Regulation of the Mitotic Exit Network Components

Tel1 and Cdc15 in *Saccharomyces cerevisiae*

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Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Biology
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Tem1 and Cdc15 in *Saccharomyces cerevisiae*

by

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Abstract

The division of a single eukaryotic cell resulting in two daughter progeny is controlled by cyclin dependent kinase activity (CDK). Mitotic cyclins associated with CDK promote the segregation of genetic material ensuring that each daughter receives a complete complement of the genome. In order for exit from mitosis into G1 to occur, mitotic CDK activity must be inactivated. In the budding yeast, *Saccharomyces cerevisiae*, a network of proteins called the mitotic exit network is essential for mitotic CDK inactivation and, therefore, exit from mitosis. The work presented herein describes the regulation of two components of the mitotic exit network, Tem1 and Cdc15. A model for activation of the mitotic exit network is proposed. The spatial separation of the GTPase Tem1 from its activating GEF, Lte1, until anaphase is suggested to be one signal leading to correct timing of mitotic exit. Additionally, the roles of distinct regions of the protein kinase, Cdc15, are examined. Domains necessary and sufficient for localization of Cdc15 to the spindle pole body, association with other Cdc15 molecules, and a putative inhibitory domain are investigated. This work investigates the regulatory mechanisms controlling two essential components of the mitotic exit network.
Dedicated to my parents
for their love and support
for 28 years
Acknowledgements

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Summary

To ensure that each daughter cell receives only one copy of each chromosome, exit from mitosis and cytokinesis must not occur prior to the completion of chromosome segregation. It is, thus, critical that mechanisms are in place that establish a dependency of mitotic exit and cytokinesis on the completion of previous events. In the budding yeast *Saccharomyces cerevisiae*, a signalling cascade known as the mitotic exit network (MEN) controls exit from mitosis and ensures that this transition occurs only after sister-chromatid separation is initiated and the genetic material has been segregated between mother and daughter cell. The thesis presented herein investigates the mechanisms regulating two essential components of the MEN signal transduction cascade governing mitotic exit: the GTPase Tem1, and the protein kinase, Cdc15.

CDKs and their role in cell-cycle progression

The cell cycle controls events leading to the formation of two cells from a single cell. In budding yeast, a single cyclin dependent kinase (CDK), Cdc28, associates with various regulatory subunits (cyclins) in a choreographed manner throughout the cell cycle to phosphorylate targets that promote cell growth and division (Box 1). During G1, the levels of G1 cyclins rise and these cyclins associate with CDKs. Activity of G1 CDKs promotes the passage of cells through START (budding yeast), also known as the restriction point (R) in fission yeast and higher eukaryotes. After passing through this point, a cell is committed to continue through the cell cycle. In budding yeast, the G1 cyclins are Cln1, Cln2 and Cln3. Cln3
is present throughout the cell cycle at low levels, and this cyclin promotes the accumulation of Cln1 and Cln2 (Nasmyth, 1993).

S phase CDKs begin to increase towards the end of G1, and are inactivated during G2 and mitosis. On reaching a critical level they promote DNA replication. Clb5 and Clb6-associated kinases are important for promoting DNA synthesis, but in their absence mitotic CDKs (Clb1, Clb2, Clb3 and Clb4-associated kinases) can support DNA replication.

Mitotic CDKs are activated at the onset of mitosis and promote chromosome condensation, formation of the mitotic spindle and breakdown of the nuclear envelope, among other processes. These functions are performed by CDKs associated with Clb1, Clb2, Clb3, Clb4 and Clb5 (perhaps also Clb6). For cells to exit from mitosis, undergo cytokinesis and enter the following G1, mitotic CDKs must be inactivated (Koepp et al., 1999; Morgan, 1997).

**Box 1: CDKs and their role in Cell Cycle**
Control of mitosis by the anaphase-promoting complex

The onset of sister-chromatid separation defines the metaphase–anaphase transition (Box 2). Until then, sister chromatids are held together by a protein complex called cohesin. Sister-chromatid separation is initiated when a protease called Separase (Esp1) cleaves one of the cohesin subunits. Until the onset of anaphase, Separase is kept inactive by its inhibitor Securin (Pds1). Securin is inactivated at the onset of anaphase by a ubiquitin-dependent proteolysis machinery, termed the anaphase-promoting complex/cyclosome (APC/C) which, together with its specificity factor Cdc20, ubiquitylates Securin, targeting it for degradation. This allows for Separase to become active and sister-chromatid separation is initiated (reviewed in Uhlmann, 2001). In budding yeast, phosphorylation of Cohesin by Cdc5 makes it a better substrate for Separase cleavage (Alexandru et al., 2001).

The cell has several safeguards to prevent either the mis-segregation of genetic material or segregation of damaged genetic material (Box 2). The mitotic spindle-assembly checkpoint blocks the activity of the APC/C–Cdc20 in response to spindle damage. The protein Mad2, with the help of other checkpoint components, binds to APC/C–Cdc20 thereby blocking its activity (reviewed in Burke, 2000; Gardner and Burke, 2000). DNA damage activates a surveillance mechanism, coined the DNA-damage checkpoint (reviewed in Zhou and Elledge, 2000). In budding yeast, activation of this checkpoint causes cell cycle arrest at metaphase by stabilizing the anaphase inhibitor Securin.
Mitotic CDK inactivation is also initiated at the metaphase–anaphase transition by APC/C–Cdc20. In higher eukaryotes, APC/C–Cdc20 appears mainly responsible for inactivating mitotic CDKs during mitosis. However, in budding yeast, a pool of mitotic CDKs persists until elongation of the mitotic spindle has been completed. Degradation of mitotic cyclins during telophase and G1 is controlled by the APC/C and the Cdh1 specificity factor (reviewed in Morgan, 1999). In higher eukaryotes, a protein called MAD2L2/MAD2B has been shown to inhibit the APC/C–Cdh1 (Chen, 2001; Pfleger, 2001), akin to the role of Mad2 in blocking the APC/C–Cdc20. It is possible that this protein, too, could function in a surveillance mechanism.

**Box 2: Control of Mitosis by the Anaphase Promoting Complex**

![Diagram of control of mitosis by the Anaphase Promoting Complex]

1. Mitotic spindle defects (Mad2, others)
2. DNA damage
3. securin
   - Dephosphorylation
   - Mitotic cyclin
      - CDK
      - Sephas
      - Metaphase → Anaphase → G1
4. Cdc20
   - APC/C
5. Cdh1
   - APC/C
6. Mad2L2/Mad2B

---

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Inactivation of mitotic CDKs in budding yeast

For cells to exit from mitosis, mitotic CDKs must be inactivated. Mitotic CDKs also need to be inactivated for cytokinesis, the final physical separation of two cells, to occur. If mitotic CDK inactivation is prevented, cells arrest in late anaphase/telophase, with DNA masses segregated and an extended mitotic spindle (reviewed in Morgan, 1999; Zachariae et al., 1998).

In all eukaryotes analyzed to date, mitotic CDK inactivation is initiated at the metaphase–anaphase transition, when chromosome segregation begins (Box 2; reviewed in McCollum and Gould, 2001; Nasmyth et al., 2000; Wassmann and Benezra, 2001). However, at least in budding yeast, the inactivation of mitotic CDKs is not completed at this transition, and a pool of active CDKs persists in the cell until spindle elongation has occurred (Jaspersen et al., 1998; Shirayama et al., 1994; Shirayama et al., 1998; Surana et al., 1993). The active pool of mitotic CDKs that is maintained beyond the metaphase–anaphase transition plays an important biological role, as mitotic CDK activity is required for maintenance of the mitotic spindle (Grandin and Reed, 1993).

Inactivation of mitotic CDKs is mediated by ubiquitin-dependent proteolysis of mitotic cyclins (reviewed in Koepp et al., 1999; Morgan, 1999; Tyers and Jorgensen, 2000). A ubiquitin ligase, the anaphase-promoting complex or cyclosome (APC/C), together with a specificity factor, ubiquitylates mitotic cyclins and targets them for degradation by the 26S proteasome (Box 2). The specificity factor Cdc20 acts during the metaphase–anaphase transition to target mitotic cyclins as well as anaphase inhibitors for degradation. Later in the cell cycle,
during exit from mitosis and G1, the specificity factor Cdh1/Hct1 works with the APC/C to target substrates for degradation, thereby promoting mitotic exit.

**Cdc14 triggers mitotic CDK inactivation**

The pool of mitotic CDKs that persists beyond the metaphase–anaphase transition needs to be inactivated to allow exit from mitosis. APC/C-mediated degradation of Clb cyclins bring about this inactivation, as does a CDK inhibitor called Sic1 (reviewed in Morgan, 1999; Zachariae, 1999).

The dual-specificity phosphatase Cdc14 is essential for mitotic CDK inactivation (Morgan, 1999). Cells mutant for CDC14 arrest in telophase with high mitotic CDK activity, whereas overexpression of CDC14 brings about ectopic mitotic CDK inactivation. Cdc14 promotes mitotic CDK inactivation by the reversal of mitotic CDK phosphorylation (Fig. 1, Visintin et al., 1998). For example, the mitotic CDK inhibitor Sic1 is degraded throughout the cell cycle in a phosphorylation-dependent manner (reviewed in Koepp et al., 1999) but is dephosphorylated by Cdc14 promoting its stabilization (Visintin et al., 1998; Jaspersen et al., 1999). Cdc14 also upregulates transcription of the SIC1 gene by dephosphorylating the transcription factor Swi5, which promotes Swi5 translocation into the nucleus (Moll et al., 1991; Knapp et al., 1996; Toyn et al., 1997; Visintin et al., 1998). Another substrate of Cdc14 is the APC specificity factor Cdh1/Hct1 (Schwab et al., 1997; Visintin et al., 1997). Dephosphorylation of Cdh1/Hct1 allows its association with APC/C, causing the APC/C to be activated (Fig. 1; Jaspersen et al., 1999; Visintin et al., 1998; Zachariae et al., 1998). Cdc14 therefore, promotes
CDK inactivation through upregulation of the CDK inhibitor, Sic1, and through degradation of the mitotic cyclin Clb2 by the APC/Cdh1. Presumably there are many Cdc14 substrates, but the essential role of Cdc14 seems to be its dephosphorylation of Sic1, Swi5 and Cdh1/Hct1.

The mitotic exit network

Control of Cdc14 activity by the MEN

The activity of Cdc14 is controlled by its inhibitor Cfi1/Net1 (Fig. 1; refs (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999) and reviewed in (Bachant and Elledge, 1999)). Throughout G1, S phase, G2 and early mitosis (metaphase), Cdc14 is held inactive by Cfi1/Net1 in the nucleolus. Cfi1/Net1 has been proposed to function by occluding the active site of Cdc14 (Traverso et al., 2001). During anaphase, Cfi1/Net1 releases Cdc14. The MEN or mitotic exit pathway is required for the sustained release of Cdc14, leading to mitotic CDK inactivation (Fig. 2; Table 1). When the signalling cascade is inactivated, cells arrest in telophase with Cdc14 sequestered in the nucleolus (Shou et al., 1999; Visintin et al., 1999).

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<td>Phosphatase</td>
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<tr>
<td>GAP components</td>
<td>Bub2, Bfa1/Byr4</td>
</tr>
<tr>
<td>GEF</td>
<td>Lte1</td>
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<tr>
<td>SPB scaffold</td>
<td>Nud1</td>
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GAP, GTPase-activating protein; GAPCenA, GAP and centrosome associated; GEF, guanine-nucleotide exchange factor; SPB, spindle pole body.
Figure 1: Cdc14 Controls Mitotic CDK Activity

Solid arrows indicate reasonably well established relationships, dashed arrows indicate more speculative ones, question marks denote more than one possible way a protein could act.

1. Cdc14 inhibits mitotic CDKs by reversing mitotic CDK-dependent phosphorylation on various proteins among which are proteins critical for downregulating mitotic CDKs such as the CDK inhibitor, Sic1, the transcription factor Swi5 and the APC/C specificity factor Cdh1/Hct1. During anaphase, Cdc14 is released from its inhibitor Cfi1/Net1. The mitotic exit network (MEN) is required to maintain the dissociation between Cdc14 and Cfi1/Net1 (Shou et al., 1999; Visintin et al., 1999). It is likely that factors other than MEN, such as the FEAR network, control the association of Cdc14 and Cfi1/Net1 (Pereira, et al., 2002; Stegmeier, et al., 2002; Yoshida et al., 2002).
How does activation of the MEN control the association of Cdc14 with Cfi1/Net1? This is not yet understood, but both Cdc14 and Cfi1 are phospho-proteins (Shou et al., 1999), raising the possibility that protein phosphorylation is important in controlling their association. A logical candidate to regulate the association of Cdc14 and Cfi1/Net1 is Dbf2, a component of the MEN (see below), as genetic evidence suggests it is downstream of the MEN components Tem1 and Cdc15, but upstream of Cdc14 (Toyn and Johnston, 1994; Grandin et al., 1998; Lee et al., 2001a; Mah et al., 2001; Visintin and Amon, 2001). However, Dbf2 may not control the association between Cdc14 and Cfi1/Net1 directly, nor is it necessarily the sole regulatory protein.

**Components of the MEN**

The MEN is a signalling cascade that consists of Tem1 (a GTPase); Cdc15, Dbf2 and Cdc5 (protein kinases); Cdc14; Mob1 (a Dbf2-associated factor); Bub2–Bfa1/Byr4 (a two-component GTPase activating protein; GAP); Lte1 (a guanine nucleotide exchange factor; GEF); and a scaffold protein, Nud1 (Table 1, Figs. 2, 3).

Several lines of evidence suggest that the MEN functions in the order outlined in figure 2. Biochemical and genetic evidence suggest that the Ras-like GTPase Tem1 functions at or near the top of the MEN (Fig. 2; Shirayama et al., 1994; Jaspersen et al., 1998; Lee et al., 2001a; Visintin and Amon, 2001). Tem1’s activity seems to be regulated both by the GEF Lte1 and the GAP complex Bub2–Bfa1/Byr4 (Fig. 2; Hoyt et al., 1991; Shirayama et al., 1994; Alexandru et al., 1999; Lee et al., 1999a; Li, 1999; Krishnan et al., 2000). During or shortly after formation of the mitotic spindle, Tem1 localizes preferentially to the cytoplasmic face of the spindle pole
body (SPB; the yeast functional homologue of the mammalian centrosome), which is destined to migrate into the bud, the future daughter cell (Fig. 3; Bardin et al., 2000; Pereira et al., 2000). During late telophase, Tem1 asymmetry is lost and is then found on both SPBs (Pereira et al., 2000). Bub2–Bfa1 localizes to SPBs in a manner similar to Tem1 (Fraschini et al., 1999; Lee et al., 1999a; Li, 1999; Pereira et al., 2000). Furthermore, the early SPB localization of Tem1 seems to depend on both BUB2 and BFA1, whereas localization of Tem1 to SPB during telophase is BUB2 and BFA1 independent (Pereira et al., 2000). The SPB component Nud1 functions as a scaffold for the Bub2-Bfa1-Tem1 complex and other components of the MEN on SPBs (Figs 2, 3). Temperature sensitive alleles of NUD1 arrest in telophase with MEN components delocalized from the SPBs (Adams and Kilmartin, 2000; Bardin et al., 2000; Gruneberg et al., 2000; Pereira et al., 2000; Visintin and Amon, 2001). (The NUD1 gene is allelic to CDC18; A. Amon, unpublished observations). CDC18 is a previously uncloned gene found in a screen for genes important for cell cycle progression, with temperature-sensitive alleles causing a telophase arrest (Hartwell et al., 1974).

Activation of Tem1 is thought to lead to the propagation of a signal to the protein kinase Cdc15 (Fig. 2; Shirayama et al., 1996). Indeed, Cdc15 also localizes to the cytoplasmic face of SPBs, although the exact localization pattern differs depending on the epitope used to tag Cdc15 and the experimental design (Fig. 3; Cenamor et al., 1999; Gruneberg et al., 2000; Xu et al., 2000; Menssen et al., 2001; Visintin and Amon, 2001). Furthermore, Cdc15 co-immunoprecipitates with Tem1 (Bardin et al., 2000) and a 82-amino-acid domain adjacent to the amino-terminal kinase domain in Cdc15 is sufficient for interaction between Cdc15 and Tem1 in a yeast two-hybrid assay (Asakawa et al., 2001). However, whether the interaction between
Cdc15 and Tem1 is direct *in vivo* and whether it occurs only when Tem1 is bound to GTP is, at present, unclear. Cdc15 is then thought to activate the protein kinase Dbf2. Indeed, Cdc15 can activate Dbf2 *in vitro* by phosphorylating it on Ser374 and Thr544 (Mah et al., 2001). Activating phosphorylation by Cdc15, requires the presence of a Dbf2-associated factor Mob1 (Komarnitsky et al., 1998; Luca and Winey, 1998; Mah et al., 2001).

The polo kinase Cdc5 is also necessary for mitotic exit (Kitada et al., 1993), although its role in the pathway appears complex and suggests that it might act at more than one point to regulate exit from mitosis (Lee et al., 2001a; Visintin and Amon, 2001). Cdc5 somehow promotes Dbf2 kinase activity (Visintin and Amon, 2001) and is thought to regulate Bub2-Bfa1, though it is disputed as to whether Cdc5 activates (Lee et al., 2001a) or inhibits Bub2-Bfa1 (Wang et al., 2000).

