic exchange of Tem1 and Lte1 in more detail and determine the cellular compartment in which Lte1 is active. However, the ultimate goal of understanding how Lte1 promotes mitotic exit can only be reached by establishing *in vitro* GEF activity assays for the protein in order to define the function of Lte1 biochemically.

Do redundant factors exist that activate Tem1 in the absence of *LTE1*?

The fact that *LTE1* is not essential for mitotic exit at room temperature has long been difficult to reconcile with the idea that Lte1 is a GEF for the Tem1 GTPase, if this is the case. Most small GTPases have a very poor GDP-off rate, which is the reason why they necessitate guanine nucleotide exchange factors for activation. *In vitro* studies suggest that Tem1 has an unusually high intrinsic exchange activity (Geymonat et al., 2002). This high GDP-dissociation rate is also a property of Sec4, a member of the Rab family of small GTPases that plays a role in polarized secretion (Kabcenell et al., 1990). However, its nucleotide exchange factor, Sec2, is essential for the process of polarized vesicle targeting (Walch-Solimena et al., 1997), suggesting that the high GDP off-rate of Sec4 does not alleviate the need for a GEF. However, since the interaction of Sec2 and the nucleotide-free form of Sec4 also contributes to the proper localization of Sec4 to secretory vesicles, it is difficult to conclude that the essential role of Sec2 in secretion is solely linked to its GEF activity (Walch-Solimena et al., 1997).

Many insights into the MEN signaling pathway have been gleaned by analogy with the related *S. pombe* septation initiation network (SIN), which controls septation and cytokinesis [reviewed in (Bardin and Amon, 2001)]. A high GDP-dissociation rate was also reported for Spg1, the Tem1 *S. pombe* homolog (Furge et al., 1998). No positive regulator of Spg1 GTP exchange has been identified thus far, leading to the hypothesis that exchange factors are superfluous for these related small GTPases. However, it is possible that a redundant factor exists to regulate Tem1 in the absence of Lte1 activity.

A 1995 study of cells lacking Ras activity found that the inability of these cells to exit from mitosis at 10°C was complemented by the overexpression of *LTE1*, *TEM1*, *DBF2*, *SPO12*, *RSR1/BUD1*, and *BUD5* (Morishita et al., 1995). The first four genes are

now known to be important for the activation of either the MEN or the FEAR networks and the inactivation of mitotic CDK activity. *BUD1* and *BUD5,* a Ras-like GTPase and its GEF, respectively, are part of a GTPase signaling module that regulates bud site selection and morphogenesis in budding yeast (reviewed in Chant, 1999). Thus far, this module has no known role in the regulation of mitotic exit. However, the fact that both *BUD1* and *BUD5* are capable of suppressing the low temperature mitotic exit defect of cells lacking Ras activity suggests that these proteins may have a role in promoting Clb-CDK inactivation that is upstream or parallel to *TEM1*. Further, Morishita and colleagues found that the deletion of *BUD1* in cells lacking Ras activity rendered these cells unable to complete mitotic exit even at 30°C (Morishita et al., 1995), suggesting that *BUD1*, and perhaps *BUD5*, perform a redundant role with Ras in promoting the exit from mitosis at this temperature.

We previously hypothesized that Ras-GTP enables the enhancement of Lte1's GEF activity on Tem1. Since *TEM1* is essential for mitotic exit, it is possible that *BUD5* encodes for an alternate GEF for Tem1 in the absence of Lte1. In this scenario, Bud1-GTP would activate Bud5, allowing the GEF to have more affinity for Tem1-GDP at its catalytic binding site. If this were true, the deletion of either *BUD5* or *BUD1* should be lethal with *lte1Δ* at 30°C, or at least exhibit severe delays in Cdc14 release and Clb-CDK degradation. Bud5 and Lte1 have significant sequence homology in their catalytic domains, (Figure 3) suggesting that the proteins could both catalyze the exchange

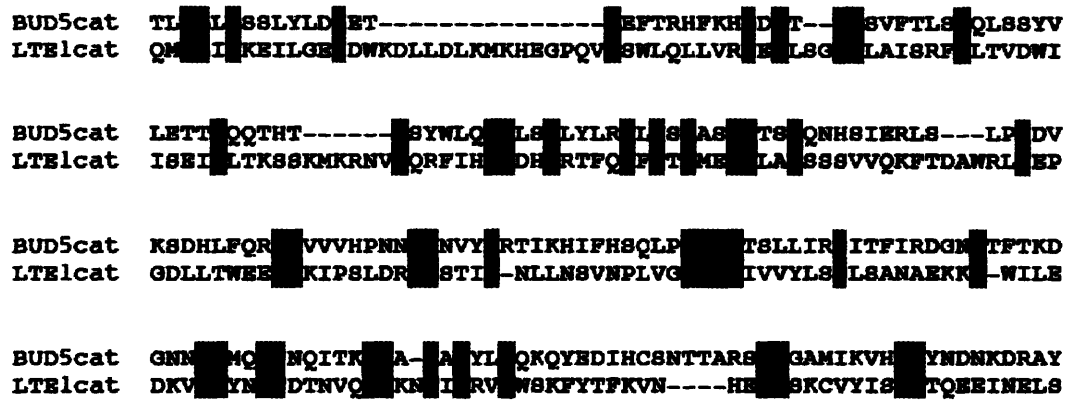

Figure 3: Alignment of Bud5 and Lte1 catalytic domains
The alignment was generated using ClustalW (Henikoff and Henikoff, 1993) to identify homology between the catalytic domains of Bud5 (amino acids 419 - 631) and Lte1 (amino acids 1201 - 1401). Strictly conserved residues are marked by red boxes, similar residues by yellow boxes. These proteins sequences exhibit 22% identity and 51% similarity within their catalytic domains.