**Signals controlling the MEN**

Recent work has illuminated some of the cellular events that control the MEN, and has given rise to a picture with more than one signal controlling this network. It appears that positive signals are required to activate the signalling cascade, and that inhibitory signals help to restrain activation of the MEN. Only when all inhibitory signals have been removed and positive signals are in place can full activation of the pathway occur, allowing exit from mitosis to proceed (Fig. 2).
Figure 2: MEN Components

Solid arrows indicate reasonably well established relationships, dashed arrows indicate more speculative ones, question marks denote more than one possible way a protein could act.

2. Nuclear position, cell polarity, mitotic spindle and DNA damage regulate the nucleotide-binding status of Tem1 (Bardin et al., 2000; Blocher et al., 2000; Daum et al., 2000; Krishnan et al., 2000; Pereira et al., 2000; Wang et al., 2000; Adames et al., 2001; Hofken et al., 2002; Jensen et al., 2002; Seshan et al., in press) either through Lte1 or Bub2-Bfa1 (Alexandru et al., 1999; Shirayama et al., 1994a) or both. Tem1 activates Cdc15 (Asakawa et al., 2001; Bardin et al., 2000) which activates Dbf2-Mob1 (Mah et al., 2001). Nud1 functions as a scaffold for Tem1 Cdc15, Dbf2, Bub2 and Bfa1 on the SPB (Bardin et al., 2000; Gruneberg et al., 2000; Pereira et al., 2000; Visintin and Amon, 2001). Cdc5 could promote Dbf2 kinase activity (Lee et al., 2001a; Visintin and Amon, 2001) and perhaps activates Bub2-Bfa1 (Lee et al., 2001a). Although, genetic evidence places Dbf2 downstream of all other MEN components, it is not known whether Dbf2-Mob1 directly controls the association of Cdc14 with Cfi1/Net1 or how it functions in cytokinesis.
Figure 3: Localization of MEN Components during the Cell Cycle

Figure 3: Localization of MEN components during the cell cycle.
Lte1 is present throughout the cell during G1, and localizes to the bud during S phase and mitosis. Nud1 functions as an anchor for MEN components (Adams and Kilmartin, 1999; Gruneberg et al., 2000). As the mitotic spindle forms, Tem1 and Bub2-Bfa1 localize to the SPB that migrates into the bud (Fraschini et al., 1999; Lee et al., 1999a; Li, 1999; Bardin et al., 2000; Pereira et al., 2000). During anaphase, Cdc15 and Dbf2-Mob1 are recruited to SPBs. The exact nature of Cdc15 and Dbf2 localization is somewhat unclear. Two reports find that Cdc15 is always associated with the mother SPB and only localizes to the daughter during anaphase and telophase (Cenamor et al., 1999; Menssen et al., 2001). Other groups report that Cdc15 localizes to both SPBs only during anaphase and telophase (Gruneberg et al., 2000; Xu et al., 2000; Visintin and Amon, 2000). Endogenous Dbf2 fused to three Myc epitopes or to a single molecule of green fluorescent protein (GFP) localizes to both SPBs during anaphase and telophase (Frenz et al., 2000; Visintin and Amon, 2001; Yoshida, 2001), whereas the expression of three copies of DBF2-GFP led to association of Dbf2 with SPBs throughout the cell cycle (Frenz et al., 2000). The Mob1 localization pattern is similar to that of Dbf2. It is found on both SPBs during anaphase and telophase (Yoshida, 2001). Cdc5 localizes to the nucleus during metaphase and anaphase and is also found on SPBs and the site of cytokinesis during anaphase (Cheng et al., 1998; Shirayama et al., 1998; Song and Lee, 2001). During late telophase, Cdc15, Dbf2 and Mob1 localize to the site of cytokinesis (Frenz et al., 2000; Xu et al., 2000; Yoshida, 2001).
**Nuclear position**

Tem1 resides preferentially on the SPB, which moves into the daughter cell where the exchange factor Lte1 is present (Bardin et al., 2000; Pereira et al., 2000 and reviewed in Hoyt, 2000). It has been proposed that translocation of the SPB (containing Tem1) into the bud (containing Lte1) is one signal that leads to the activation of Tem1 (Bardin et al., 2000; Pereira et al., 2000). In mutants where the mitotic spindle is misaligned and anaphase occurs in the mother cell (nuclear-position defect), both the release of Cdc14 from the nucleolus and exit from mitosis are delayed until the spindle has been repositioned to reach into the daughter cell (Yeh et al., 1995; Muhua et al., 1998; Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Pereira et al., 2000; Wang et al., 2000; Adames et al., 2001). Overexpression of Lte1, which forces high levels of this protein into the mother cell, can partially alleviate this delay (Bardin et al., 2000).

Bub2–Bfa1 is also required to prevent mitotic exit when the mitotic spindle is misaligned (Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Pereira et al., 2000; Wang et al., 2000; Adames et al., 2001). However, it is unclear whether and how the activity of the complex is regulated. Phosphorylation of Bfa1 changes during the cell cycle, suggesting that Bub2–Bfa1 activity is subject to cell cycle regulation (Wang et al., 2000; Lee et al., 2001a). One signal controlling the activity of Bub2-Bfa1 has been suggested to be the presence of cytoplasmic microtubules in the bud neck (Adames et al., 2001).

It is curious that the deletion of either GAP component (BUB2 or BFA1) or the GEF LTE1 does not result in an extreme cell-cycle phenotype at room temperature. Deletion of BUB2 or BFA1 has no detectable phenotype at any temperature. LTE1 is essential for mitotic exit at low
temperatures but less important at higher temperatures. It has been proposed that, at room temperature, the positioning of the nucleus is fast relative to cytokinesis, and so a phenotype is not detected in cells (Pereira et al., 2000). It has also been suggested that the high GDP release rate of Tem1 in vitro at room temperature accounts for the non-essential function of Lte1 (Geymonat, et al., 2002). It also is possible that yet undiscovered regulators have redundant roles in activating and inactivating Tem1. In fact, deletion of SPO12, a component of the FEAR network (cdc fourteen early anaphase release, see below) which can also promote Cdc14 release, is synthetically lethal with lte1Δ, suggesting that these genes have partially redundant functions (Stegmeier, et al., 2002). Alternatively, regulation of MEN components other than Tem1 may account for the correct timing of Cdc14 release from Cfi1/Net1 in the absence of the GAP and GEF activities.

*Microtubule integrity and DNA damage*

Surveillance mechanisms termed the spindle-assembly checkpoint and the DNA-damage checkpoint monitor for problems such as defects in the attachment of microtubules to the kinetochore or DNA damage, respectively (for reviews see refs. Gardner and Burke, 2000; Zhou and Elledge, 2000). Activation of either checkpoint prevents the degradation of Pds1, leading to metaphase arrest (Box 2). Interestingly, DNA damage can also stabilize Pds1 in cells arrested in late anaphase/telophase (Tinker-Kulberg and Morgan, 1999; Wang et al., 2000). Stabilized Pds1 inhibits both sister-chromatid separation and activation of Cdc14 under these conditions. The mechanism whereby Pds1 controls Cdc14 activation is not known. This might be a consequence of inhibition of the FEAR component Esp1 by Pds1, or Pds1 could play an additional inhibitory role on Cdc14.
The two-component GAP Bub2–Bfa1 is also needed to inhibit the MEN during metaphase arrest induced by a defective mitotic spindle (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999; Wang et al., 2000). Furthermore, Bfa1 is hyperphosphorylated when the mitotic spindle checkpoint is activated, raising the possibility that the activity of Bub2–Bfa1 is under the control of the checkpoint (Lee et al., 2001a). Cdc5 and Cdc14 have been proposed to regulate the phosphorylation status and activity of Bfa1 (Hu et al., 2001; Pereira et al., 2002). Two reports have addressed the role of Bub2-Bfa1 in inhibiting MEN in response to DNA damage. Whereas Wang et al. (2000) conclude that Bub2-Bfa1 is required for metaphase arrest in response to DNA damage, Krishnan et al. (2000) conclude that they are not. The reasons for this discrepancy are at present unclear, but may reflect differences in strain backgrounds and/or experimental procedures employed.

**Cell polarity**

Regulators of cell polarity are important for localization to and sequestration of the GEF Lte1 in the daughter bud (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et al., in press). Lte1 becomes phosphorylated coincident with the formation of the daughter bud suggesting a possible link between phosphorylation and localization (Bardin et al., 2000). Recent work has demonstrated that localization of Lte1 is under control of Cdc42 signaling via the PAK kinase Cla4. Cla4 likely directly phosphorylates Lte1 and promotes its localization to the bud (Seshan et al., in press). Transport to the bud is partially dependent on the actin cytoskeleton, however maintenance in the bud is independent of actin, but dependent on the cortical protein Kell (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et al., in press). The septins, which form a ring around the bud neck, are also required to hold Lte1 in the bud, presumably by
forming a diffusion barrier for membrane lipid associated proteins (Hofken and Schiebel, 2002; Seshan et al., in press). As the cell cycle progresses through START, Cdc28 activates Cdc42 which allows the localization of proteins involved in bud formation. Activation of Cla4 by Cdc42 stimulates phosphorylation and recruitment of Lte1 to the bud where it is spatially separated from the GTPase, Tem1, preventing early activation of MEN. In addition to promoting mitotic exit through Cla4 and Lte1, Cdc42 has a second mitotic exit function. Activation of Ste20 by Cdc42 also positively regulates mitotic exit, although the function of Ste20 in exit from mitosis is unknown (Hofken and Schiebel, 2002).

**Other regulators of mitotic exit: The FEAR Network**

The securin Pds1 is a negative regulator of the seprase Esp1, which is responsible for separating sister chromatids at the metaphase–anaphase transition (Box 2; reviewed in ref. Nasmyth et al., 2000). Interestingly, not only sister-chromatid separation but also exit from mitosis is delayed in esp1 mutants or cells overexpressing a stabilized version of Pds1. This finding suggests that ESP1 is somehow required for the efficient activation of Cdc14 and/or that PDS1 prevents the activation of Cdc14 (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). The inhibitory effects of a stabilized version of Pds1 on mitotic exit and the requirement of ESP1 for mitotic exit can be alleviated by deleting BUB2 suggesting that ESP1 functions upstream or parallel to BUB2 (Shirayama et al., 1999; Wang et al., 2000; Lee et al., 2001a).

The analysis of Cdc14 localization in cells with mutations in MEN components revealed that Cdc14 undergoes a transient release after metaphase that is independent of MEN. In MEN
mutants Cdc14 is, re-sequestered after the MEN independent early anaphase release and cells arrest in telophase. Therefore, MEN is required for sustained release of Cdc14 leading to mitotic exit. The finding that Cdc14 release can occur independent of MEN lead to the identification of a second network that is important for Cdc14 release. The early anaphase MEN independent release was found to depend on ESP1, SLK19, SPO12, and CDC5, named the FEAR network (cdc fourteen early anaphase release; Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). Esp1 and Cdc5 are known to become active prior to sister chromatid separation in metaphase, and therefore FEAR may be another means to delay inactivation of mitotic CDKs before chromosome segregation has occurred. Slk19 is a substrate of Esp1 and is important for stability of the mitotic spindle. Spo12’s function is unknown. Whether these genes act together and how they promote Cdc14 release is not understood. It is also unclear if Cdc14 functions to inactivate mitotic CDKs during early anaphase, or whether MEN is required for this function.

Inactivation of mitotic CDKs in fission yeast

Like in S. cerevisiae, inactivation of mitotic CDKs is required for exit from mitosis in the fission yeast S. pombe (Yamano et al., 1996). However, degradation of the mitotic cyclin cdc13 is likely to be brought about by APC/C and the Cdc20 homologue, slp1. Whereas, the Cdh1/Hct1 homologue ste9 and the CDK inhibitor rum1 (a functional homologue of Sic1) are not important for mitotic CDK inactivation but are required for keeping mitotic CDK levels low during G1 (Moreno et al., 1994; Kitamura et al., 1998; Kominami et al., 1998).
Clp1/flp1 regulates mitotic CDK activity

Recently, a homologue of *S. cerevisiae* Cdc14 — clp1/flp1 — has been identified in *S. pombe* (Cueille et al., 2001; Trautmann et al., 2001). The *clp1+/flp1+* gene is not essential, but deletion of this gene does cause cells to enter mitosis prematurely. Conversely, overexpression of *clp1+/flp1+* causes cells to arrest in G2, suggesting that this gene inhibits mitotic CDK activity (Cueille et al., 2001; Trautmann et al., 2001). However, unlike its *S. cerevisiae* homologue, clp1/flp1 seems to regulate the inactivation of mitotic CDKs by controlling the phosphorylation status of tyrosine 15 of cdc2, the sole CDK in *S. pombe*.

In *S. pombe* and higher eukaryotes, mitotic CDKs are kept inactive during G2 through phosphorylation of a highly conserved tyrosine residue (Y15) within the CDK subunit (reviewed in Morgan, 1997). The protein kinase wee1 phosphorylates Y15, whereas the protein phosphatase cdc25 dephosphorylates this residue. The clp1/flp1 protein is thought to promote tyrosine phosphorylation of cdc2 by inhibiting cdc25, by activating wee1, or by promoting both events. The molecular mechanisms of clp1/flp1 action are currently unknown. Thus, it appears that clp1/flp1 functions primarily during G2 to regulate mitotic CDK activity, rather than Cdc14, which functions to regulate mitotic CDK activity during late stages of mitosis.

*clp1+/flp1+* in some way is also important for cytokinesis. A small percentage of cells deleted for *clp1+/flp1+* display defects in septum formation (Cueille et al., 2001; Trautmann et al., 2001). The role of *clp1+/flp1+* in cytokinesis remains to be determined.
Figure 4: clp1/flp1 Controls Mitotic CDK Activity

Solid arrows indicate reasonably well established relationships, dashed arrows indicate more speculative ones, question marks denote more than one possible way a protein could act.

4. clp1/flp1 regulates mitotic CDKs either by inhibiting cdc25, activating wee1, or promoting both events (Cueille et al., 2001; Trautmann et al., 2001). The similarity in localization of Cdc14 and clp1/flp1 raises the possibility that clp1/flp1 is regulated by a catalytic inhibitor analogous to Cfi1/Net1, which resides in the nucleolus. An active septation initiation network (SIN) is needed to maintain clp1/flp1 in its released state. Whether it promotes export out of the nucleolus or inhibits the return of clp1/flp1 thereto is unclear. As the release of clp1/flp1 from the nucleolus is independent of the SIN, other cellular factors must exist that control the release of clp1/flp1 from the nucleolus (Cueille et al., 2001; Trautmann et al., 2001).
Figure 5: SIN Components

Solid arrows indicate reasonably well established relationships, dashed arrows indicate more speculative ones, question marks denote more than one possible way a protein could act. See Table 1 for homologous *S. cerevisiae* genes.

5. spg1 is inhibited by the two-component GAP, cdc16–byr4 (Furge et al., 1998; Jwa and Song, 1998) but no GEF has been identified thus far. plo1 regulates spg1, possibly via cdc16–byr4. spg1–GTP activates cdc7 which activates sid1–cdc14, which in turn activates sid2–mob1(Schmidt et al., 1997; Sohrmann et al., 1998; Sparks et al., 1999; Guertin et al., 2000; Hou et al., 2000; Salimova et al., 2000;). sid4 and cdc11 function as anchors for SIN components on the SPB (Chang and Gould, 2000; Krapp et al., 2001). After sid2–mob1 kinase is activated at SPBs, it localize to the medial ring to promote septum formation (Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). Mitotic CDKs inhibit the SIN through sid1–ccc14 (Chang and Gould, 2000). How SIN controls cytokinesis and the localization of clp1/flp1 is not known.
Figure 6: Localization of SIN components during the cell cycle.

6. sid4 and cdc11 function as anchors of SIN components on the SPB (Chang and Gould, 2000; Krapp et al., 2001). pl01, a regulator of spg1 function localizes to both SPBs when cells enter mitosis, and subsequently, as the mitotic spindle forms thereon, pl01 is also seen at the medial ring during metaphase and anaphase (Ohkura et al., 1995; Mulvihill et al., 1999; Tanaka et al., 2001). spg1 localizes to SPBs throughout the cell cycle but is not active until mitosis as it is inhibited by the two-component GTPase-activating protein (GAP), cdc16-byr4 during G1, S phase and G2. When the metaphase spindle has formed, cdc16-byr4 dissociates from both SPBs, leading to recruitment of the cdc7 to both SPBs. At the onset of anaphase, the GAP cdc16-byr4 localizes to one SPB, leading to the loss of cdc7 at this SPB (Cerutti and Simanis, 1999; Furge et al., 1998; Jwa and Song, 1998; Sohrmann et al., 1998). During anaphase, sid1-cdc14 localizes to the cdc7-containing SPB (Guertin et al., 2000). sid2-mob1 localizes to SPBs throughout the cell cycle but, after the sid2-mob1 kinase is activated at SPBs in a cdc7- and sid1-cdc14-dependent manner also localizes to the medial ring (Balasubramanian et al., 1998; Sparks et al., 1999; Hou et al., 2000; Salimova et al., 2000).
Control of clp1/flp1 by the SIN

The subcellular localization of clp1/flp1 changes during the cell cycle. Whereas clp1/flp1 is present in the nucleolus and on SPBs during G1 and S phase, it is released from the nucleolus during early mitosis. It then localizes to the mitotic spindle and medial ring (Cueille et al., 2001; Trautmann et al., 2001). An active septation initiation network (SIN) is needed to maintain clp1/flp1 in its released state (Fig. 4, Table 1). How clp1/flp1 is held in the nucleolus and how the SIN regulates the subcellular localization of clp1/flp1 is not understood.

The septum initiation network

Control of formation of the septum is governed by the SIN — a signalling cascade of proteins with homology to those of the MEN (Table 1). The SIN consists of spg1 (a GTPase); cdc7, sid1, sid2 and plo1 (protein kinases); cdc14 (a sid1-associated factor); mob1 (a sid2-associated factor); and cdc16–byr4 (GAP components). Inactivation of the SIN results in the formation of elongated, multinucleated cells that cannot form a division septum. When the SIN is hyperactivated, cells form multiple septa (reviewed in Le Goff et al., 1999; McCollum and Gould, 2001).

The order in which the components of the SIN function is well understood and remarkably similar to that of the MEN (Fig. 5). The GTPase spg1 is thought to function near or at the top of the SIN (Fig. 5; Schmidt et al., 1997). The spg1 protein localizes to SPBs throughout the cell cycle, and this localization is likely to be important for function (Fig. 6; Sohrmann et al., 1998). spg1 is inhibited by the two-component GAP, cdc16–byr4, until the initiation of mitosis (Minet et al., 1979; Fankhauser et al., 1993; Song et al., 1996; Furge et al.,
When the metaphase spindle has formed, cdc16–byr4 dissociates from both SPBs, and this is believed to lead to the activation of spg1 and recruitment of cdc7 to both SPBs. Upon the onset of anaphase, cdc16–byr4 localizes to only one SPB (Fig. 6). As cdc16–byr4 move on and off SPBs, cdc7 is always associated with the SPB from which the cdc16–byr4 complex is absent, suggesting that cdc16–byr4 prevents the recruitment of cdc7 (Cerutti and Simanis, 1999; Li et al., 2000a). Localization of spg1, cdc7 and byr4 to the SPB depends on two components of the SPB, sid4 and cdc11, which are thought to function as anchors for these proteins (Chang and Gould, 2000; Krapp et al., 2001). cdc11 binds to and requires sid4 to localize to the SPB, and both proteins are needed to anchor SIN components (Krapp et al., 2001). During anaphase, the protein kinase sid1, complexed with cdc14 (no relation to its S. cerevisiae namesake), localizes to the cdc7-containing SPB — but only once mitotic kinase activity has been lowered below a critical threshold (Guertin et al., 2000).

The sid2 protein kinase and its associated factor mob1 localize to SPBs throughout the cell cycle (Balasubramanian et al., 1998; Sparks et al., 1999; Hou et al., 2000; Salimova et al., 2000). However, it is thought that only after the sid2 kinase is activated at SPBs (in a cdc7- and sid1-cdc14-dependent manner) can sid2 and mob1 localize to the medial ring (Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). The mob1 and sid2 proteins are the only pathway components that are seen at the site of the division septum, leading to the hypothesis that these proteins carry the ‘make a septum’ message to the site of cytokinesis (Fig. 6).
The polo kinase encoded by the \textit{plo1}+ gene is essential for septation (Ohkura et al., 1995). Furthermore, overexpression of \textit{plo1}+ can drive septation in interphase, allowing transient recruitment of \textit{cdc7} to the SPB. This has lead to the idea that \textit{plo1} acts in a positive regulatory manner upstream of the SIN pathway (Ohkura et al., 1995; Mulvihill et al., 1999; Tanaka et al., 2001) and that, perhaps, \textit{plo1}+ is involved in removing \textit{cdc16–byr4} from SPBs.

**Signals controlling the SIN**

To date, there are two signals known to control the SIN: mitotic CDK activity and the cytokinesis checkpoint.

**Mitotic CDK activity**

Inactivation of mitotic CDKs is required for formation of the division septum (Guertin et al., 2000). The sid1–\textit{cdc14} complex seems to be a sensor for the state of mitotic CDK activity within cells, and only when the CDK activity has dropped below a certain low threshold can sid1–\textit{cdc14} localize to the SPB containing spg1 (Guertin et al., 2000). Through this dependency, the cell ensures that septum formation does not occur during mitosis, when mitotic CDK activity is high. Consistent with this idea, hyperactivation of the SIN leads to ectopic formation of a septum, but only during those stages of the cell cycle when mitotic CDK activity is low — during interphase and late stages of mitosis (Ohkura et al., 1995; Schmidt et al., 1997).

**The cytokinesis checkpoint**

The cytokinesis checkpoint in \textit{S. pombe} was uncovered through the identification of mutations in the \textit{cps1+} \textit{drc1+} gene, a subunit of the 1,3-\textbeta-glucan synthase (Liu et al., 1999; Le
Goff et al., 1999; Liu et al., 2000). *cpsI/drcI* mutants fail to assemble a septum, but nevertheless enter a new cell cycle, go through one round of DNA replication, and then arrest in G2 of the following cell cycle. The checkpoint arrest depends on polymerized F-actin, wee1, clp1/flp1, as well as the SIN (Liu et al., 1999; Le Goff et al., 1999; Liu et al., 2000; Cueille et al., 2001; Trautmann et al., 2001). It is unclear how the SIN senses defects in medial-ring formation and how the SIN prevents cell-cycle progression under conditions where a septum fails to form. However, it is tempting to speculate that, in analogy to the MEN functioning through Cdc14 to downregulate mitotic CDKs, in this checkpoint the SIN functions through clp1/flp1 to promote Y15 phosphorylation of cdc2, leading to inhibition of mitotic CDK activity and a G2 arrest.

**Higher eukaryotes**

Finding true homologues for components of Ras-like signalling cascades is challenging as the large amount of conservation among family members can make it difficult to detect conservation of homologues between species. However, the GTPases Tem1 and spg1 are most similar to the Rab family of GTPases. To our knowledge, no known Rabs in higher eukaryotes have been found that have a defect in exit from mitosis or cytokinesis, or localize to the centrosome. Likewise, true Cdc15/cdc7 or sid1 homologues have yet to be found.

One intriguing possibility for a homologue of the two-component GAP Bub2/cdc16–Bfa1/byr4 is the human protein GAPCenA (for GAP and centrosome associated; Cuif et al., 1999). GAPCenA was identified through a yeast two-hybrid screen as interacting with the GTPase Rab6, which is involved in transport between the Golgi body and the endoplasmic reticulum (ER) (Martinez et al., 1997). GAPCenA has GAP activity towards Rab6.
(Cuif et al., 1999), and it shares homology with Bub2 and cdc16. Interestingly, a pool of GAPCenA localizes to centrosomes. Whether GAPCenA regulates an as-yet-unidentified Tem1/spg1 homologue on the centrosome, or whether Rab6 is the Tem1/spg1 homologue, is not known.

The *Drosophila melanogaster* gene WARTS was identified as a tumour suppressor (Justice et al., 1995; Xu et al., 1995) and has similarity to Dbf2/sid2. Interestingly, human WARTS (LATS1) has been shown to interact directly with CDC2 in mitosis (Tao et al., 1999). h-WARTS/LATS1 localizes to the centrosome in interphase, translocates to the mitotic spindle in metaphase and anaphase, and then localizes to the midbody in telophase (Nishiyama et al., 1999). The Dbf2/sid2-associated factor Mob1/mob1 also has numerous homologues in higher eukaryotes (Luca and Winey, 1998; Moreno et al., 2001).

Cdc5/plo1 are members of the polo kinase family, which includes *Drosophila* Polo, *Xenopus laevis* Plx1 and mammalian Plk, Snk, Sak and Fnk/Prk (reviewed in (Glover et al., 1998)). Work in *Xenopus* has demonstrated that Plx1 activity is needed for exit from mitosis in egg extracts (Descombes and Nigg, 1998). Various polo kinases have been found to localize to the centrosome and cleavage furrow, and recently it was demonstrated that murine Sak (a polo kinase family member) also localizes to the nucleolus (Lee et al., 1998; Lee et al., 1999b; Moutinho-Santos et al., 1999; Hudson et al., 2001). Together these data suggest that polo kinases are likely to be involved in controlling exit from mitosis and cytokinesis.
Human homologs of Cdc14/clp1/flp1 — Cdc14A and Cdc14 B have been identified (Li et al., 1997; Bembenek and Yu, 2001; Kaiser et al., 2002). Cdc14A localizes to the centrosomes, whereas Cdc14B is nucleolar. Although not extensively studied, it seems that hCdc14 can cause CDK inactivation as well as dephosphorylation and activation of human Cdh1-APC (Li et al., 2000b; Listovsky et al., 2000; Bembenek and Yu, 2001). The conditional overproduction of Cdc14A results in premature centrosome splitting and RNAi mediated down-regulation of Cdc14A causes mitotic defects including impaired centrosome splitting and inability to undergo cytokinesis (Kaiser et al., 2002; Mailand et al., 2002). Overexpression of hCdc14A in murine NIH3T3 cells alleviates a telophase block imposed by expression of a non-degradable version of cyclin B and allows for mouse Cdh1 to associate with the APC/Cyclosome (Listovsky et al., 2000). It is not yet known whether hCdc14 can also regulate mitotic CDKs through tyrosine phosphorylation. Analysis of a Cdc14 homolog in C. elegans has shown that CeCdc14 localizes to the central spindle during anaphase and the midbody during telophase. CeCdc14 is required for cytokinesis during the early embryonic cell cycles, although is dispensable for cell cycle progression in adult animals (Gruneberg et al., 2002).

p53 may also be a hCdc14 substrate. Both human Cdc14A and Cdc14B have been shown to associate with the tumor suppressor p53 in a yeast two-hybrid assay and by co-immunoprecipitation and can dephosphorylate p53 on Ser 315. Li and coworkers (Li et al., 2000b) proposed that this dephosphorylation might activate p53, possibly by directing p53 into the nucleus. Clearly, it will be interesting to evaluate the relevance of hCdc14 in mitotic CDK inactivation and p53 regulation in vivo.
A role for the centrosome?

Recent work has implicated the centrosome in controlling of cytokinesis in mammalian cells (Khodjakov and Rieder, 2001; Piel et al., 2001). Each centrosome is composed of an old centriole (from the previous cell cycle) and new centriole (duplicated during the current cell cycle). Analysis of green fluorescent protein (GFP)-labelled centrioles has demonstrated that during cytokinesis there is a rapid movement of the old centriole into the intercellular bridge, the thin region of cytoplasm separating the two daughter cells. Ablation of the centrosome does not affect nuclear division, but leads to defects in cytokinesis (Khodjakov and Rieder, 2001; Piel et al., 2001). Is the centrosome, like the SPB in *S. cerevisiae* and *S. pombe*, anchoring signalling networks needed for completion of the cell cycle?

Comparing the SIN and the MEN

At a first glance, one might think that the functions of the SIN and the MEN are quite different — the SIN regulates formation of the division septum after mitotic CDK inactivation, whereas the MEN controls mitotic CDK inactivation. Recent evidence, however, indicates that the MEN also regulates cytokinesis and there are some hints that the SIN, at least under some circumstances, controls mitotic CDK activity through $clp1+/flp1+$. 

The MEN and cytokinesis

When the mitotic-exit function of Tem1 is bypassed by a *net1-1* allele, *tem1* mutants show defects in cytokinesis (Shou et al., 1999). Furthermore, a specific *CDC15* allele, *cdc15-
lyt1, is defective in cytokinesis (Jimenez et al., 1998). Finally, GFP-tagged versions of Cdc15, Dbf2, Mob1 and Cdc5 are detected at the bud neck in late mitosis (Frenz et al., 2000; Song et al., 2000; Song and Lee, 2001; Xu et al., 2000; Yoshida, 2001). Interestingly, the accumulation of Dbf2 and Mob1 at the bud neck depends on CDC14 (Frenz et al., 2000; Yoshida, 2001). We speculate that in budding yeast, the role of the MEN in mitotic CDK inactivation and cytokinesis represents sequential steps. The MEN first promotes the dissociation of Cdc14 from Cfi1/Net1, and thus loss of mitotic CDK activity. The absence of mitotic CDKs and the presence of active Cdc14, then causes dephosphorylation of CDK consensus sites leading to dephosphorylation of Cdc15, and perhaps other components of the MEN (Jaspersen and Morgan, 2000). This allows components of the MEN to localize to the bud neck, where they regulate cytokinesis. It is possible that the MEN controls the activity of IQGAP (Iqg1/Cyk1 in budding yeast), a key regulator of actomyosin ring assembly and contraction as Tem1 has been shown to interact with this protein in vitro (Shannon and Li, 1999). Furthermore, TEM1 is required for proper actomyosin and septin dynamics (Frenz et al., 2000; Lee et al., 2001b; Lippincott et al., 2001) suggesting that Tem1 could be a key regulator of actomyosin ring assembly and contraction. Clearly, determining the mechanism whereby the MEN regulate cytokinesis will likely provide key insights into how cytokinesis is regulated.

The SIN and mitotic CDK inactivation

The SIN is required to maintain a low mitotic CDK state when the cytokinesis checkpoint is activated, indicating that the SIN can inhibit mitotic CDKs (Cueille et al., 2001; Trautmann et al., 2001). The maintenance of a low mitotic CDK state in this checkpoint arrest also depends on clp1/flp1 and tyrosine phosphorylation of cdc2 (Cueille et al., 2001; Trautmann
et al., 2001). Perhaps, under conditions when the cytokinesis checkpoint is activated, like the MEN in *S. cerevisiae*, the SIN can induce activation of clp1/flp1 by facilitating nucleolar release, which then promotes tyrosine phosphorylation of cdc2, bringing about the inactivation of mitotic CDKs.

An inappropriately active SIN also triggers inactivation of mitotic CDKs in cells that are arrested in mitosis due to activation of the mitotic spindle checkpoint (reviewed in Amon, 1999). The *cdc16-116* mutation allows cells to bypass the arrest as the cells can inactivate mitotic CDKs and undergo cytokinesis (Fankhauser et al., 1993; Guertin et al., 2000; Sohrmann et al., 1998). Perhaps the lack of functional *cdc16*-byr4 GAP initiates a positive-feedback loop in which a hyperactivated SIN promotes clp1/flp1 activity, leading to inactivation of mitotic CDKs. Loss of mitotic CDK activity in turn causes further activation of the SIN. This hypothesis predicts that the bypass of the mitotic spindle checkpoint arrest seen in *cdc16-116* mutants depends on *clp1+/flp1+*.

**Comparing the Control of Cdc14 or clp1/flp1 localization**

In fission yeast, clp1/flp1 becomes released from the nucleolus during early mitosis in a SIN-independent manner (Cuelle et al., 2001; Trautmann et al., 2001). However, the SIN is needed to keep clp1/flp1 released — at least when the cytokinesis checkpoint is activated (Trautmann et al., 2001). This is in contrast to Cdc14 in *S. cerevisiae*, where temperature-sensitive alleles of the MEN have Cdc14 tightly sequestered in the nucleolus which led to the proposal that the MEN is required for the release of Cdc14 from the nucleolus (Shou et al., 1999; Visintin et al., 1999). Recent data, however, has shown that Cdc14 is transiently released from
the nucleolus during early mitosis in a MEN-independent manner and that the MEN is required to maintain Cdc14 in a released state (Pereira, et al., 2002; Stegmeier et al., 2002; Yoshida et al, 2002). This observation raises the interesting possibility that the control of Cdc14 and clp1/flp1 release is similar — that is, the MEN, like SIN activity, is needed to hold Cdc14 out of the nucleolus.

**Cyclin degradation versus tyrosine phosphorylation**

Both Cdc14 and clp1/flp1 promote mitotic CDK inactivation, but by different means. Whereas *S. pombe* clp1/flp1 inhibits mitotic CDKs by promoting tyrosine phosphorylation on cdc2, budding yeast Cdc14 inhibits mitotic CDKs by promoting the degradation of mitotic cyclins. These findings add to the body of evidence that the critical regulation of mitotic CDK activity in *S. pombe* is through the phosphorylation status of cdc2, whereas control of mitotic CDK activity in *S. cerevisiae* is in large part mediated by cyclin degradation (reviewed in Morgan, 1997; Tyers and Jorgensen, 2000). Budding yeast Cdc14 seems to have a high affinity for CDK phosphorylation consensus sites (Visintin et al., 1998; Jaspersen et al., 1999; Jaspersen and Morgan, 2000). Whether clp1p/flp1p uses a similar mechanism to promote cdc2p inactivation remains to be determined.
Conclusions and perspectives

A great amount of progress has been made by the work of numerous groups in the last five years toward understanding how mitotic exit is accomplished. The work in this thesis has contributed to an understanding of the MEN in several ways. Analysis of localization and regulation of the GTPase Tem1, its GEF, Lte1, and its GAP, Bub2-Bfa1, lead to a model whereby nuclear movement into the daughter bud during anaphase promotes activation of the MEN. The work presented here also investigates functional domains of the MEN protein kinase, Cdc15, that promote SPB localization, self-association, and auto-inhibition. This work indicates that several layers of regulation occur on these two-components and suggests that, perhaps, other levels of control are awaiting discovery.