reaction on Tem1. Tem1 and Bud1 are also similar in the sequence of their switch1 and switch 2 domains, which are involved in the nucleotide exchange reaction (Boriack-Sjodin et al., 1998). In addition, Bud5 has a GEFN domain that is homologous to the Sos Rem domain. The predicted secondary structure of Bud5 also indicates that this region consists of six alpha helices, indicating that Bud5 could be regulated by Bud1-GTP in a manner similar to the regulation of Sos by Ras-GTP. It will be interesting to see whether the activation of GEFs by GTPases is a general mechanism for mediating crosstalk between different GTPase modules.

Another possible mechanism by which *BUD1* and *BUD5* could regulate mitotic exit is through the Cdc42 GTPase. It has been shown that both *CDC42* and *CDC24* mutants are defective in mitotic exit in combination with a deletion of *LTE1*, and that this is due to a role for the PAK-like kinase Ste20 in promoting mitotic exit (Hofken and Schiebel, 2002). The mechanism by which *STE20* promotes mitotic exit is thus far unknown. However, it is possible that this kinase participates in the early anaphase release of Cdc14 from the nucleolus since synthetic lethality with *lte1Δ* is a hallmark of FEAR mutants. Cdc42 is positively regulated by its exchange factor, Cdc24, and the action of this GTPase module initiates actin cytoskeleton polarization (Gulli and Peter, 2001). Recent studies suggest that the Bud1 GTPase links the polarization machinery to bud site markers by interacting with both Cdc24 and Cdc42 (Kozminski et al., 2003; Park et al., 1997; Zheng et al., 1995). Thus, it is also possible that Bud1 and Bud5 promote mitotic exit by activating Cdc42. Whether the FEAR release Cdc14 is impaired in *ste20Δ* is unclear, but if Bud1 acts to promote FEAR, then a *bud1Δ lte1Δ* double mutant should

be defective in the early anaphase release of Cdc14. If, however, Bud1 and Bud5 act to promote GDP-exchange on Tem1, the FEAR release of Cdc14 should be unaffected in the double mutant. It will be interesting to determine the bud site selection GTPase module acts within the FEAR or the MEN to promote mitotic exit.

The regulation of Lte1 by cell polarity determinants provides additional temporal links between mitotic exit and cytokinesis.

The establishment of temporal links between mitotic exit and cytokinesis is critical for the maintenance of genomic stability. More and more evidence supports the hypothesis that the MEN signaling pathway plays a direct and essential role in cytokinesis following the completion of mitosis. These findings suggest that late mitotic events are temporally coupled by employing one pathway to control multiple events. For example, several groups previously reported that the MEN protein kinases Cdc5, Cdc15, Dbf2 and Dbf20 and the Dbf2-associated factor Mob1 localize to the SPB during mitotic exit and at the bud neck during cytokinesis (Frenz et al., 2000; Song et al., 2000; Xu et al., 2000; Yoshida, 2001). That the translocation of these proteins to the bud neck is of functional importance for the completion of cytokinesis came from the observation that *mob1-77* mutants that overexpress the CDK inhibitor *SIC1*, hence alleviating the need for *MOB1* in mitotic exit, are still impaired in cytokinesis (Luca et al., 2001).

Not only are MEN proteins required for the initiation of cytokinesis, but also it appears that, like in *S. pombe*, mitotic CDK inactivation is likely a prerequisite for cytokinesis, providing another temporal link between these two processes. Dbf2 and a

related protein Dbf20 localize to the mother – bud neck concomitant with mitotic CDK inactivation (Lim et al., 2003). Furthermore, mitotic kinase inactivation is required for the translocation of Dbf2 and Dbf20 to the bud neck. Consistently, ectopic inactivation of CDKs is sufficient to promote the association of Dbf2 and Dbf20 with the bud neck (Lim et al., 2003). The dependence of this translocation on CDK inactivation suggests an elegant mechanism for ensuring that cytokinesis follows exit from mitosis.

The recent discovery of a requirement for polarity determinants in maintaining Lte1 localization revealed a novel functional connection between mitotic exit and cell polarity factors. In particular, the Rho GTPase Cdc42 impinges on the process of mitotic exit at several levels. For example, Cdc42 activates the protein kinase Cla4, which is required for the localization Lte1 to the bud cortex (Chiroli et al., 2003; Hofken and Schiebel, 2002; Seshan et al., 2002). Cells lacking either *CDC42* or *CLA4* are impaired for Cdc14 release at 10°C (Hofken and Schiebel, 2002; Seshan et al., 2002), suggesting that Cdc42, through Cla4, regulates Lte1 activity as well (see above). Two related Cdc42 effectors, Gic1 and Gic2, also activate mitotic exit through the MEN pathway. Gic1 interferes with the binding of the two-component GAP complex, Bub2/Bfa1, to the Tem1 GTPase (Hofken and Schiebel, 2004). This regulation occurs at anaphase onset, when Gic1 is released from the bud cortex into the cytoplasm, providing a second mechanism by which nuclear migration and mitotic exit can be temporally coupled (Hofken and Schiebel, 2004). Yet another Cdc42 effector, the PAK-like kinase Ste20, also regulates mitotic exit. Mutants in components of the FEAR pathway, which contributes to the timely release of Cdc14 during anaphase, are normally non-essential. However, in combination with a mutation in a non-essential MEN component, such as *LTE1*, these

cells are not viable. In a screen for genes that act redundantly with the MEN pathway, Hofken and Schiebel identified *STE20* (Hofken and Schiebel, 2002). It is unclear how the Ste20 protein contributes to Cdc14 release, though it has been shown that this kinase does not regulate Lte1 localization or phosphorylation (Hofken and Schiebel, 2002; Seshan et al., 2002).