Kominami, K., Seth-Smith, H. and Toda, T. (1998) Apc10 and Ste9/Srw1, two regulators of the APC-cyclosome, as well as the CDK inhibitor Rum1 are required for G1 cell-cycle arrest in fission yeast. *Embo J*, 17, 5388-5399.


Chapter II:

A mechanism for coupling exit from mitosis
to partitioning of the nucleus

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Dr. Rosella Visintin contributed to work in this chapter.
Summary

Exit from mitosis must not occur prior to partitioning of chromosomes between daughter cells. We find that the GTP-binding protein Tem1, a regulator of mitotic exit is present on the spindle pole body that migrates into the bud during S phase and mitosis. Tem1’s exchange factor, Lte1, localizes to the bud. Thus, Tem1 and Lte1 are present in the same cellular compartment (the bud) only after the nucleus enters the bud during nuclear division. We also find that the presence of Tem1 and Lte1 in the bud is required for mitotic exit. Our results suggest that the spatial segregation of Tem1 and Lte1 ensures that exit from mitosis only occurs after the genetic material is partitioned between mother and daughter cell.
Introduction

In budding yeast, entry into mitosis and mitotic spindle formation rely on the mitotic cyclin-dependent kinases (Cdk1 complexes containing Clb1 to Clb5). After formation of the mitotic spindle in the mother cell the nucleus migrates to the neck between the mother and the daughter cell where it awaits the onset of anaphase. Entry into anaphase is controlled by the ubiquitin-protein ligase APC/C (Anaphase Promoting Complex/Cyclosome; reviewed in King et al., 1996). The APC/C initiates anaphase by degrading the anaphase inhibitor Pds1 (Cohen-Fix et al., 1996). Proteolysis of Pds1 liberates Esp1, allowing it to initiate cleavage of protein complexes (cohesins) that hold sister chromatids together (Guacci et al., 1997; Michaelis et al., 1997; Ciosk et al., 1998; Uhlmann et al., 1999). When the bonds between sister chromatids have been dissolved, nuclear division commences with the nucleus extending into the daughter cell. After nuclear division is completed, mitotic kinases are inactivated which triggers exit from mitosis and cytokinesis (reviewed in Morgan, 1999).

The highly conserved phosphatase Cdc14 plays a pivotal role in promoting inactivation of mitotic kinases and thus exit from mitosis (Visintin et al., 1998; Jaspersen et al, 1999). Cdc14 dephosphorylates the APC/C -specificity factor Cdh1/Hct1 thereby stimulating APC-dependent degradation of mitotic cyclins. By dephosphorylating the CDK inhibitor Sic1 and its transcription factor Swi5, Cdc14 induces stabilization of Sic1 and SIC1 transcription, respectively.

Cdc14 activity is regulated by Cfi1/Net1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Cfi1/Net1 sequesters and inhibits Cdc14 in the nucleolus during G1, S phase and early mitosis. During nuclear division, Cdc14 is released from Cfi1/Net1 in the nucleolus, allowing it to reach its targets in the nucleus (Sic1 and Cdh1) and cytoplasm (Swi5). The release
of Cdc14 from the nucleolus is regulated by the mitotic exit pathway. This pathway includes the Ras-like GTP binding protein Tem1, the putative exchange factor Lte1, the two component GTPase-activating enzyme (GAP) Bub2-Bfa1/Byr4, the protein kinases Cdc5, Cdc15, Dbf2 and Dbf20 and the Dbf2-associated protein Mob1 (reviewed in Morgan, 1999). Genetic interactions among these genes indicate that they function in a common pathway and that TEM1 and LTE1 act together at or near the top of this signaling cascade (Shirayama et al., 1994a; Jaspersen et al., 1998; Morgan, 1999). Pds1 and Esp1 are also required for release of Cdc14 from the nucleolus and inactivation of mitotic kinases (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999; Shirayama et al., 1999). How these proteins control the exit pathway is not known. Release of Cdc14 from the nucleolus is not sufficient for it to trigger inactivation of mitotic kinases. Clb5-Cdc28 kinase, a potent antagonist of Cdc14, also needs to be inactivated for Cdc14 to efficiently dephosphorylate its targets. This is achieved by the APC, which degrades Clb5 during anaphase (Shirayama et al., 1999).

The components of the mitotic exit pathway are highly conserved among eukaryotes but it is not known how they function to promote Cdc14 release. We report here that Tem1 is synthesized and localized to the spindle pole body that migrates into the bud concomitant with mitotic spindle formation. Tem1’s exchange factor is present throughout the cell during G1, but localizes to the bud as soon as it forms. Owing to the changes of Tem1 protein levels during the cell cycle and the localization patterns of Tem1 and Lte1, the two proteins are only present in the same cellular compartment after the nucleus enters the bud during nuclear division. Indeed, we find that Tem1 and Lte1 need to be in the same compartment to initiate mitotic exit. We also show that Tem1’s GAP Bub2 contributes to restraining mitotic exit when the nucleus is not partitioned between mother and daughter cell. Our results suggest that one function of the mitotic
exit pathway is to ensure that exit from mitosis occurs only after the genetic material has been partitioned between mother and daughter cell, thereby guaranteeing maintenance of ploidy.

Results

Tem1 and Lte1 protein levels during the cell cycle.

Epistasis analyses indicate that TEM1 and LTE1 function at or near the top of the mitotic exit pathway. Overexpression of CDC15 and CDC14 rescues the mitotic exit defect of temperature sensitive (ts) tem1-3 mutants. In contrast, overexpression of TEM1 does not rescue the ts lethality of cdc14-1 and cdc15-2 mutants (Jaspersen et al., 1998) but suppresses the cold-sensitive lethality of cells deleted for LTE1 (Shirayama et al., 1994b). We, thus, hypothesized that analysis of Tem1 and Lte1 may provide insight into how the mitotic exit pathway is controlled. The chromosomal copy of TEM1 was tagged with three Myc epitopes (TEM1-MYC). Tem1-Myc levels were low during G1 and early S phase. Tem1-Myc accumulated during mitosis (15 minutes later than the mitotic cyclin Clb2) and reached peak levels during telophase (Figure 1A, B).

We also tagged the chromosomal copy of LTE1 with a HA epitope (LTE1-HA). Lte1-Ha levels fluctuated weakly, if at all, during the cell cycle (Figure 1C, D). However, we did note that Lte1 extracted from G1 unbudded cells migrated faster on SDS-PAGE than Lte1 obtained from budded cells (compare Figure 1C and D). Addition of calf intestinal phosphatase (CIP) to Lte1-Ha immunoprecipitates led to the presence of only a fast migrating form of Lte1 indicating that phosphorylation was responsible for the change in Lte1’s electrophoretic mobility (Figure 1E).
Figure 1: Tem1 and Lte1 Protein Levels during the Cell Cycle

Figure 1: Tem1 and Lte1 protein levels during the cell cycle. Cells either carrying a TEM1-MYC tag (A1828; A, B) or an LTE1-HA fusion (A1949; C, D) were arrested in G1 with α-factor (5 μg/ml) followed by release into medium lacking pheromone. The amount of Tem1-myc (A) or Lte1-Ha (C) protein and the percentage of budded cells and cells containing telophase spindles (B, D) were analyzed. Clb2 was analyzed to determine when cells were in mitosis. Kar2 was used as internal loading control.

(E) Lte1-Ha was immunoprecipitated using an anti-Ha antibody and either immediately processed for SDS PAGE (IP) or incubated with CIP buffer alone (IP+buffer) or with 80 units of calf intestinal alkaline phosphatase (CIP) (IP+CIP). An immunoprecipitation from cells without an LTE1-HA fusion is shown in lane labeled “no tag”.

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**Tem1 is present on one spindle pole body during mitosis.**

Next, we analyzed the subcellular localization of Tem1 in exponentially growing cells by indirect in situ immunofluorescence using an anti-Myc antibody. Consistent with our Western blot analysis little or no Tem1 staining was detectable in G1 cells or in cells that had not yet formed a mitotic spindle (Figure 2B). In 75 percent of cells containing a mitotic spindle Tem1 was present as a single dot at one end of spindles (Figure 2B). In the remaining 25 percent of mitotic cells, a strong Tem1 signal was observed at one end of the mitotic spindle and a faint one at the other end (data not shown).

The subcellular localization of Tem1 was similar to that of components of spindle pole bodies (SPBs). Indeed Tem1 colocalized, although not in its entirety, with the γ-tubulin Tub4, which localizes to the inner and outer plaques of the SPB (Figure 2C; Rout and Kilmartin, 1990; Sobel and Snyder, 1995; Knop et al., 1997). To determine more precisely where on the SPB Tem1 was localized, we examined Tem1 localization in nud1-66 mutants, where the outer plaque of the SPB dissociates from the rest of the organelle (Adams and Kilmartin, 1999). Although similar amounts of Tem1 protein were present in nud1-66 as in wild-type cells (data not shown), only 10 percent of nud1-66 cells showed localization of Tem1 at the end of mitotic spindles. In 40 percent of cells Tem1 was present as a “dot” in the cytoplasm (for examples see Figure 2D). Tem1 was undetectable by indirect immunofluorescence in 49 percent of mitotic cells. Our results suggest that Tem1 is present on the outer plaque of one SPB or a structure associated with it during late S phase, G2 and mitosis.
Figure 2: Subcellular localization of Tem1-Myc during the cell cycle.

(A) Exponential growing wild-type cells (K699) without a TEM1-MYC fusion (no tag).
(B) Tem1-Myc and microtubules of strain A1828 were visualized using anti-Myc antibodies (α-Myc) and anti-tubulin antibodies (α-Tubulin), respectively. 4',6-diamidino-2-phenylindole (DAPI) was used to stain DNA. Abbreviations: early S = early S phase, late S = late S phase, G2/Meta = G2/Metaphase, Telo = Telophase. Due to the small size of the Tem1 signal and space limitations, individual cells representing different cell cycle stages rather than fields of cells are shown.

(C) Colocalization of Tem1-Myc with the spindle pole body component Tub4. To clearly separate the two SPBs we performed this analysis in a cdc15-2 mutant, which arrests in telophase.

(D) Tem1-Myc localization in nud1-66 mutants (A1964) 120 minutes after release from a pheromone-induced G1 arrest at 37°C. Arrowhead indicates mislocalized Tem1-Myc.
Next we determined whether the localization of Tem1 was controlled by components of the mitotic exit pathway. Tem1 was present on one spindle pole body in cdc15-2, cdc5-1, dbf2-2 and cfi1::URA3 mutants (Figure 3A, B and data not shown) showing that components of the mitotic exit pathway are not required for Tem1 localization. We also analyzed whether microtubules were important for Tem1 localization. Interestingly, in cells treated with the microtubule-depolymerizing drug nocodazole although Tem1 was present at high levels as judged by Western blot analysis the protein was only weakly present at the SPB (data not shown). Thus, either microtubules are important for localizing Tem1 to the SPB or Tem1 is masked by other proteins that assemble onto the SPB in response to spindle damage or proteins present on this organelle that are modified in response to microtubule depolymerization.

**Tem1 is present at the spindle pole body that migrates into the bud.**

Because Tem1 was present on predominantly one SPB, we asked if the Tem1-bearing SPB migrated into the bud or remained in the mother cell. To address this question we treated cells with α-factor pheromone until they formed a mating projection (shmoo). When cells were released into the cell cycle the newly formed bud was spherical whereas the mother cell remained shmoo-shaped. In 135 out of 136 cells Tem1 was present in the bud (for an example see Figure 3C), demonstrating that Tem1 localized to the SPB destined to migrate into the bud.

**Tem1 is associated with Cdc15 in mitosis but not during G1.**

In *S. pombe* the Cdc15 and Tem1 homologs cdc7p and spg1p are found to be associated (Schmidt et al., 1997). Cdc15 also coimmunoprecipitated with Tem1 in exponential growing cultures, cells arrested in metaphase and telophase (Figure 3D) when Tem1 is found on the spindle pole body. During G1, Tem1 protein levels are low and no Tem1 protein is detected on
the SPB (Figure 1A, 3D). We were nevertheless able to detect immunoprecipitated Tem1 but Cdc15 was not found in these immunoprecipitates. The absence of Cdc15 in Tem1 immunoprecipitates obtained from G1 cells was not due to low levels of Tem1 obtained in the immunoprecipitation. The amount of Tem1 immunoprecipitated from G1 cells was 3 to 4 fold lower than the amount obtained from exponentially growing cultures, yet Cdc15 could not be detected in Tem1 immunoprecipitates even in long exposures (Figure 3D). Similar amounts of Cdc15 protein were present in various arrests (Figure 3D) excluding the possibility that the lack of a Cdc15-Tem1 interaction during G1 was due to low levels of Cdc15 protein in this cell cycle stage (Figure 3D). These results suggest a correlation between the ability of Tem1 to associate with Cdc15 and the presence of Tem1 on the SPB. During mitosis, Tem1 is localized on the SPB and associates with Cdc15. During G1, Tem1 is not found on the SPB, and an association between Tem1 and Cdc15 is not detected.

**Lte1 is localized to the bud.**

Next we determined the subcellular localization of Lte1-Ha using an anti-Ha antibody. This analysis revealed that Lte1-Ha was present throughout the cell during early G1 (Figure 4B). As soon as the bud emerged, Lte1 was found only in the bud. The asymmetric localization of Lte1 persisted until the completion of nuclear division. After the disassembly of the mitotic spindle Lte1 asymmetry was lost and the protein was present in both mother cell and bud (Figure 4B). The localization of Lte1-Ha was not affected by mutations in components of the mitotic exit pathway (data not shown) indicating that components of the mitotic exit pathway are not required for Lte1 localization.
Figure 3: Tem1-Myc localizes to the Bud-destined Spindle Pole Body and interacts with Cdc15.

(A, B) cdc15-2 (A; A1904) or dbf2-2 mutants (B; A1943) carrying a TEM1-MYC fusion arrested (C) Tem1-Myc cells were treated with α-factor pheromone (20 μg/ml) and released from the block. Tem1-Myc localization was determined in late anaphase cells. M marks the mother cell, that has a mating projection, D marks the bud (spherical).

(D) Tem1-myc was immunoprecipitated and the presence of Cdc15 in immunoprecipitates was determined by Western blot analysis. Cdc15-Ha fusion, Tem1-myc cells (A2193) were either arrested in G1 using α-factor (αF, lane 4) or grown to exponential phase (cyc, lane 5). A cdc23-1 (A2128, lane 6) or cdc14-3 (A2040, lane 7) mutant carrying Cdc15-Ha and Tem1-myc were employed to arrest cells in metaphase and telophase, respectively. Lane 1: Tem1-immunoprecipitation from an untagged strain (K699; no tag). Lanes 2: Tem1-myc immunoprecipitation from a Cdc15-Ha strain (A1787; Cdc15 only). Lane 3: Tem1-myc immunoprecipitation from a Tem1-myc strain (A1828; Tem1 only), respectively. The top panel on the left shows a 1 minute exposures of western blots, the bottom left panel shows a 12 hr exposure. The panel on the right shows the amount of Tem1-myc and Cdc15-Ha present in total extracts. The asterisk indicates a yeast protein that is recognized by the Ha antibody that is not Cdc15-Ha.
Figure 4: Subcellular localization of Lte1-Ha during the Cell Cycle

(A) Exponentially growing wild-type cells (A2578) without an LTE1-Ha fusion (no tag).
(B) Lte1-Ha and microtubules of strain A1949 were visualized using anti-Ha antibodies (α-Ha) and anti-tubulin antibodies (α-tubulin), respectively. DAPI was used to stain DNA. Due to space limitations, individual cells representing different cell cycle stages rather than fields of cells are shown.
Abbreviations: early S, early S phase; late S, late S phase; G2/Meta, G2/Metaphase; Ana, anaphase; Telo, telophase.
(C) Lte1-Ha staining of cells expressing LTE1-Ha from the endogenous (left panel, A1949) or the GAL1-10 promoter (A2067; right panel).
Exit from mitosis is inhibited when nuclear division occurs in the mother cell.

Our localization data indicate that Tem1 and Lte1 are spatially segregated until the onset of nuclear division, but are in the same compartment after the nucleus translocates into the bud during nuclear division, and continue to remain so until cells exit mitosis. If the presence of Tem1 and Lte1 in the same compartment were required for mitotic exit to occur, mutants in which the nucleus does not translocate into the bud during nuclear division should not release Cdc14 from the nucleolus and exit mitosis. Cells lacking cytoplasmic dynein (DYN1/DHCL) are defective in positioning the nucleus correctly during nuclear division (Saunders et al., 1995; Yeh et al., 1995; Carminati and Stearns, 1997; Shaw et al., 1997). As a result of this defect, a small portion (about 10 percent) of dyn1/dhc1Δ cells undergo nuclear division in the mother cell. Most of such cells eventually correct the defect and half of the already divided nucleus is moved into the bud.

If entry of the Tem1-bearing spindle pole body into the bud were important for exit from mitosis, dyn1/dhc1Δ cells in which nuclear division had occurred in the mother cell (henceforth dyn1/dhc1Δ binucleate mother cell) should not release Cdc14 from the nucleolus and exit from mitosis should be inhibited. The appearance of binucleate mother cells is a transient phenomenon in dyn1/dhc1Δ mutants as most binucleate mother cells eventually move half of the divided nucleus into the bud. We, therefore, manipulated cells to undergo a synchronous cell cycle to analyze Cdc14 localization and cell cycle position. Wild-type and dyn1/dhc1Δ cells were arrested in early S phase with hydroxyurea at 30°C and then released into the cell cycle at 16°C (the nuclear positioning defect of dyn1/dhc1Δ mutants is more pronounced at lower temperatures; Yeh et al., 1995). After release from the hydroxyurea block, α-factor pheromone was added to cultures to prevent reentry into the next cell cycle. We then analyzed the localization of Cdc14 when dyn1/dhc1Δ cells where in telophase and distinguished between two classes of dyn1/dhc1Δ cells;
(1) dyn1/dhc1Δ cells, in which partitioning of the nucleus between mother and daughter cells was not perturbed (henceforth dyn1/dhc1Δ normal telophase cells) and (2) dyn1/dhc1Δ cells where nuclear division occurred in the mother cell (dyn1/dhc1Δ binucleate mother cells). Time points between 4 to 6.5 hours after release from the hydroxyurea arrest were chosen for this analysis when cells with telophase spindles were present in cultures (Figure 5B).

In the wild-type strain Cdc14, was found to be released throughout the nucleus and cytoplasm in 98 percent of cells in telophase (mean value of all time points analyzed) and to be sequestered in the nucleolus in 2 percent of cells (Figure 5D). Cdc14 was also present throughout the cell during telophase in 99.6 percent of dyn1/dhc1Δ normal telophase cells (Figure 5D). In contrast, in 77 percent of dyn1/dhc1Δ binucleate mother cells Cdc14 was sequestered in the nucleolus during telophase (Figure 5E; for an example see Figure 5A). The fraction of binucleate mother cells with telophase spindles was also high (82.5 percent) suggesting that these cells failed to or were severely delayed in exit from mitosis (Figure 5C). We noted that a small proportion of dyn1/dhc1Δ binucleate mother cells, after a long delay, exited mitosis and formed a mother cell with two nuclei and a daughter cell without a nucleus (Figure 5G). Low levels of Lte1 in the mother cell, another exchange factor, or slow Tem1-intrinsic exchange activity could be responsible for this slow mitotic exit. Our results are consistent with the idea that entry of the Tem1-bearing SPB into the bud is required for exit from mitosis.
Figure 5: Analysis of Mitotic Exit in dyn1/dhc1Δ Mutants

A  α-Cdc14-Ha  α-Tubulin  DAPI

B

C

D

E

F

G

○ Wild-type  ● dyn1::URA3
□ GAL-LTE1 ■ dyn1::URA3, GAL-LTE1
Figure 5: Analysis of mitotic exit in dyn1/dhc1Δ mutants.

(A) Cdc14-Ha localization in cells lacking DYN1/DHC1 (A2077) grown at 16°C for 12 hours. The photographs show one telophase cell where nuclear division occurred normally and Cdc14-Ha was released (top cell) and one telophase cell (bottom cell), where nuclear division occurred in the mother cell and Cdc14-Ha remained sequestered in the nucleolus.

(B-F) Wild-type (open circles; A1411), dyn1/dhc1Δ (closed circles; A2077), GAL-LTE1 (open squares; A2058) and dyn1/dhc1Δ, GAL-LTE1 (closed squares; A2079) cells were arrested in early S phase using 5mg/ml hydroxyurea at 30°C in YEP medium containing raffinose and galactose (YEPRaf+Gal). When arrest was complete (after 2.25 hrs) cells were released at 16°C into YEPRaf+Gal medium containing α-factor (5μg/ml) to ensure that cells progress through only one cell cycle.

(B) Percentage of cells with telophase spindles.

(C) Percentage of telophase spindles in dyn1/dhc1Δ and dyn1/dhc1Δ, GAL-LTE1 cells where nuclear division occurred in the mother (binucleate mother cells).

(D) Percentage of Cdc14 sequestered in the nucleolus in telophase wild-type, dyn1/dhc1Δ, GAL-LTE1 and dyn1/dhc1Δ, GAL-LTE1 cells where the nucleus was positioned correctly between mother and daughter cell (normal telophase cells).

(E) Percentage of Cdc14 sequestered in the nucleolus in telophase dyn1/dhc1Δ and dyn1/dhc1Δ, GAL-LTE1 in binucleate mother cells.

(F) Percentage of Cdc14 being released from the nucleolus (present throughout the nucleus and cytoplasm) in cells other than being in anaphase or in telophase. The cartoons of cells within the graphs indicate the cell type scored.

(G) Percentage of anucleate (black bars) and multinucleate (gray bars) cells in wild-type, GAL-LTE1, dyn1/dhc1Δ and dyn1/dhc1Δ, GAL-LTE1 cells grown at 16°C in YEPRaf+Gal medium for 24 hours.
Overexpression of LTE1 induces Cdc14 release and exit from mitosis in dyn1/dhc1Δ binucleate mother cells.

To determine whether the presence of Tem1 and Lte1 in the same compartment was indeed required for release of Cdc14 from the nucleolus and mitotic exit, we analyzed the consequences on Cdc14 localization and mitotic exit in dyn1/dhc1Δ binucleate mother cells when Lte1 was forced into the mother cell. In cells overexpressing LTE1 from the galactose inducible GALI-10 promoter, the majority of Lte1 protein localized to the bud, but some protein was also present in mother cells (Figure 4D). High levels of Lte1 had little effect on cell cycle progression (data not shown), but a small proportion of cells (5-10 percent) showed premature release of Cdc14 from the nucleolus at 16°C (Figure 5F).

Overexpression of LTE1 had a dramatic effect on Cdc14 localization in dyn1/dhc1Δ binucleate mother cells (Figure 5E). Only 16.5 percent of dyn/dhc1Δ binucleate mother cells (mean value of all time points analyzed) had Cdc14 sequestered in the nucleolus. Overexpression of LTE1 also caused a decrease in the percentage of dyn/dhc1Δ binucleate mother cells with telophase spindles (Figure 5C) and an increase in multinucleate and anucleate cells (but not binucleate mother cells) after longer incubation times (Figure 5G). These findings indicate that overexpression of LTE1 induced exit from mitosis in these cells. We conclude that the presence of Tem1 and Lte1 in the same compartment is required for release of Cdc14 from the nucleolus and exit from mitosis.
Nuclear position correlates with the ability of esp1-1 cells to exit from mitosis.

To further test the hypothesis that entry of the Tem1-bearing SPB into the bud was important for exit from mitosis we analyzed Cdc14 localization and exit from mitosis in esp1-1 mutants. Cells impaired for ESP1 function progress through mitosis normally until metaphase. In more than 90 percent of esp1-1 cells the undivided nucleus migrates into the bud (Jansen et al., 1996; Figure 6A). Cells then disassemble the metaphase spindle, exit mitosis without having undergone nuclear division, enter a new cell cycle, and form a bud and replicate their DNA (McGrew et al., 1992; Ciosk et al., 1998). If entry of the Tem1-bearing SPB into the bud were important for the initiation of mitotic exit, esp1-1 cells should exit mitosis only when the nucleus is present in the bud but not when it remains in the mother cell. esp1-1 mutants were incubated with α-factor pheromone until they formed a mating projection to distinguish mother cells from buds, followed by synchronous release into the cell cycle. Metaphase spindle formed in the mother cell 90 minutes after release and, concomitantly, the nucleus translocated into the bud in the majority of cells (Figure 6A). Cdc14 was released from the nucleolus 15 minutes after translocation of the nucleus into the bud (Figure 6A) which is consistent with the idea that the Tem1-bearing SPB has to enter the bud for Cdc14 to be released from the nucleolus.

Next we analyzed the localization of Cdc14 in esp1-1 metaphase cells (time points between 105 to 225 min after release from the α-factor arrest were chosen for this analysis) and distinguished between two classes of cells; (1) esp1-1 cells, in which the nucleus resided in the mother cells and (2) esp1-1 cells where the nucleus was in the bud or in the bud neck. As observed in the dyn1/dhc1Δ mutant analysis (Figure 5), there was a remarkable correlation
between nuclear position and Cdc14 localization. Cdc14 was sequestered in the nucleolus during metaphase in cells, in which the nucleus resided in the mother cell (Figure 6D). In contrast, Cdc14 was released from the nucleolus in a high proportion of esp1-l cells where the metaphase nucleus was found in the bud or bud neck (Figure 6C). Furthermore, more than 90 percent of cells where the nucleus was found in the bud formed a new bud, indicating that cells had exited mitosis and entered a new cell cycle (Figure 6E, for an example see figure 6G). In contrast, rebudding was severely inhibited in cells where the nucleus remained in the mother cell (Figure 6F).

Overexpression of LTE1 led, although with a delay, to release of Cdc14 from the nucleolus in mother cells (Figure 6D) and allowed these mother cells to exit from mitosis as judged by the formation of a new bud (Figure 6F). As observed in the dyn1/dhc1Δ mutant analysis (Figure 5B), overexpression of LTE1 in wild-type cells delayed progression through mitosis (Figure 6B) suggesting that GAL-LTE1 causes, albeit minor, cell cycle defects. Our results indicate that entry of the Tem1-bearing SPB into the bud, where Lte1 resides, is required for release of Cdc14 from the nucleolus and exit from mitosis.

**BUB2 is required to prevent mitotic exit when nuclear division occurs in the mother cell.**

To determine whether Tem1's GAP, Bub2-Bfa1 participates in inhibiting exit from mitosis when nuclear division occurred in the mother cell we analyzed Cdc14 localization and cell cycle position in dyn1/dhc1Δ and dyn1/dhc1Δ, bub2::HIS3 cells. Cells were arrested in early S phase with hydroxyurea at 30°C and then released into the cell cycle at 16°C in the presence of α-factor. A portion of dyn1/dhc1Δ cells was delayed in disassembly of the mitotic spindle (Figure 5B, 7A) which was partially abolished by deleting BUB2 (Figure 7A). The analysis of Cdc14 localization and the status of the mitotic spindle in dyn1/dhc1Δ and dyn1/dhc1Δ, bub2::HIS3 normal telophase and binucleate mother cells is shown in Figures 7B - D. Cdc14 was sequestered
in the nucleolus in a large proportion of dyn1/dhc1Δ binucleate mother cells (Figure 7D) and the fraction of telophase spindles was high in these cells (Figure 7B). Deletion of BUB2 led to a decrease in binucleate mother cells with Cdc14 sequestered in the nucleolus and telophase spindles (Figure 7B, D) and an increase in multinucleate and anucleate cells after longer incubation times (Figure 7E). Deletion of BUB2 had a less dramatic effect than overexpressing LTE1 on dyn1/dhc1Δ binucleate mother cells (compare Figures 5 and 7). These findings suggest that BUB2 participates in ensuring that exit from mitosis only occurs after the nucleus has translocated into the bud. However, deletion of BUB2 has less dramatic consequences than forcing Lte1 into the mother cell.

Figure 6: Mitotic exit in esp1-1 mutants.

esp1-1 (A2277) and esp1-1, GAL-LTE1 (A2278) cells were arrested in G1 using 20µg/ml α-factor at 23°C in YEP medium containing raffinose and galactose (YEP Raf+Gal). When cells had formed a mating projection (after 3.25 hrs) cells were released at 37°C into YEP Raf+Gal medium.

(A) Percentage of (1) esp1-1 cells in metaphase (open triangles), (2) esp1-1 cells with Cdc14 released from the nucleolus (closed triangles) and (3) esp1-1 cells with the nucleus located in the bud or bud neck (small squares).

(B) Percentage of esp1-1, GAL-LTE1 cells (1) in metaphase (open triangles), (2) with Cdc14 released from the nucleolus (closed triangles) and (3) the nucleus located in the bud or bud neck (small squares).

(C) Percentage of Cdc14 released from the nucleolus in esp1-1 (open squares) and esp1-1, GAL-LTE1 (closed squares) cells with metaphase nuclei in the bud or bud neck.

(D) Percentage of Cdc14 released from the nucleolus in esp1-1 (open circles) and esp1-1, GAL-LTE1 (closed circles) cells with metaphase nuclei in the mother cell.

(E) Percentage of rebudded cells with nuclei in the bud or bud neck in esp1-1 (open squares) and esp1-1, GAL-LTE1 (closed squares) mutants.

(F) Percentage of rebudded cells with nuclei in the mother cells in esp1-1 (open circles) and esp1-1, GAL-LTE1 (closed circles) mutants.

The cartoons of cells within the graphs indicate the cell type scored.

(G) The photographs show one esp1-1 cell where the nucleus remained in the mother cell and rebudding did not occur (right cell) and one cell where the nucleus translocated into the bud which subsequently formed a new bud. M marks, the mother cell, that has a mating projection, D marks the bud (spherical).
Figure 6: Mitotic Exit in esp1-1 Mutants

A

esp1-1

B

esp1-1, GAL-LTE1

Δ Cells in metaphase
■ Cells with nucleus in bud or budneck
▲ Cells with Cdc14 released from the nucleolus

C

D

Percent Cdc14 released

Percent Cdc14 released

E

F

G

DAPI

Nomarski

esp1-1, nucleus in mother
esp1-1, nucleus in bud or budneck
esp1-1 GAL-LTE1, nucleus in mother
esp1-1 GAL-LTE1, nucleus in bud or budneck

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Figure 7: Analysis of Mitotic Exit in dyn1/dhc1Δ and dyn1/dhc1Δ bub2::HIS3 Mutants

Wild-type (open circles; A1411), dyn1/dhc1Δ (closed circles; A2077), bub2::HIS3 (open squares; A1901) and dyn1/dhc1Δ bub2::HIS3 (closed squares; A2270) cells were arrested in early S phase using 5mg/ml hydroxyurea at 30°C in YEPD medium. When arrest was complete (after 2.25 hrs) cells were washed and released into YEPD medium containing α-factor (5mg/ml) at 16°C.

(A) Total amount of cells in telophase.
(B) Percentage of telophase spindles in dyn1/dhc1Δ and dyn1/dhc1Δ, bub2::HIS3 binucleate mother cells.
(C) Percentage of Cdc14 sequestered in the nucleolus in telophase wild-type, dyn1/dhc1Δ, bub2::HIS3 and dyn1/dhc1Δ, bub2::HIS3 normal telophase cells.
(D) Percentage of Cdc14 sequestered in the nucleolus in telophase dyn1/dhc1Δ and dyn1/dhc1Δ, bub2::HIS3 binucleate mother cells.

The cartoons of cells within the graphs indicate the cell type scored.

(E) Percentage of anucleate (black bars) and multinucleate (gray bars) cells in wild-type, bub2::HIS3, dyn1/dhc1Δ and dyn1/dhc1Δ bub2::HIS3 cells grown at 16°C for 24 hours. dyn1/dhc1Δ, bub2::HIS3 cells contain almost as many anucleate and multinucleate cells as dyn1/dhc1Δ GAL-LTE1 cells despite the effects of deleting BUB2 on Cdc14 release from the nucleolus and spindle disassembly were not as dramatic as that caused by overexpression of LTE1. This is because glucose was used as a carbon source instead of raffinose and galactose in this experiment, which allowed for more cell divisions to occur.
Discussion

The mitotic exit pathway is highly conserved among eukaryotes (Morgan, 1999), yet its role in promoting exit from mitosis has been unclear. Our studies on Tem1 and Lte1 shed light on this question. We find that Tem1 and Lte1 need to be in the same compartment in order to induce mitotic exit and that this occurs as the nucleus migrates into the bud during nuclear division. In wild-type cells under optimal growth conditions entry of the nucleus into the bud coincides with the onset of nuclear division. As exit from mitosis requires Tem1 and Lte1 to be in the same compartment, the spatial segregation of Tem1 and Lte1 until the onset of nuclear division ensures that exit from mitosis does not occur prior to nuclear division. Under some conditions (e.g. cold) nuclear division can occur entirely in the mother cell. Even under these aberrant conditions the mode of Tem1 and Lte1 localization ensures that the nucleus is partitioned between mother and daughter cell prior to exit from mitosis. Thus, the mitotic exit pathway guarantees that the outcome of cell division is productive and two cells, each with a nucleus, are generated.

The localization of Tem1.