What is the significance of Cdc42 having multiple roles in mitotic exit? Perhaps Cdc42 and its effectors provide an additional temporal link between mitotic exit and cytokinesis. Cdc42 and its effectors regulate the changes in the actin cytoskeleton that execute polarized growth. Cytokinesis in budding yeast occurs by the action of an acto-myosin based contractile ring located at the mother – bud neck. The recruitment of ring components and the formation of the septum require the presence of a functional septin ring (Tolliday et al., 2003). The proper functioning of the septin ring, in turn, depends upon the Cdc42 GTPase and its effector Cla4, as well as the MEN. Thus the MEN and the cell polarity regulator Cdc42 both regulate cytokinesis by promoting septin ring function.

The septin ring provides a spatio-temporal link between mitotic exit and cytokinesis

Recent work has shown that the septin ring fluctuates in its fluidity in a cell cycle regulated manner (Dobbelaere et al., 2003). In G1, the ring is "fluid" and characterized by rapid turnover within the ring structure. However, from S phase to the end of M phase, the septin ring takes on an immobile or "frozen" character. Cytokinesis involves changes

in septin ring dynamics from frozen to fluid states, ending with a fluid state in G1 (Dobbelaere et al., 2003). The rigid frozen state of the septin ring is induced by the phosphorylation of a septin subunit by Cla4. Activation of the MEN is then required for the activation of the PP2A phosphatase that dephosphorylates the septin subunit and restores the fluid state (Dobbelaere et al., 2003). Thus, MEN activation is temporally coupled to the execution of cytokinesis.

The septins also have a dual role in coupling nuclear migration to mitotic exit. The spatial restriction of Tem1 and Lte1 is important for preventing exit from mitosis in cells with misaligned spindles. This idea is consistent with the finding that the presence of Lte1 in the mother cell, either by overexpression of *LTE1* or by the disruption of the septin ring using a *SHS1* deletion, causes inappropriate mitotic exit in an *LTE1*-dependent manner (Bardin et al., 2000; Castillon et al., 2003). In addition, the septin *CDC10* has an *LTE1*-independent role in preventing inappropriate mitotic exit (Castillon et al., 2003). Previously, Adames et al. suggested that astral microtubules interacting with the bud neck act as a sensor for spindle position, as inappropriate exit of cells with misaligned spindles correlated with loss of cytoplasmic microtubules from the bud neck (Adames et al., 2001). The mechanism by which the septin scaffold inhibits MEN in such cells is not known, but it may act through the two-component GAP Bub2/Bfa2, since cells with mispositioned spindles are unable to restrain mitotic exit in the absence of *BUB2* (Bardin et al., 2000; Pereira et al., 2000).

The initiation of a new cell cycle in the absence of cytokinesis is detrimental to the maintenance of genomic stability. Therefore, the cell likely has mechanisms to monitor and prevent this from occurring. The septin ring appears to play a role in

delaying cell cycle initiation in the absence of cytokinesis. Mitotic exit mutants such as *cdc15-1*, *tem1-3* and *cdc14-3* characteristically arrest with a long spindle and high mitotic CDK activity. Although cytokinesis does not occur in these cells at 37°C, prolonged incubation at the restrictive temperature causes a distal apical projection to form in the daughter cell due to a commitment of the arrested cells to the initiation of a new cell cycle (Jimenez et al., 1998). This apical projection constitutes a failed budding attempt that causes the cells to eventually lyse and is accompanied by the initiation of S phase (Jimenez et al., 1998). The formation of this distal projection can be abolished in START defective double mutants such as *cdc15-1, cdc28-4* (Jimenez et al., 1998). Interestingly, in *cdc15-1* mutant cells lacking a component of the septin ring, *CDC10* (*cdc15-1 cdc10Δ* cells), several rounds of budding were initiated before lysis, indicating that a septin mutation protects the MEN mutant from lysis (Jimenez et al., 1998). These results suggest that a septin-dependent checkpoint prevents cytokinesis-defective cells from initiating new rounds of replication and forming chains – a deleterious condition indeed for multi-cellular organisms. It will be interesting to determine the molecular mechanisms of this checkpoint and to note whether a similar checkpoint exists in higher eukaryotes. Thus, the septin scaffold acts as a spatio-temporal timer to couple nuclear migration to mitotic exit, and subsequently to link cytokinesis to the initiation of a new cell cycle.

How do cells ensure that mitotic exit is completed before cytokinesis?

The activities of the MEN and Cdc42 effectors are required for the completion of both mitotic exit and cytokinesis, allowing these two processes to be temporally coupled

within cell cycle progression. However, sharing requirements for specific protein activities would only allow both processes to occur at the same time, not in a specific sequential order. One way to make sure that mitotic exit occurs before cytokinesis is to create a lower sensitivity to MEN levels for the completion of cytokinesis than for exiting from mitosis. If this were true, then by the time MEN activity had reached sufficient levels for cells to undergo cytokinesis, the exit from mitosis would have to have been initiated.

Potential examples of cytokinesis needing greater MEN activity than mitotic exit are alleles of *DBF2* and *MOB1* that are lethal at 37°C due to an inability to initiate mitotic exit. These mutants are able to degrade Clb-CDKs at a semi-permissive temperature (31°C) but are incapable of forming a septum and therefore, cannot undergo cytokinesis. Such cells accumulate as large groups of cells attached to each other after eventually arresting (Lim et al., 2003), It is possible that all of these temperature-sensitive MEN mutations simply prevent the partially defective proteins from being able to translocate to the division site, where they perform their cytokinetic function. However, this can be easily tested by examining the localization patterns of tagged versions of these alleles at the semi-permissive temperature.

Another example of cytokinesis being less sensitive to MEN activity than mitotic exit is the temporal lag observed between spindle disassembly and actomyosin ring contraction in the absence of *LTE1*. Normally, a tight coupling is observed between spindle disassembly and contraction of the actomyosin ring at the division site (Adames et al., 2001; Segal et al., 2002). However, cells lacking *LTE1* exhibit a delay in the constriction of the actomyosin ring following spindle disassembly even when grown at

30°C, a temperature at which there is not a significant delay in the timing of mitotic exit observed (Jensen et al., 2004). This temporal uncoupling of mitotic exit and cytokinesis can be abolished by the deletion of *BUB2* or by the overexpression of *CDC14*, but NOT by the overexpression of the CDK-inhibitor *SIC1*, indicating that MEN plays a role in the temporal regulation of cytokinesis that is independent of its role in mitotic CDK inactivation, and that the role of *LTE1* in coupling spindle disassembly to actomyosin ring contraction is via its ability to activate the MEN (Jensen et al., 2004). The requirement of higher levels of MEN activity for cytokinesis than for mitotic exit allows for a stringent ordering of these two processes, resulting in the completion of mitotic exit prior to the onset of cytokinesis.

The hypothesis that mitotic exit and cytokinesis are sensitive to varying MEN activation levels assumes that the MEN signaling pathway exhibits a 'graded' response, and not a 'switchlike' response. In a graded response, intermediate states of activation are observed, whereas such states are never observed in switchlike responses (Ferrell, 2000). One example of a signaling cascade that exhibits switchlike behavior is the MAPK oocyte maturation pathway in *Xenopus* (Ferrell and Machleder, 1998). MAPK activity is induced by the addition of the hormone progesterone to G_2 arrested oocytes, resulting in oocyte maturation. In a seminal study examining individual oocytes, Ferrell and Machleder discovered that the exposure of immature oocytes to intermediate progesterone levels results in near-complete activation of MAPK in some oocytes, and near-zero activation in others (Ferrell and Machleder, 1998). Thus, although populations of oocytes exhibited an intermediate level of MAPK activation in response to intermediate levels of progesterone, this was not observed in individual oocytes.

In contrast to the *Xenopus* MAPK pathway, the yeast MAPK mating cascade was found to behave in a graded manner. Using GFP under the control of the mating-induced *FUS1* promoter, Poritz and colleagues observed intermediate states of GFP fluorescence when cells were treated with intermediate amounts of the alpha-factor pheromone (Poritz et al., 2001). Importantly, intermediate *FUS1* promoter activation was observed not only at the population level, but at the single-cell level as well. The authors used flow cytometry to analyze the transcriptional response in individual cells (Poritz et al., 2001).

In order to test whether the MEN signaling pathway exhibits a graded or switchlike response at the single-cell level, one could employ a GFP-reporter system similar to the one used by Poritz and colleagues. The *SWI5* transcription factor regulates the Clb-CDK-inhibitor *SIC1*. Swi5 is localized to the cytoplasm from S phase to anaphase, and translocates to the nucleus upon Cdc14-mediated dephosphorylation of the protein (Visintin et al., 1998). Therefore, the activation of *SWI5* promoter-regulated genes is a marker for MEN activation. One can place GFP under the control of the *SWI5* promoter and examine whether intermediate states of promoter activation exist in cells that are exiting from mitosis. By arresting cells prior to mitotic exit at 37°C using a *cdc15-2* mutation and subsequently releasing these cells at the permissive temperature to allow synchronous mitotic exit, one can collect cells for flow cytometry analysis and examine the levels of MEN activation in single cells.

Perspectives on mitotic exit in higher eukaryotes

The elucidation of the mechanisms that regulate mitotic exit in budding yeast will likely lead to the understanding of this process in higher eukaryotes. There is mounting evidence that the process of mitotic exit in mammalian cells is more similar to that in budding yeast than was previously appreciated. For example, in budding yeast, APC^{Cdh1} plays a key part in the degradation of substrates that are important for the completion of mitotic exit (Visintin et al., 1998; Zachariae et al., 1998). In contrast, the essential role of APC^{Cdh1} in mammalian cells was thought to be the repression of mitotic cyclins in order to establish the G1 phase [reviewed in (Peters, 2002)]. However, a recent study examining the timing of APC^{Cdh1} substrate degradation revealed that key mitotic regulators are degraded at distinct times during mitotic exit, suggesting that APC^{Cdh1} activity is modulated during mitotic exit (Lindon and Pines, 2004). Specifically, the authors found that the degradation of the polo-like kinase Plk1 by APC^{Cdh1} is necessary for timely exit from mitosis (Lindon and Pines, 2004). These findings suggest that although the proteolysis of cyclin B is completed at the metaphase – anaphase transition by APC^{Cdc20}, APC^{Cdh1} is important for the execution of exit from mitosis in human cells.