Two lines of evidence suggest that the presence of Tem1 on the SPB is critical for the protein to promote exit from mitosis. First, unlike most other mutants that disrupt SPB function, which arrest in G2 (Geissler et al., 1996; Knop et al., 1996; Theesfeld et al., 1999), nud1-66 mutants arrest in telophase (Adams and Kilmartin, 1999), indicating that they fail to exit from mitosis. Although other explanations are possible, the nud1-66 phenotype may be a consequence of a failure to localize Tem1 on the SPB. Second, Tem1 interacts with Cdc15 only in cell cycle stages when Tem1 is present on the SPB, suggesting that SPB localization of Tem1 is required for the protein to associate with Cdc15.
Remarkably, in most cells Tem1 is found on only the SPB that migrates into the bud. How this asymmetry is generated is not known. As Tem1 protein levels fluctuate during the cell cycle, it is tempting to speculate that degradation of Tem1 during exit from mitosis and G1 clears Tem1 from the SPB. If Tem1 were then only capable of loading onto the newly assembled SPB, asymmetry would be generated. Whether the absence of Tem1 on the spindle pole that remains in the mother cell is important for the regulation of exit from mitosis is also unclear. 25 percent of mitotic spindles show, albeit weakly, Tem1 localization on the spindle pole body that remains in the mother cell. In S. pombe, the Tem1 homolog spg1p is present on both SPBs (Schmidt et al., 1997) but its two component GAP cdc16p-Bfa1p localizes to only one SPB where it keeps spg1p inactive (Cerutti and Simanis, 1999). Why cells choose to keep Tem1/spg1p activity restricted to one spindle pole body is a mystery. However, the finding that this regulatory mechanism is conserved at least among yeasts, indicates that it is important for the regulation of exit from mitosis.

**Importance of the spatial segregation of Lte1 and Tem1.**

Genetic evidence suggests that **LTE1** and **TEM1** function in a common pathway and that **LTE1** functions as the exchange factor for **TEM1**. Deletion of **TEM1** is lethal causing cells to arrest in telophase (Shirayama et al., 1994b). Deletion of **LTE1** also causes arrest in telophase at low temperatures (Shirayama et al., 1994a) and a transient delay in telophase at 25°C (20 min; A. B. unpublished observations). Furthermore, overexpression of **TEM1** suppresses the cold-sensitive lethality of cells deleted for **LTE1** (Shirayama et al., 1994b). However, unlike **TEM1**, **LTE1** is only essential for viability at low temperatures. Whether other exchange factors exist that substitute for **LTE1** at higher temperatures or whether Tem1 contains an intrinsic exchange activity, is at present unclear.
Our analysis of *dyn/dhc1Δ* and *esp1-1* mutants indicates that the presence of Tem1 and Lte1 in the bud is required for release of Cdc14 from the nucleolus and exit from mitosis. In *dyn1/dhc1Δ* cells where the nucleus was partitioned between mother and daughter cell and *esp1-1* mutants where the nucleus was in the bud or in the bud neck, Cdc14 was released from the nucleolus and exit from mitosis occurred. In contrast, in *dyn1/dhc1Δ* binucleate mother cells and *esp1-1* mutants in which the nucleus resided in the mother cell, Cdc14 was sequestered in the nucleolus and exit from mitosis was inhibited. Overexpression of *LTE1* allowed a large proportion, but not all, of such *dyn1/dhc1Δ* or *esp1-1* cells to release Cdc14 from the nucleolus and exit from mitosis. We can envision two reasons or a combination thereof for this incomplete rescue. Lte1 may not be as active in the mother cell due to insufficient amount of protein accumulating or due to lack of phosphorylation that occurs only upon its the translocation of Lte1 into the bud. Alternatively, activation of other components of the mitotic exit pathway may be necessary to efficiently trigger exit from mitosis. This idea is consistent with the following two observations. (1) Overexpression of *LTE1* had little effect on cell cycle progression in wild-type cells and (2) Cdc14 release from the nucleolus did not immediately occur after the nucleus translocated into the bud in *esp1-1* mutants, but was delayed by 15 minutes.

It is important to note that restriction of Lte1 to the bud does not only ensure that mitotic exit is induced after the nucleus is partitioned between mother and daughter cell. Cells lacking the actin motor *MYO2* fail to form a bud and the mother cell grows unusually large (Johnston et al., 1991; Govindan et al., 1995). In such cells release of Cdc14 from the nucleolus is delayed. The delay in Cdc14 release is, to a large extent, abolished by overexpression of *LTE1* (A. B.
unpublished observations). Thus, restricting Lte1 also ensures that sufficient amounts of Lte1 are present to activate Tem1 once the nucleus enters the bud during nuclear division.

**The role of Bub2 in restraining mitotic exit.**

Our analyses showed that Bub2, which localizes to both SPBs (Fraschini et al., 1999; Li, 1999) also participates in restraining exit from mitosis when nuclear division occurred in the mother cell. Whether Bub2-Bfa1 activity is regulated or whether its GAP activity is constitutive and antagonized by Lte1 once Tem1 enters the bud is unknown. However, it is clear that Bub2-Bfa1 is critical to prevent mitotic exit when cell cycle progression is halted in response to spindle damage (Hoyt et al., 1991; Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). Indeed, we found that Cdc14 was released from the nucleolus in a small portion (one percent) of metaphase cells (A. B., unpublished observations). We speculate that Bub2-Bfa1 functions as a safeguard to prevent low levels of Lte1 in the mother cell, another exchange factor, or slow Tem1-intrinsic exchange activity to activate the mitotic exit pathway when onset of anaphase is inhibited due to mitotic spindle defects.

**Signals for exit from mitosis.**

Inhibition of cytokinesis was previously observed when nuclear division occurred in the mother cell and shown to be due to activation of the spindle-positioning checkpoint (Yeh et al., 1995; Muhua et al., 1998). Our results show that this is likely to be due to inhibition of Cdc14 release from the nucleolus and that this is in large part due to Lte1 and Tem1 not being in the same compartment. Mutants have been identified that abolish the delay in mitotic exit when the mitotic spindle is mispositioned (Muhua et al., 1998). It will be interesting to determine whether these mutants affect Tem1, Lte1 or Bub2 localization.
Improper nuclear position is probably not the only event that prevents exit from mitosis. Defects in mitotic spindle formation inhibit mitotic exit through Bub2-Bfa1 (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). Two regulators of sister-chromatid separation, Pds1 and Esp1, also inhibit exit from mitosis and Cdc14 release from the nucleolus (Cohen-Fix and Koshland, 1999; Shirayama et al., 1999; Tinker-Kulberg and Morgan, 1999). Whether these proteins regulate Tem1 activation or other aspects of the mitotic exit pathway is, at present, not known. Thus, it appears that the mitotic exit pathway is responsive to a variety of signals, such as onset of nuclear division, correct positioning of the nucleus, the activity of proteins that control sister-chromatid separation and the integrity of the mitotic spindle and only triggers mitotic exit when all these processes have occurred properly.

A model for how exit from mitosis is coupled to partitioning of the nucleus between mother and daughter cell.

Our results provide a mechanism for ensuring that exit from mitosis does not occur prior to partitioning of the genetic material between mother and daughter cell (Figure 8). During G1, Lte1 is localized uniformly throughout the cell. During this cell cycle stage, Tem1 protein levels are very low and it fails to associate with the mitotic exit pathway component Cdc15, suggesting that Tem1 is not capable of activating the mitotic exit pathway during G1. Perhaps, the presence of Bub2-Bfa1 on the SPB acts as a safeguard to prevent premature activation of Tem1. During early S phase, when the bud forms, Lte1 is recruited into the bud. Tem1 protein accumulates during late S phase when the mitotic spindle forms and, after mitotic spindle formation, is loaded onto the bud-destined SPB. As nuclear division begins, the nucleus moves to the bud neck and is pulled into the bud as chromosome segregation commences. This leads to the presence of Tem1
and Lte1 in the same compartment, allowing for its activation. How activation of Tem1 at the spindle pole body leads to release of Cdc14 in the nucleolus is an important question that remains to be addressed. As cells exit mitosis, Tem1 is cleared from the SPB, perhaps by protein degradation, preventing further stimulation of the mitotic exit pathway and allowing for the rapid return of Cdc14 into the nucleolus.

Our results point towards a novel mechanism for regulating the activity of signal transduction pathways: the coupling of activation of a signal transduction pathway to the physical movement of an organelle. In the case of the mitotic exit pathway, this mechanism establishes a dependency of mitotic exit on partitioning of the genetic material between mother and daughter cell.
Figure 8: A Model for Activation of the Mitotic Exit Pathway

In G1, Lte1 (in green) is diffuse throughout the cell, Tem1 protein levels are low, it is not SPB localized and it does not interact with Cdc15. During S phase, Lte1 becomes compartmentalized into the daughter bud and in late S phase, Tem1 (in red) is loaded onto the SPB destined for the daughter cell. Bfa1 and Bub2 play a role inhibiting Tem1 until anaphase. During anaphase, the nucleus extends into the daughter cell placing Tem1 and Lte1 in the same cellular compartment, thereby facilitating activation of the mitotic exit pathway and allowing Cdc14 release (in blue). Release of Cdc14 into the nucleus and cytoplasm during anaphase and telophase promotes inactivation of mitotic CDKs and mitotic exit. In late telophase, Lte1 becomes diffuse between the mother and daughter cell, Tem1 SPB localization is lost, and Cdc14 becomes resequenced in the nucleolus.
Experimental procedures

Plasmids and strains

All strains were derivatives of strain W303 (K699). To generate a GAL-LTE1 fusion a Nco1-BspH1 fragment carrying LTE (Nco1 at the AUG) was cloned under the control of the GAL1-10 promoter by blunt end ligation. HA or MYC tags were introduced into LTE1 and TEM1, respectively as described by Schneider et al. (1995) and were fully functional.

Growth conditions

Conditions for growth and release of synchronous cultures from arrest by α-factor were as described by Surana et al.(1993). Cells were arrested with hydroxyurea and nocodazole by adding 5mg/ml hydroxyurea or 15μg/ml nocodazole to cultures, respectively. To release cells from an hydroxyurea-induced arrest, cells were washed with 5 volumes of medium and transferred into medium lacking the drug.

Techniques

Immunoblot analysis of the total amount of Clb2, Sic1 and Kar2 was performed as described in by Cohen-Fix et al. (1996). Antibody dilutions were used as in Visintin et al. (1997). Co-immunoprecipitations were performed as described by Amon et al. (1993) using 1 mg of cell extracts. Indirect-immunofluorescence analyses methods and antibody concentrations are described in Visintin et al. (1999).
Literature Cited


Chapter III:

Mitotic exit regulation through distinct domains within the protein kinase Cdc15.

A version of this manuscript has submitted for publication by Bardin, A. & Amon, A.
Abstract

The mitotic exit network (MEN), a Ras-like signaling cascade, promotes the release of the protein phosphatase Cdc14 from the nucleolus and is essential for cells to exit from mitosis in *S. cerevisiae*. We have characterized functional domains of one of the components of MEN, the protein kinase Cdc15, and investigated the role of these domains in mitotic exit. We show that a region adjacent to Cdc15's kinase domain is required for self-association, for binding to spindle pole bodies, and that this domain is essential for *CDC15* function. Furthermore, we find that overexpression of *CDC15* lacking the C-terminal 224 amino acids results in hyperactivation of MEN and premature release of Cdc14 from the nucleolus, suggesting that this domain within Cdc15 functions to inhibit MEN signaling. Our findings suggest multiple modes of MEN regulation through the protein kinase Cdc15.
Introduction

Exit from mitosis is the final cell cycle transition that leads to the production of two genetically identical daughter cells. Proper execution of this transition is essential for each of the two daughter cells to receive a complete complement of the genome and, thus, to maintain ploidy. In all eukaryotes examined to date, exit from mitosis is brought about by the inactivation of mitotic cyclin dependent kinase (mitotic CDK) activity (reviewed in Morgan, 1999). In budding yeast, the protein phosphatase Cdc14 induces the inactivation of mitotic CDKs by reversing CDK-dependent phosphorylation on proteins important for promoting mitotic CDK inactivation (Jaspersen et al., 1998; Visintin et al., 1998; Jaspersen et al., 1999; et al.; reviewed in Bardin and Amon, 2001).

Cdc14 is regulated by an inhibitor, Cfi1/Net1. Cfi1/Net1 inhibits and sequesters Cdc14 in the nucleolus throughout most of the cell cycle (Shou et al., 1999; Visintin et al., 1999). Only during anaphase and telophase is Cdc14 released from its inhibitor (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). To date, two pathways have been identified that control Cdc14 localization: the FEAR network (Cdc fourteen early anaphase release) and the MEN (Mitotic Exit Network) (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002; reviewed in Bardin and Amon, 2001; McCollum and Gould, 2001; Jensen et al., 2002). The FEAR network appears to be activated at the metaphase - anaphase transition and promotes Cdc14 release from the nucleolus during early anaphase. MEN is thought to be activated when the nucleus migrates into the daughter cell during anaphase (Bardin et al., 2000; Pereira et al., 2000) and is essential
for sustaining release of Cdc14 from the nucleolus during late stages of anaphase. Temperature sensitive mutations in MEN components cause cells to arrest in telophase, upstream of Cdc14 activation, with Cdc14 sequestered in the nucleolus and high levels of mitotic CDK activity (Surana et al., 1993; Shirayama et al., 1994; Jaspersen et al., 1998; Shou et al., 1999; Visintin et al., 1998).

MEN is a signal transduction pathway comprised of a GTPase, Tem1; the guanine nucleotide exchange factor (GEF), Lte1; the two-component GTPase activating protein (GAP) complex Bub2 – Bfa1; the protein kinases Cdc15, Dbf2, and Cdc5; a Dbf2 associated factor, Mob1; and a scaffold protein, Nud1 (reviewed in (Bardin and Amon, 2001)). Localization of MEN components to spindle pole bodies (SPBs) and in vitro assays for Dbf2 kinase activation have been used to order MEN components into a pathway (Cenamor et al., 1999; Bardin et al., 2000; Frenz et al., 2000; Xu et al., 2000; Lee et al., 2001; Luca et al., 2001; Visintin and Amon, 2001; Yoshida, 2001). These analyses revealed that Tem1 binds to and functions upstream of Cdc15, which activates Dbf2 in a Mob1-dependent manner. The polo kinase Cdc5 regulates the activity of several MEN components (Lee et al., 2001; Visintin and Amon, 2001).

Most MEN components have been shown to localize to SPBs (reviewed in Bardin et al., 2001). Tem1, Bub2 and Bfa1, predominantly localize to the SPB that migrates into the daughter cell during anaphase (Fraschini et al., 1999; Bardin et al., 2000; Daum et al., 2000; Pereira et al., 2000). Cdc5 localizes to both SPBs during metaphase and anaphase (Shirayama et al., 1998), whereas Cdc15 and Dbf2 localize to both SPBs only during anaphase (Cenamor et al., 1999; Frenz et al., 2000; Xu et al., 2000; Menssen et al., 2001; Visintin and Amon, 2001). Localization
of Dbf2 to SPBs coincides with activation of Dbf2 kinase activity, suggesting that SPB association of Dbf2 is a prerequisite for Dbf2 activation (Fesquet et al., 1999; Visintin and Amon, 2001). Localization of most MEN components to SPBs requires the SPB component Nud1. Cells carrying a temperature sensitive allele in NUD1 arrest in telophase with Tem1, Cdc15, and Dbf2 mislocalized suggesting that SPB localization of MEN components is important for promoting mitotic exit (Bardin et al., 2000; Gruneberg et al., 2000; Visintin and Amon, 2001).

Three cellular events have been identified that control MEN activation: spindle position, mitotic spindle damage, and DNA damage. All are thought to regulate the GTP/GDP binding status of Tem1 through Lte1 and Bub2 – Bfa1 (reviewed in Hoyt, 2000; Bardin and Amon, 2001; McCollum and Gould, 2001). However, although Bub2 – Bfa1 and Lte1 are important for controlling MEN activity, several lines of evidence suggest that MEN is regulated at additional levels. Neither LTE1 nor BUB2 nor BFA1 are essential for viability. Furthermore, in an unperturbed cell cycle, neither deletion of BUB2 or BFA1 nor overexpression of LTE1 causes a cell cycle defect or premature release Cdc14 from the nucleolus (Bardin et al., 2000). Thus, other components of MEN are likely subject to regulation.

One MEN component known to be a target of regulation is the protein kinase Cdc15. Cdc14 released from the nucleolus by the FEAR network during early anaphase causes dephosphorylation of Cdc15 allowing it to become a more potent activator of mitotic exit (Jaspersen and Morgan, 2000; Xu et al., 2000; Menssen et al., 2001; Stegmeier et al., 2002). In order to identify additional modes of Cdc15 regulation, we have determined how different
domains of Cdc15 function to regulate mitotic exit. We mapped the region of Cdc15 required for association with SPBs to a domain adjacent to the kinase domain. Our analyses also show that Cdc15 “self-associates” as determined by the ability to co-immunoprecipitate itself. It is not known whether this is due to a direct interaction, or whether it is mediated through interaction with other proteins. However, we find that self-association is mediated through a region that overlaps the SPB-localization domain of Cdc15. This domain is essential for viability suggesting that SPB association and/or self-association are required for CDC15 function. Finally, we find that overexpression of CDC15 lacking the C-terminal 224 amino acids, but not overexpression of full length CDC15, causes hyperactivation of MEN as judged by elevated levels of Dbf2 kinase activity and premature release of Cdc14 from the nucleolus. Our results suggest the presence of at least three functional domains within Cdc15 aside from the kinase domain: a SPB association domain, a self-association domain, and a domain that inhibits MEN signaling.

Results

The kinase and C-terminal domains of Cdc15 are dispensable for its localization to the spindle pole body.

The association of Cdc15 with spindle pole bodies is cell cycle regulated. SPB localization occurs during anaphase and depends on TEM1 (Cenamor et al., 1999; Xu et al., 2000; Menssen et al., 2001; Visintin and Amon, 2001). However, the findings that Tem1 primarily associates with the SPB destined for the daughter cell while Cdc15 localizes to both SPBs, and that Cdc15 can, when overexpressed, localize to SPBs in the absence of Tem1 (Figure
2B; Cenamor et al., 1999; Bardin et al., 2000; Pereira et al., 2000), raised the possibility that Cdc15 also associates with SPBs by means other than binding to Tem1. To identify regions within Cdc15 that were required for its association with SPBs we examined the localization of various GFP-Cdc15 truncation mutants (Figure 1A). As endogenous Cdc15 is exceedingly difficult to detect in cells, we employed the GALI promoter to increase expression of the CDC15 truncations (Figure 1B).

The association of the overexpressed full-length GFP-Cdc15 with SPBs was indistinguishable from that of the endogenous Cdc15 protein tagged with either 3 HA or 13 MYC epitopes (Visintin and Amon, 2001; data not shown). GFP-Cdc15 localized to SPBs during anaphase and telophase but not significantly during other cell cycle stages (Figure 2A). Live cell analysis showed that GFP-Cdc15 also localized to the bud neck, which is not detected by indirect in situ immunofluorescence using Cdc15-3HA or Cdc15-13MYC fusions (Table 1; Cenamor et al., 1999; Menssen et al., 2001; Visintin and Amon, 2001; Xu et al., 2000). However, in contrast to endogenous Cdc15, overexpressed GFP-Cdc15 could localize to SPBs in the absence of TEM1 and suppressed the lethality associated with deletion of TEM1 (Figure 2B; and data not shown Cenamor et al., 1999).

Having established that overexpressed GFP-Cdc15 associates with SPBs in a cell cycle-dependent manner, we made a series of truncations to determine which region in Cdc15 was required for its association with this organelle. Because CDC15 is an essential gene, the Cdc15 truncations employed in this analysis were expressed in cells also containing full length CDC15 under its endogenous promoter (summarized in Figure 1A). GFP-Cdc15 lacking the N-terminal
kinase domain (GFP-Cdc15 [267 – 974]) localizes to both SPBs (Figure 2A, Table 1). A C-terminal deletion of this construct demonstrated that amino acids 751 – 974 were also dispensable for SPB localization as GFP-Cdc15 [267-750] localized to both SPBs (Figure 2A, Table 1). Truncation GFP-Cdc15 [267-702] also localized to SPBs, but was reduced, compared to truncation GFP-Cdc15 [267-750] (Table 1). Further removal of the N-terminus or C-terminus resulted in defects or loss of SPB localization (Table 1). GFP-Cdc15 [267-550] localized to SPBs but was also found in a punctate pattern along the mitotic spindle (data not shown). Notably, a Cdc15 truncation that has previously been shown to include amino acids sufficient for Tem1 binding (GFP-Cdc15 [267-376], Asakawa et al., 2001) did not localize to SPBs (Table 1, data not shown). Our results demonstrate that amino acids 267-702 of GFP-Cdc15 are necessary and sufficient for a wild-type pattern of SPB association when expressed from the GAL1 promoter. Thus, the region within Cdc15 required for SPB association is much larger than that reported to be sufficient for Tem1 binding, suggesting that mechanisms other than Tem1 binding are necessary for a stable association of Cdc15 with SPBs.

The use of GFP-Cdc15 fusions also enabled us to determine which region within Cdc15 was necessary for its association with the bud neck. We found that GFP-Cdc15 [267-702] was the smallest fragment able to localize to the bud neck (Table 1). We conclude that SPB and bud neck localization are mediated through a similar region within Cdc15.
Table 1: Cdc15 localization and self-association in cells overexpressing various CDC15 truncations.

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<th>Cdc15 on bud neck</th>
<th>Cdc15-Cdc15</th>
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* localizes weakly to SPBs but also along the mitotic spindle
** localizes weakly to SPBs but also forms large aggregates in cytoplasm
NA not analyzed
Figure 1: Cdc15 functional domains

(A.) Cdc15 Functional Domains. Cdc15 is a 974aa protein with an N terminal kinase domain (aa1-267, purple). We show that Cdc15 has a domain (aa361-702, in yellow) which is necessary and sufficient for interaction with other Cdc15 molecules. Additionally, SPB localization is mediated by a domain in the middle of Cdc15 (aa267-702, in red). We further demonstrate that a C-terminal domain (aa751-974, in green) functions to inhibit MEN activity. Removal of aa751-974 results in a dominant active Cdc15 that can ectopically activate Dbf2 kinase activity and cause Cdc14 nucleolar release. (B.) Expression of a subset of GFP-CDC15 constructs. Anti-GFP western blot analysis of 50 mg of extract from strains grown in YEP + 2% raffinose, induced with 2% galactose for 2.5 hours. (* denotes cross-reacting bands, x denotes non-specific mark on membrane). The following strains were used: A4683 (GFP-CDC15 [1-974, full length]), A5575 (GFP-CDC15 [1-750]), A5576 (GFP-CDC15 [1-702]), A4692 (GFP-CDC15 [1-550]), A5578 (GFP-CDC15 [1-464]), A4678 (GFP-CDC15 [1-376]), A4690 (GFP-CDC15 [1-273]), A4685 (GFP-CDC15 [267-974]), A5464 (GFP-CDC15 [361-974]), A5466 (GFP-CDC15 [395-974]), A6456 (GFP-CDC15 [751-974]), A5909 (GFP-CDC15 [361-702]).
Figure 2. Cdc15 N-terminal kinase domain and C-terminus as well as TEM1 are dispensable for SPB localization of GFP-Cdc15 expressed from the GAL1 promoter. Cells were grown to mid-log phase in YEP + 2% raffinose and induced for 2.5 hours with 2% galactose. (A.) Fluorescence microscopy of strains expressing GFP-Cdc15 [1-974, full length] (A4441), GFP-Cdc15 [267-974] (A4376), GFP-Cdc15 [267-750] (A4403), or GFP-Cdc15 [361-974] (A5254). (B.) GFP-Cdc15 can localize to SPBs in the absence of Tem1 protein. Cells are grown continuously in 2% raffinose, 2% galactose. Fluorescence microscopy of GFP-CDC15 in a tem1D background (A6956). (Note: fixation in EtOH destroys bud neck localized GFP signal.)
Cdc15 self-associates and is mediated by amino acids 361–702.

The importance of dimerization in the regulation of kinases has been highlighted in recent years with the discovery that Raf is positively regulated through dimerization and that PAK is negatively regulated through a trans auto-inhibitory dimerization mechanism (Farrar et al., 1996; Parrini et al., 2002). To determine whether Cdc15 could associate with itself, we performed co-immunoprecipitation analyses with a GFP-Cdc15 expressed from the GAL1 promoter and endogenous Cdc15 tagged with 3 HA epitopes. GFP-Cdc15 immunoprecipitated Cdc15-3HA and vice versa (Figure 3A, data not shown). Endogenous Cdc15-3HA also immunoprecipitated Cdc15-13MYC produced at endogenous levels (Figure 3B) indicating that the association between overproduced GFP-Cdc15 and Cdc15-HA was not an artifact of overexpression.

To determine the region of Cdc15 that mediates interaction with itself, various GFP-Cdc15 truncations produced from the GAL1 promoter were tested for their ability to immunoprecipitate Cdc15-3HA. Removal of the first 360 amino acids of GFP-CDC15 did not significantly affect the interaction of GFP-Cdc15 with Cdc15-3HA (Figure 3A, Table 1), indicating that the kinase domain and the Tem1 binding domain (as defined by Asakawa et al., 2001) were dispensable for this interaction. The removal of the C-terminus (aa 703–974) also did not significantly affect self-association as GFP-Cdc15 [361–702] efficiently immunoprecipitated Cdc15-3HA (Figure 3A, Table 1). Further deletion of C-terminal and N-terminal regions reduced the ability of Cdc15 to self-associate. GFP-Cdc15 [1-550] retained some ability to bind to Cdc15-3HA, however, this was abolished when GFP-Cdc15 [1-429] was
expressed (Figure 3A, Table 1). Our results show that region aa 361 – 702 is sufficient for Cdc15 to self-associate.

To further define the self-association domain within Cdc15 we removed two regions within Cdc15, amino acids 362 – 464 (Cdc15-3HA [Δ362-464]) and 465 - 550 (Cdc15-3HA [Δ465-550]), and assayed the ability of these constructs expressed from the endogenous promoter to bind to Cdc15-13MYC also expressed from the endogenous promoter. Removal of amino acids 362-464 or 465-550, dramatically reduced the interaction of Cdc15-3HA with Cdc15-13MYC (Figure 3B). Deletion of these regions also affected CDC15 function as the cdc15-3HA [Δ362-464] and cdc15-3HA [Δ465-550] constructs did not complement the temperature sensitive lethality of a cdc15-2 allele (Figure 3D). Loss of function was not due to degradation of an unstable protein at 37°C since protein levels of Δ362-464 and Δ465-550 were comparable to that of wild-type protein at 37°C (data not shown). Furthermore, truncations Cdc15-3HA [Δ362-464] and Cdc15-3HA [Δ465-550] retained the ability to immunoprecipitate Tem1 (Figure 3C) as well as retained in vitro kinase activity (data not shown), indicating that the proteins were not grossly mis-folded. We conclude that aa 361 – 702 are required and sufficient for Cdc15 to bind to self-associate and that this region is essential for Cdc15 function.
**Figure 3A:** Cdc15 contains a domain that mediates self association

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Total Cdc15-3HA

IP: GFP-Cdc15
W: Cdc15-3HA

**Figure 3A:** Cdc15 contains a domain that mediates self-association and is essential for viability.
Cells were grown in YEP + 2% raffinose and induced with 2% galactos for 2.5 hours. GFP-CDC15 constructs were immunoprecipitated from 1mg of extract and the amount of associated Cdc15-3HA is assayed by anti-HA.11 western blot. The upper panel is the input amount of Cdc15-3HA in 50 μg of extract. The middle panel is co-immunoprecipitated Cdc15-3HA, and the lower panel is the amount of immunoprecipitated GFP-Cdc15 construct. (*) denotes non-specific mark on the membrane. The following genotypes were used: A2587 (W303, wildtype), A3932(CDC15-3HA); A4441 (GFP-CDC15[1-974]); A4683 (GFP-CDC15[1-974,full length],CDC15-3HA).A5575 (GFP-CDC15[1-751],CDC15-3HA).A5576 (GFP-CDC15[1-702],CDC15-3HA)
A4692 (GFP-CDC15[1-550],CDC15-3HA), A5578 (GFP-CDC15[1-464],CDC15-3HA), A4678 (GFP-CDC15[1-376],CDC15-3HA), A4690 (GFP-CDC15[1-273],CDC15-3HA), A4685 (GFP-CDC15[267-974],CDC15-3HA), A5464 (GFP-CDC15[361-974],CDC15-3HA), A5466 (GFP-CDC15[395-974],CDC15-3HA), A6456 (GFP-CDC15[751-974],CDC15-3HA), A5909 (GFP-CDC15[361-702],CDC15-3HA).
Figure 3:  Cdc15 contains a domain that mediates self-association and is essential for viability.

B

| CDC15-13MYC | - | - | + | + | + |
| CDC15-3HA   | Full length | Full length | 465-464 | 465-550 |

Total Cdc15-13MYC
IP: Cdc15-HA
W: Cdc15-MYC
IP: Cdc15-HA
W: Cdc15-HA

C

| TEM1-3MYC | - | + | + | + | + |
| CDC15-3HA | - | - | Full length | Full length | 465-464 | 465-550 |

Total Tem1-MYC
IP: Cdc15-HA
W: Tem1-MYC
IP: Cdc15-HA
W: Cdc15-HA

D

cdc15(Δ362-464), cdc15-2

Figure 3B-3D:
Cdc15 contains a domain that mediates self-association and is essential for viability.

(B.) Loss of aa362-464 or 465-550 disrupts the ability of Cdc15-3HA to immunoprecipitate Cdc15-13MYC. Cells were grown in YPD to mid-log phase. 1mg of extract was used for immunoprecipitation with an anti-HA.11 antibody. The upper panel is the input amount of Cdc15-13MYC in 50mg of extract. The middle panel indicates the amount of associated Cdc15-13MYC assayed by anti-MYC Western blot. The lower panel shows the amount of immunoprecipitated Cdc15-3HA construct. The following strains were used: A2587 (W303, wild-type); A3932 (CDC15-3HA); A5401 (CDC15-13MYC); A6501 (CDC15-3HA, CDC15-13MYC); A6502 (CDC15(Δ362-464)-3HA, CDC15-13MYC); A6503 (CDC15 (Δ465-550)-3HA CDC15-13MYC). (C.) Loss of self-association domains does not affect the ability of Cdc15 to interact with Tem1. Cells were grown in YPD to mid-log phase. 1mg of extract was used for immunoprecipitation with an anti-HA.11 antibody. The upper panel indicates the amount of Tem1-3MYC protein in the starting extract. The middle panel shows the amount of Tem1-3MYC protein that is co-immunoprecipitated. The lower panel is the amount of Cdc15-13HA construct that is immunoprecipitated. The following strains were used: A2587 (W303, wild type); A1828 (TEML-3MYC); A6542 (TEML-3MYC, CDC15-3HA); A6543 (TEML-3MYC, CDC15(Δ362-464)-3HA); A6544 (TEML-3MYC, CDC15(Δ465-550)-3HA). (D.) Loss of self-association domain results in lack of complementation of cdc15-2 at 37°C. Strains expressing either wild-type CDC15 (A6546 (cdc15-2, CDC15-3HA)) or CDC15 lacking amino acids required for self-association (A6547 (cdc15-2, CDC15(Δ362-464)-3HA); A6548 (cdc15-2, CDC15(Δ465-550))) were streaked to single colonies on YPD plates and grown at 37°C for 36 hours.

98
Kinase activity immunoprecipitated with a Cdc15-3HA fusion is increased when a GFP-
CDC15 fusion is overexpressed.

To investigate whether self-association of Cdc15 negatively regulates its kinase activity, as previously described for Pak1 kinase (Lei et al., 2000), we determined the consequences of overexpressing GFP-CDC15 on the kinase activity associated with immunoprecipitated Cdc15-3HA. We reasoned that increased levels of GFP-Cdc15 would increase the total amount of Cdc15-3HA that self-associates with other Cdc15 molecules. When full length GFP-CDC15 was overexpressed, the kinase activity associated with Cdc15-3HA immunoprecipitates was increased by 2.0 fold (Figure 4). The increase was due to expression of GFP-CDC15 as an uninduced control culture showed kinase levels similar to the wild-type (Figure 4, lane 3). The increase in Cdc15-3HA associated kinase activity was consistently seen in multiple experiments and was dependent on the kinase domain of GFP-CDC15 since overexpression of GFP-CDC15 [267 – 974] did not lead to an increase in kinase activity. Additionally, the increase was dependent on the self-association domain since GFP-CDC15[1 – 376], a construct which has wild-type kinase activity (Figure 6B), but lacks the self-association domain (Table 1, Figure 3A) also did not lead to an increase in kinase activity associated with Cdc15-3HA (Figure 4). Our results suggest that Cdc15 multimers are not inhibited for kinase activity such as Pak1, but in fact, do possess in vitro kinase activity.
Figure 4: Increased Kinase Activity of Cdc15-HA upon overproduction of GFP-CDC15

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Kinase activity precipitated with Cdc15-Ha

|                | 0.0 | 1.0 | 1.2 | 2.0 | 1.0 | 1.1 |

Figure 4. The over-expression of full length GFP-CDC15, but not GFP-CDC15 lacking the kinase domain or GFP-CDC15 lacking the self-association domain, results in an increase in associated kinase activity of Cdc15-3HA. Cells were grown in YEP +2% raffinose overnight to mid-log phase. Cultures were diluted to OD600=0.2 and arrested in metaphase with 15mg/ml nocodazole for 1.5 hours. An additional 15mg/ml of nocodazole was added to prevent cells from escaping the metaphase arrest and cells were induced for 2.5 hours with 2% galactose (except lane 3 which is uninduced). The amount of kinase activity associated with Cdc15-3HA was then assayed by immunoprecipitation followed by kinase assay using myelin basic protein (MBP) as an artificial substrate. The following strains were used: A2587 (W303, wild-type), A3932 (CDC15-3HA); A4683 (CDC15-3HA, GAL-GFP-CDC15 [1-974]); A4686 (CDC15-3HA, GAL-GFP-CDC15 [267-974]), A4678 (CDC15-3HA, GAL-GFP-CDC15 [1-376]).
Overexpression of \textit{CDC15} lacking the kinase domain causes cell cycle arrest in telophase.

During our localization studies we noticed that overexpression of \textit{CDC15} lacking the kinase domain (\textit{GFP-CDC15 [267-974]}) upon release from a pheromone-induced G1 block, caused cells to arrest in late anaphase/telophase with DNA masses segregated and long mitotic spindles (Figure 5A, Table 1). The fact that these cells also expressed full length \textit{CDC15} from its endogenous promoter suggested this construct functioned in a dominant negative manner.