Mammalian homologs of MEN and SIN components have been identified. In at least one instance, the subcellular localization of the homolog as well as its role in regulating late mitotic events appears to be conserved. Centriolin encodes the mammalian homolog of the *S. cerevisiae* Nud1 protein and *S. pombe* Cdc11p protein, which act as scaffolds for MEN and SIN components, respectively, at the SPB. Centriolin localizes specifically to the mother centriole, which displays the unique property of moving towards the midbody at the end of mitosis, triggering abscission of the daughter cells.

Centriolin is likely to be important for this abscission event to occur, as reducing centriolin expression using siRNA disables cell separation (Gromley et al., 2003).

Interestingly, one isoform of the human homolog of *S. cerevisiae* Cdc14, hCdc14A, localizes to centrosomes during telophase and cells lacking hCdc14A have cytokinesis defects (Kaiser et al., 2002). An attractive hypothesis is that hCdc14A localizes specifically to the mother centriole and plays a role in regulating the events that lead to cell separation. Although no homolog of *LTE1* has yet been identified, future studies of how this protein is regulated, both genetically and biochemically, will likely prove to be useful in the understanding of how Ras and Ras-related pathways collaborate to regulate mitotic events.

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Appendix A: Structure-function analysis of the Cfi1/Cdc14 interaction domains

Introduction

The phosphatase Cdc14 is the ultimate effector of the MEN cascade, which directs the inactivation of mitotic CDKs at the end of mitosis. The activity of Cdc14 throughout the cell cycle is regulated by an inhibitor. Prior to metaphase, Cdc14 is held inactive by its inhibitor, Cfi1/Net1, in the nucleolar compartment (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). At the metaphase – anaphase transition, upon activation of APC^{Cdc20}, the transient release of Cdc14 from Cfi1 is initiated by the FEAR network (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida and Toh-e, 2002). In mid – late anaphase, the MEN is activated, in part by the movement of the daughter SPB into the bud compartment (Bardin et al., 2000; Pereira et al., 2000).

The precise regulatory mechanisms that govern the release of Cdc14 and Cfi1 are beginning to be understood. However, a mechanistic understanding of the physical interaction between these two proteins is still lacking. Without more detailed information about the nature of the interaction between these two key regulators of mitotic exit, it will be difficult to get a clear picture of the process of mitotic exit in budding yeast. To this end, we have undertaken a structure – function analysis of Cdc14 and Cfi1. We constructed large C-terminal and N-terminal truncations of tagged version of Cfi1 and C-terminal truncations of Cdc14 in order to dissect the regions of these proteins that are important for their interaction *in vivo*.

Materials and Methods

Cfi1 truncations were constructed using the PCR method described in Longtine et al., 1998 (Longtine et al., 1998). C-terminal truncations were tagged with a 13-MYC epitope for immunofluorescence and western visualization. N-terminal truncations were constructed at the endogenous *CFI1* locus, tagged with a 3-HA epitope, and placed under the control of the *GAL1-10* galactose inducible promoter. Cdc14 truncations were constructed by cloning a PCR product from a wild-type strain containing N-terminally tagged Cdc14 (A1411) into the HinDIII site of the Ycplac22 *TRP1*-marked vector. Each plasmid contains a region of *HA-CDC14* under its endogenous promoter.

Immunofluorescence and western analysis, and co-immunoprecipitation experiments were performed as described in Seshan and Amon, 2005. One exception was made in co-IP analysis: $MgCl_2$ was not added to the co-IP buffer when analyzing the immunoprecipitation of Cdc14-HA with Cfi1-13MYC.

Results and Conclusions

CFI1 truncation analysis

The results of the Cfi1 truncation analysis are summarized in Figure 1. We identified a region of *CFI1* that is required for the protein's localization to the nucleolus. This region is located at the C-terminus of the protein from amino acids 2678 – 3569. The deletion of this region causes Cfi1 to localize to the entire nucleus rather than the nucleolus. It will be interesting to attempt to identify interactors of Cfi1 that bind to this region, as they are likely to be important for tethering the protein in the nucleolus. On the other hand, the

reasons for nucleolar rather than nuclear localization of the Cfi1-Cdc14 complex seem at present unclear. Constructs C7 and C6 lack the nucleolar localization domain of *CFI1* but still contain the nuclear localization domain, located at amino acids 1 – 293, as well as the Cdc14 interaction domain, which is located from amino acid 1 – 589. Interestingly, both constructs C7 and C6 are capable of performing the function of full-length *CFI1*, at least at a gross phenotypic level. This is conistent with the finding that a truncation of *CFI1* consisting of amino acids 1-676 is able to inhibit Cdc14 activity *in vitro* (Traverso et al., 2001). Thus, it seems that the key role of Cfi1 lies in its ability to bind to Cdc14, and that the ability of regulators of the Cdc14 – Cfi1 interaction to dissociate the inhibitory complex and activate Cdc14 is not wholly impaired by the localization of the complex to the nucleus instead of the nucleolus. However, it would be worth examining in more detail the subtle effects on mitotic exit that the C7 truncation imposes.

We additionally identified a region of *CFI1* that is important for the protein's localization to the nucleus. The deletion of the N-terminal 293 amino acids of Cfi1 cause the truncated protein to be excluded from the nucleus. In addition, the absence of nuclear localization is detrimental to the ability of Cfi1 to inhibit Cdc14. Perhaps this nuclearly-excluded protein cannot associate with Cdc14, which is kept in the nucleus by some unknown mechanism. Another possibility is that Cdc14 is tethered to the mis-localized Cfi1 truncation in these cells, which would most likely disrupt the regulatotion of Cdc14-Cfi1 complex formation, and perhaps render Cdc14 unable to dissociate from Cfi1 during anaphase. It would therefore be of great interest to examine the localization of Cdc14 in cells expressing the N2 Cfi1 truncation.

In addition, phosphorylation of Cfi1 has been shown to be important for the regulation of the Cdc14 – Cfi1 complex formation (Azzam et al., 2004; Visintin et al., 2003; Yoshida and Toh-e, 2002). The activation of the MEN promotes the phosphorylation of Cfi1, while the phosphorylation of Cdc14 seems to be modulated by the FEAR. The efficient dissociation of these two proteins and activation of Cdc14 requires both Cfi1 and Cdc14 phosphorylation (Visintin et al., 2003). Therefore, it would be of interest to examine the phosphorylation status of these truncations to see whether any of the phenotypic changes are linked to the absence of key phosphorylated residues in Cfi1. This could be done by comparison with the Cfi1 phospho-residues that have been mapped by Azzam et al.

CDC14 truncations

The results of the C-terminal *CDC14* truncation analysis are summarized in Table 1. Not surprisingly, it is clear that the phosphatase domain of Cdc14 is required for its ability to complement the mitotic exit defect of a *cdc14-3* mutant at the restrictive temperature of 37°C. Truncations lacking the phosphatase domain are unstable and the protein is not detectable by western analysis. The truncation of the C-terminal 136 amino acids does not seem to be detrimental to the function of Cdc14, since this truncation can support growth of the *cdc14-3* mutant. It will be of interest to examine the region between the phosphatase domain and amino acid 414 more carefully and determine the minimal region of the C-terminus of Cdc14 that is required for its function.

A mechanism for the regulation of Cdc14 independently of its association with Cfi1 is sure to exist, since the deletion of *CFI1* leads to an impairment, but not a inability,

of Cdc14's activity to be regulated. The overexpression of *CDC14* under the *GAL1-10* promoter is lethal, causing cells to arrest in G1 due to the inability of these cells to accumulate Clb-CDKs (Visintin et al., 1999). However, in cells lacking *CFI1*, the accumulation of the mitotic cyclin Clb2 is delayed, but eventually does occur (Visintin et al., 1999). This suggests that there may be other negative regulators of Cdc14 in the absence of Cfi1 function. The finer mapping of Cdc14's domains should be coupled with an analysis of the residues in Cdc14 required for its interaction with Cfi1. This could lead to an identification of a region of Cdc14 that regulates its activity independently of Cfi1.

Table 1: CDC14 C-Terminal Truncation Constructs

#1 CDC14 promoter-HA_____♣_____550

#2 CDC14 promoter-HA_____♣_____414

#3 CDC14 promoter-HA_____274

#4 CDC14 promoter-HA_____140

** The symbol ♣ indicates the location of the Cdc14-phosphatase domain.

	#1	**#2**	**#3**	**#4**
Length(aa)	550	414	274	140
Est. Protein Size (kD)	66	52	Undetectable	undetectable
Localization	Like wt	Like wt	undetectable	undetectable
Comple-ments *cdc14-3*?	yes	yes	no	no
# of isolates tested	2	2	2	2

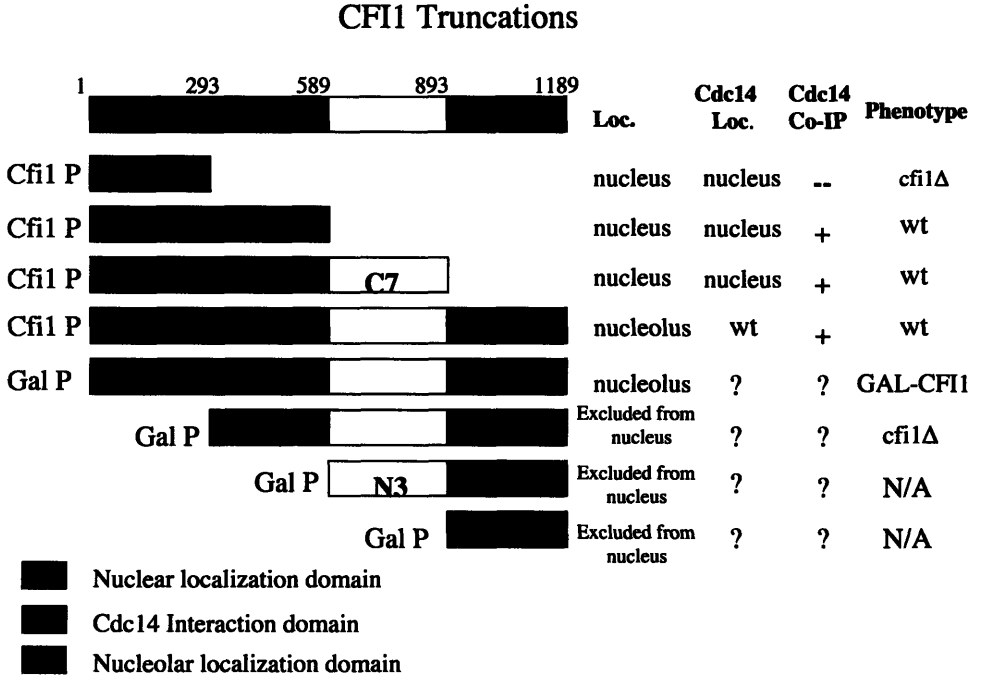

Figure 1: Truncation analysis of *CFI1*

A summary of results obtained for each truncation is presented with regards to localization of Cfi1 (column 1), localization of Cdc14-HA (column 2), the ability of each protein to associate with Cdc14-HA by co-immunoprecipitation analysis (column 3), and the phenotypic analysis of cells containing the truncation construct as examined by cellular morphology under the light microscope. For analysis of *GAL-CFI1* constructs, cells were grown in YEP + 2% Raffinose for 2 hours and subsequently collected for immunofluorescence and phenotypic analysis. The numbers above the schematic indicate the amino acid at which each truncation begins/ends. Note that for N-terminal constructs N3 and N4, phenotypic analysis was precluded by the fact that the endogenous protein under the endogenous *CFI1* promoter was still being produced. Therefore, in cells

containing the N3 truncation, a region of *CFI1* that corresponds to the C6 truncation is still present, and in N4 truncated cells, a region of *CFI1* that corresponds to C7 is still present. Because both C6 and C7 are able to function as wild-type *CFI1*, the effects of deleting the first 1766 or 2678 amino acids on *CFI1* function are not possible.

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Appendix B: Two-hybrid screen using Lte1 as bait

Introduction

Lte1 is a positive regulator of the MEN pathway, which executes the destruction of mitotic CDKs at mitotic exit. *LTE1* is only essential for mitotic exit at low temperatures (Shirayama et al., 1994), and is required in cells that are impaired for the FEAR network (Stegmeier et al., 2002). The localization pattern of Lte1 throughout the cell cycle is highly regulated. In G1, the protein is present throughout the cytoplasm. In S phase, concomitant with bud formation, Lte1 associates with the incipient bud cortex by the dual action of the Ras2 GTPase and the Cdc42 GTPase, through its effector, the Cla4 kinase (Bardin et al., 2000; Hofken and Schiebel, 2002; Pereira et al., 2000; Seshan and Amon, 2005; Seshan et al., 2002; Yoshida et al., 2003). The phosphorylation of Lte1 by Cla4 is thought to mediate its ability to associate with the bud cortex (Hofken and Schiebel, 2002; Seshan et al., 2002). In addition, the bud cortex association of Lte1 may regulate the protein's ability to initiate mitotic exit. The sequestration of Lte1 at the bud cortex is contingent upon the function of the septin ring, which acts as a diffusion barrier at the mother – bud neck (Faty et al., 2002; Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et al., 2002). Upon exit from mitosis, Cdc14 initiates the dephosphorylation and dissociation of Lte1 from Ras and the cell cortex (Jensen et al., 2002; Seshan and Amon, 2005; Seshan et al., 2002).

Several proteins that regulate the localization, and potentially the activity, of Lte1 have now been identified. However, the mechanism by which Lte1 activates mitotic exit is poorly understood. Lte1 has both N-terminal and a C-terminal GEF homology domains. In addition, it has been shown genetically to function upstream of the Tem1

GTPase (Shirayama et al., 1994), leading to the hypothesis that Lte1 is a GEF for Tem1. However, the requirement of Lte1's GEF domain for its mitotic exit promoting function is controversial (Seshan and Amon, 2005; Yoshida et al., 2003) and a region of Lte1 in the middle of the protein (aa 659 to 926) that does not contain the GEF homology domains has been proposed to be sufficient for the function of Lte1 in activating mitotic exit at low temperatures (Yoshida et al., 2003). In order to perform an unbiased screen for regulators of Lte1, we have performed a 2-hybrid screen using three different regions of Lte1 as baits: Lte1N (aa 1-500); Lte1mini (aa 659-926); and Lte1C (aa 926-1435).

Materials and Methods

The bait plasmids are A892 (LTE1-N), A893 (LTE1-C) and A894 (Lte1-mini). Greater than 6 X 10^8 colonies were screened for each bait. Only one library was screened (C1) to saturation. In order to complete the screen, both C2 and C3 libraries must be mated to the baits and examined. Colonies were originally plated on –Ade plates, and after 10 days, these plates were replica-plated to –His, -Leu, -Ura, -Ade plates to select for cells that could activate the His reporter as well, and that still contained the bait plasmid (URA-marked) and the library prey plasmid (LEU-marked). Colonies that grew on these plates were streaked to single colonies and patched to –Ade plates. Each patch was then patched to 5-FOA plates and subsequently re-tested on –Ade in order to test whether loss of the Ura-marked bait plasmid prevented the activation of the *ADE* reporter gene, indicating bait dependence and eliminating self-activating library constructs. DNA was isolated from true positives and transformed into *E. coli*. Plasmid DNA was obtained and

sequenced using primer that primes 300bp upstream of multi-cloning site in pGAD-two-hybrid vector (#1745).

Results and Conclusions

The results of this incomplete 2-hybrid screen are noted in Table 1. Thus far, the only known regulator of Lte1 identified is *KEL2*. The Kelch-repeat protein Kel2 is an interactor of Kel1, which may regulate mitotic exit. Kel1 does not appear to be required for the localization of Lte1 but does associate with Lte1 throughout the cell cycle (Hofken and Schiebel, 2002; Jensen et al., 2002), and may also negatively regulate mitotic exit (Hofken and Schiebel, 2002). Kel1 and Kel2 both localize to the bud tip during bud growth, and to the bud neck during cytokinesis (Philips and Herskowitz, 1998). These poorly characterized proteins do not regulate actin cytoskeletal dynamics, as is the case for their homologs in *D. melanogaster*. However, Kel1 and Kel2 do seem to play a role in cell fusion during mating, but their role in mitotic exit, if any, is not understood (Philips and Herskowitz, 1998).

The *GSY2* gene products acts as a glycogen synthase and is induced under conditions of glucose-limitation and nitrogen starvation (Parrou et al., 1999). This protein is negatively regulated by the Ras-activated cAMP pathway (Thevelein and de Winde, 1999). A role for these proteins in mitotic exit has not yet been discovered. However, the interaction with Gsy2 may highlight a previously unappreciated role for Lte1 in mediating the response of budding yeast cells to nutritional conditions. The role of Ras in

regulating Lte1 is clearly independent of its role in cAMP production and nutrient signaling, since the constitutive activation of protein kinase A by the deletion of its negative regulatory subunit *BCY1* does not rescue the mitotic exit defects of cells lacking Ras activity (Morishita et al., 1995; Yoshida et al., 2003). However, Lte1 is more highly phosphorylated in alpha-factor arrested cells grown in synthetic complete media than those grown in YPD (Figure 1). In addition, this higher basal phosphorylation level is only apparent in alpha-factor arrested cells, not in S phase arrested cells (unpublished data). The absence of *CLA4* abolishes all basal Lte1 phosphorylation (Figure 1). The significance of this G1 phosphorylation is yet to be discovered, but it is clear that regulators of nutrient signaling do impinge on Lte1 and that this may be important for the regulation of mating or other environmentally regulated processes. In support of this, spores lacking *LTE1* are much less efficient in germinating (unpublished observations).

BUD14 encodes a protein of unknown function that regulates (Ni and Snyder, 2001) bud-site selection in diploids (Ni and Snyder, 2001). A GTPase module regulates bud site-selection differently in haploids and diploids. Haploids follow an axial budding pattern, while diploids bud in a bipolar fashion (Chant and Pringle, 1995). The Ras-like GTPase Bud1 regulates the polarization of the cytoskeleton in response to cortical landmarks (Michelitch and Chant, 1996). Diploid cells lacking *BUD14* exhibit a random budding pattern. A role for bud-site selection regulators in mitotic exit has been hinted at by the finding that the overexpression of *BUD1* or its GEF *BUD5* rescues the mitotic exit phenotype of cells lacking Ras activity (Morishita et al., 1995). However, no direct connections have been made between the mitotic exit machinery and the bud-site selection GTPase module. Haploid cells lacking *BUD14* are not defective in Lte1

localization and are also not synthetic lethal with a *SPO12* deletion (unpublished data), suggesting that if Bud14 does interact with and regulate Lte1, it may do so redundantly.

Ras and Rho GTPases collaborate to regulate the localization, and perhaps the function, of Lte1. Therefore, Lte1 is a junction at which different pathways converge to regulate the timing of mitotic exit. The usefulness of a 2-hybrid screen in illuminating the regulators and the function of Lte1 in mitotic exit is evident. The completion of this screen should allow the establishment of novel connections between mitotic exit and bud-site selection, as well as nutrient signaling.

Table 1: Results of 2-hybrid using Lte1N, Lte1C, and Lte1mini as baits

Lte1 bait	Interactor	# times obtained
Lte1C	GSY2	4
Lte1C	TEL2	2
Lte1C	LTE1(Cterminus)	1
Lte1mini	BUD14	1
Lte1mini	YAP6/HAL7	2
Lte1mini	KEL2	1

Note: The Lte1-full length bait construct was constructed, but was not visible by western blot analysis. All contructs contain an N-terminal HA tag, and therefore can be visualized by western blot analysis and immunofluorescence.

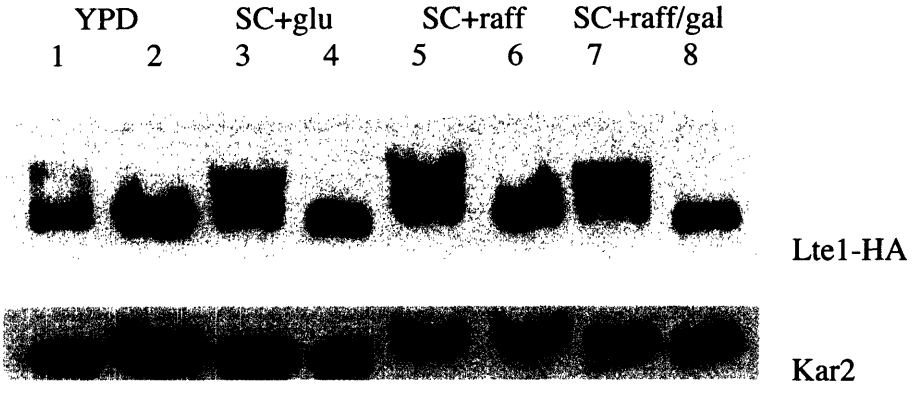

Figure 1: Phosphorylation status of Lte1 in changes in response to different media
Lanes 1, 3, 5, and 7 are wild type cells containing an *LTE1-HA* fusion, and lanes 2, 4, 6, and 8 are *cla4Δ* containing an *LTE1-HA* fusion. Cells in lanes 1 and 2 were grown in YPD overnight; cells in lanes 3 and 4 were grown in SC+ 2% glucose; cells in lanes 5 and 6 were grown in SC+ 2% raffinose; cells in lanes 7 and 8 were grown in SC + 2% raffinose + 2% galactose. All cultures were arrested in 5μg/ml alpha-factor pheromone for 2 hours and 30 minutes. Cells were then collected for western blot analysis. Kar2 serves as an internal loading control.

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