Overexpression of truncations \textit{GFP-CDC15 [267–750]}, \textit{GFP-CDC15 [267–702]}, and \textit{GFP-CDC15 [267–550]} also caused a late anaphase/telophase arrest (Figure 5B, Table 1). In contrast, overexpression of full length \textit{GFP-CDC15} or the same constructs with the kinase domain present did not cause a telophase arrest (Figure 5, Table 1). Our data suggest that truncations \textit{GFP-CDC15 [267–750]}, \textit{GFP-CDC15 [267–702]}, and \textit{GFP-CDC15 [267–550]}, henceforth called class 1 truncations, while retaining the ability to bind to at least some important binding partners, must out-compete endogenous full length Cdc15 and sequester the binding partners into a kinase inactive complex, thereby preventing exit from mitosis.

Our analysis of the consequences of overexpressing various \textit{GFP-CDC15} truncations on cell cycle progression revealed two more classes of \textit{CDC15} truncations. Class 2 truncations (\textit{GFP-CDC15 [1-429]}, \textit{GFP-CDC15 [267–429]}, \textit{GFP-CDC15 [1-376]}, \textit{GFP-CDC15 [267–376]}, \textit{GFP-CDC15-3HA [Δ362-464]} and \textit{GFP-CDC15-3HA [Δ465-550]}) also caused a late anaphase/telophase arrest when overexpressed (Figure 5B, data not shown). In contrast to class 1 truncations, however, cell cycle arrest (or delay in the case of \textit{GFP-CDC15 [267-376]}) was also observed when the kinase domain was present in the truncation (Figure 5B, Table 1, data not shown). Class 2 truncations that contained the N-terminal kinase domain possessed kinase
activity as judged by their ability to phosphorylate Myelin Basic Protein (MBP) \textit{in vitro} (Figure 6B, data not shown). Thus, loss of kinase activity is not likely to be responsible for the dominant negative phenotype caused by overexpressing these truncations. Perhaps, class 2 truncations retain the ability to bind one or more binding partners and therefore compete out the endogenously expressed Cdc15-3HA. However, they seem to lose the ability to interact with other binding partners important for mitotic exit as the presence of the kinase domain is not sufficient to alleviate the telophase arrest.

Class 3 truncations, in contrast to class I and II truncations, did not interfere with cell cycle progression. This class included \textit{GFP-CDC15 [1-273]}, \textit{GFP-CDC15 [361-974]}, \textit{GFP-CDC15 [361-702]}, \textit{GFP-CDC15 [395-740]}, \textit{GFP-CDC15 [395-702]}, \textit{GFP-CDC15 [551-974]} and \textit{GFP-CDC15 [751-974]} (Figure 5B, Table 1). We have found two modes of dominant negative inhibition by overexpression of Cdc15 truncated proteins: one that results simply from a lack of the kinase domain (class I), and a second which is independent of the presence or absence of the kinase domain (class II). The class II arrest seems to result from the loss of aa 430-550, since \textit{GFP-CDC15[1-550]} does not cause a telophase arrest but \textit{GFP-CDC15[1-429]} does cause a telophase arrest (Figure 5B, Table 1).
Figure 5. **Dominant negative phenotypes of GFP-CDC15 truncation constructs**

(A) Strains were arrested for 3 hours in G1 with the alpha factor pheromone (5μg/ml) and induced with 2% galactose for the last hour. Cells were synchronously released into YEP + 2% raffinose, 2% galactose, samples were taken at indicated time points. Dashed lines represent metaphase spindles, solid lines represent anaphase/telophase spindles (blue = wild-type (A3932), green = GFP-CDC15 [1-974, full length] (A4441), red = GFP-CDC15 [267-974] (A4376)). (B) The percent of anaphase and telophase spindles are analyzed in a synchronous release from G1 arrest (performed as described above in 5A). The first panel depicts Class I CDC15 constructs. (blue diamonds=wild-type (A3932), yellow squares=GFP-CDC15 [1-750] (A5574), purple asterisks=GFP-CDC15 [267-750] (A4695), red closed circles=GFP-CDC15 [1-702] (A5576), black open circles=GFP-CDC15 [267-702] (A5051), green closed triangles=GFP-CDC15 [1-550] (A4691), blue open triangles=GFP-CDC15 [267-550] (A4687). Class II CDC15 constructs are shown in the second panel. (blue diamonds=wild-type (A3932), pink diamonds=GFP-CDC15 [1-429] (A5580), red squares=GFP-CDC15 [267-429] (A4680), green circles=GFP-CDC15 [1-376] (A4677), light blue triangles=GFP-CDC15 [267-376] (A6373).) The third panel depicts Class III CDC15 constructs. (blue=wild-type (A3932), pale blue open circles=GFP-CDC15 [361-974] (A5464), black squares=GFP-CDC15 [361-702] (A5909), purple closed triangles=GFP-CDC15 [395-974] (A5466), green open triangles=GFP-CDC15 [395-702] (A5914), red closed circles=GFP-CDC15 [751-974] (A6456).) Orange open circles=GFP-CDC15 [1-273] (A4690).
An inhibitory domain within the C-terminus of CDC15.

Two truncations caused defects in progression through early stages of the cell cycle when overexpressed. Overexpression of CDC15 lacking the C-terminal 224 amino acids (GFP-CDC15 [1 – 750]) led to defects in mitotic entry as judged by the formation of metaphase and anaphase spindles in these cells (Figure 5B, 6A). Truncation GFP-CDC15 [1 – 702] also impaired progression through mitosis when overexpressed, albeit to a lesser extent (Figure 5B). This finding raised the possibility that the MEN was hyperactive in these cells. Since MEN antagonizes CDK activity, cells in which MEN is overactive have delayed entry into and progression through mitosis (reviewed in Bardin and Amon, 2001). To test this idea we first determined whether GFP-Cdc15 [1-750] was more active as a kinase than full length GFP-Cdc15. Cdc15 kinase activity does not fluctuate during the cell cycle as judged by in vitro kinase assays (Jaspersen and Morgan, 2000). However, since expression of some constructs causes arrest in telophase, cells were first arrested in metaphase using nocodazole to ensure that cells were in the same cell cycle stage when kinase activity was assayed. The specific activity of GFP-Cdc15 [1-750] was 2.6 fold higher than that of full length GFP-Cdc15 (Figure 6B) suggesting that the protein was indeed hyperactive as a protein kinase. We also noted that overexpressed GFP-Cdc15 [1-750] caused hyperphosphorylation of endogenous full length Cdc15 (Figure 3A). Whether this phosphorylation affects Cdc15 kinase activity or other aspects of Cdc15’s function is, at present, unclear.

To determine whether overexpression of GFP-CDC15 [1 – 750] caused hyperactivation of MEN, we compared Dbf2-associated kinase activity in GFP-CDC15 [1 – 750] expressing cells with that of cells overexpressing full-length CDC15 (GFP-CDC15[1-974]). Cultures of
wild-type, *GFP-CDC15 [1-974]*, *GFP-CDC15 [1-750]* and *GFP-CDC15 [267-750]* (lacking the kinase domain) were released from a pheromone-induced G1 arrest in the presence of galactose to induce production of the various *CDC15* constructs. In wild-type cells, Dbf2-associated kinase activity was, as previously reported (Toyn and Johnston, 1994), low during G1 and S phase and early mitosis but high during anaphase and exit from mitosis (105 – 120 minutes after release; Figure 7A). Overexpression of full-length *CDC15* did not affect Dbf2-associated kinase activity in vitro (Figure 7A) indicating that overexpression of *CDC15* does not further activate Dbf2 kinase. In contrast, overexpression of *GFP-CDC15 [1-750]* caused a dramatic increase in Dbf2-associated kinase activity. Dbf2 was activated as early as 45 minutes after release from the pheromone block and stayed high throughout the remainder of the experiment (Figure 7A). The increase in Dbf2 kinase activity was not due to higher expression of *GFP-CDC15 [1-750]* as the amount of GFP-Cdc15 [1-750] protein produced upon overexpression was actually lower than that of full-length Cdc15 (Figure 1B, 6B). Furthermore, the increase in Dbf2 kinase activity was dependent on the presence of the kinase domain of *GFP-CDC15 [1-750]*. Overexpression of *GAL-GFP-CDC15 [267-750]* lacking the kinase domain in fact prevented activation of Dbf2, which is consistent with the observation that this construct causes a dominant negative telophase arrest (Figure 5B). These data show that *GFP-CDC15 [1-750]* is, when overexpressed, capable of causing hyperactivation of Dbf2 associated kinase activity.
Figure 6: GFP-Cdc [1-750] Produces a Delay in Mitosis and has Higher Specific Activity Relative to Wild-type

**A**

- Graph showing percent metaphase spindles over time (min) for different constructs of GFP-Cdc15.

**B**

- Western blot analysis of GFP-Cdc15 constructs in vitro kinase activity towards MBP.

**Figure 6**: Expression of CDC15 lacking the C-terminus causes a delay in entry into mitosis and this truncated form of Cdc15 [1-750] has a higher specific activity than Cdc15 [1-974, full length]. (A.) Strains were arrested for 3 hours in G1 with the alpha factor pheromone (5μg/ml) and induced with 2% galactose for the last hour. Cells were then synchronously released into YEP + 2% raffinose, 2% galactose and samples were taken at indicated time points. The percent of metaphase spindles were counted as a marker for entry into mitosis. (circles=wild-type (A3932), squares= GFP-CDC15 [1-974] (A4441), triangles=GFP-CDC15 [1-750] (A5574), and diamonds=GFP-CDC15 [267-750] (A4695)). (B.) Cultures were prepared as described in Figure 4. GFP-Cdc15 constructs were immunoprecipitated and associated kinase activity was measured using MBP as an artificial substrate. The following strains were used: A3932 (wild-type), A4683 (GAL-GFP-CDC15 [1-974, full length]), A5575 (GAL-GFP-CDC15 [1-750]), A4692 (GAL-GFP-CDC15 [1-550]), A4678 (GAL-GFP-CDC15 [1-376]), A4690 (GAL-GFP-CDC15 [1-273]), A4686 (GAL-GFP-CDC15 [267-974]). The upper panel shows Western blot analysis of the amount of GFP-Cdc15 construct present in immunoprecipitate kinase assay (top portion of kinase gel). The second panel is an autoradiogram of 32P labeled MBP from in vitro kinase assays on the indicated immunoprecipitated GFP-Cdc15 construct (lower portion of kinase gel). The third panel is the auto-phosphorylation of immunoprecipitated GFP-Cdc15 construct (top portion of kinase gel). The amount of 32P is quantitated relative to amount of GFP-Cdc15 construct present in the kinase assay.
**Figure 7:** GFP-Cdc15 [1-750] can hyperactivate Dbf2 and Ectopically release Cdc14 from the nucleolus

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**B**

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**Figure 7.** Removal of a C-terminal domain of Cdc15 results in hyperactivation activation of Dbf2 kinase activity and ectopic release of Cdc14 from the nucleolus. (A.) Strains were arrested for 3 hours in G1 with the alpha factor pheromone (5μg/ml) and induced with 2% galactose for the last hour. Cells were synchronously released into YEP + 2% raffinose, 2% galactose, samples were taken at indicated time points. Dbf2 associated kinase activity toward Histone H1 was measured. Dbf2 and Cdc28 (loading control) protein levels are shown. The following strains were used: A6131 (wild-type, *DBF2-3MYC*); A6130 (*DBF2-3MYC, GAL-GFP-CDC15 [1-750] ); A5568 (*DBF2-3MYC, GFP-CDC15 [1-974, full length] ); A5571 (*DBF2-3MYC, GAL-GFP-CDC15 [267-750] ). (B.) Strains were grown to mid-log phase in YEP + 2% raffinose, arrested in metaphase with 15μg/ml nocodazole for 1.5 hours. An additional 7.5μg/ml nocodazole was added to maintain the metaphase arrest, and GFP-Cdc15 constructs were induced with 2% galactose. Samples were taken at indicated time points. The percent of cells having Cdc14 released from the nucleolus was quantitated. (C.) Cdc14 staining and merge with DAPI nuclear stain. Images are from 90' timepoint of timecourse in 7B. The following strains were used: A1411 (wild-type, *CDC14-3HA*), A5965 (*CDC14-3HA, GAL-GFP-CDC15 [1-974 full length] ), A5966 (*CDC14-3HA, GAL-GFP-CDC15 [1-750] ), A5968 (*CDC14-3HA, GAL-GFP-CDC15 [267-750] ).
Cdc14 is released prematurely from the nucleolus in cells overexpressing GFP-CDC15 [1-750].

Activation of the MEN ultimately triggers Cdc14 release from its inhibitor Cfi1/Net1 in the nucleolus. We therefore tested whether overexpression of GFP-CDC15 [1-750] promoted release of Cdc14 from the nucleolus at times when Cdc14 is normally sequestered. Wild-type cells, cells overexpressing full-length GFP-CDC15, GFP-CDC15 [1-750] and GFP-CDC15 [267–750] lacking the kinase domain were arrested in metaphase using the microtubule depolymerizing drug, nocodazole (Figure 7B, 7C). Overexpression of GFP-CDC15 [1-750] in the arrest caused release of Cdc14 from the nucleolus, in a manner dependent on its kinase activity, while overexpression of GFP-CDC15 [full length] did not (Figure 7B, 7C). Our results show that removal of the C-terminal 224 amino acids leads to CDC15 being hyperactive when overexpressed, suggesting that the C-terminus of Cdc15 functions in the inhibition of MEN signaling.

We also examined whether GFP-CDC15 [1-750] expressed from the endogenous promoter caused hyperactivation of MEN. When expressed at endogenous levels GFP-CDC15 [1-750] failed to complement the temperature sensitive lethality of a cdc15-2 mutation (data not shown). However, GFP-CDC15 [1-750] expressed from the GAL1 promoter not only suppressed the temperature sensitive lethality of a cdc15-2 mutants but also supported cell division as the sole source of CDC15 in the cell (data not shown). Several reasons could account for the phenotypic difference between the overexpressed GFP-CDC15 [1-750] and endogenously expressed CDC15[1-750]. The truncation GFP-Cdc15 [1-750] appears to be less stable than full length GFP-Cdc15 as judged by the ability of the protein to accumulate in cells when produced
from the *GAL1* promoter (Figure 1B, 5B). A less stable truncated protein could account for the failure of the Cdc15 [1-750] expressed at endogenous levels to sustain cell division.

Alternatively, it is possible that the reduction in SPB localization and the formation of aggregates observed when the protein is overexpressed (Table 1) are responsible for the loss-of-function phenotype observed when this truncation is expressed at endogenous levels.

**Discussion**

Cdc15 is similar to members of the Ste20/PAK family of protein kinases which are regulated by modular domains present in their non-catalytic region (reviewed in Dan et al., 2001). In this study, we defined various functional domains within Cdc15 and determined their role in exit from mitosis. We show that a region of Cdc15 adjacent to the kinase domain promotes SPB binding and self-association. Our deletion analysis also identified a region within the C-terminus of Cdc15 which when deleted causes an increase in specific activity of Cdc15, hyperactivation of the MEN protein kinase Dbf2 and premature release of Cdc14 from the nucleolus, at least when the protein is overexpressed. Our findings suggest the presence of a MEN inhibitory function in Cdc15's C-terminus.

When expressed at endogenous levels Cdc15 is difficult to detect within the cell by indirect *in situ* immunofluorescence (Cenamor et al., 1999; Xu et al., 2000; Menssen et al., 2001; Visintin and Amon, 2001). To facilitate Cdc15's detection and construction of N-terminal truncations we expressed *GFP-CDC15* fusions from the *GAL1* promoter. Overexpression of *CDC15* has previously been employed to facilitate detection (Cenamor et al., 1999; Xu et al.,...
2000; Menssen et al., 2001). We carefully compared the localization of endogenous Cdc15 with that of overexpressed Cdc15 and noted two differences in localization pattern. In contrast to endogenous Cdc15, GFP-Cdc15 expressed from the GAL1 promoter was also detected in the cytoplasm, and associated with SPBs in the absence of Tem1. These differences are likely to be due to overexpression of CDC15 and/or limits of detection of endogenous Cdc15. However, importantly, the cell cycle regulated association of Cdc15 with SPBs and the bud neck was not affected when CDC15 was overexpressed indicating that at least some aspects of Cdc15 regulation are preserved under conditions of overexpression.

**SPB localization of Cdc15 is mediated through a large region of Cdc15 adjacent to the kinase domain.**

GFP-Cdc15 [267-750] staining on SPBs was even brighter than that of full length Cdc15 or GFP-Cdc15 [267-974]. GFP-Cdc15 [267-702] also localized to SPBs but to a lesser extent than GFP-Cdc15 [267-750], although brighter than wild-type or GFP-Cdc15 [267-974]. GFP-Cdc15 [267 – 550] associated with SPBs but was also found in a punctate pattern along the mitotic spindle. Further deletion, either N-terminally or C-terminally caused complete loss of SPB association. Thus, while GFP-Cdc15 [267 – 550] retains some ability to associate with SPBs, only region 267-702 is sufficient for wild-type levels of SPB association. A previous study showed that removal of the C-terminal 135 amino acids caused loss of SPB localization of overexpressed Cdc15 – GFP (Menssen et al., 2001). Our result that GFP-Cdc15 [267-702] localizes to the SPB demonstrates that a domain sufficient for localization is present in this

Previous work has shown, that Cdc15-3HA expressed from the endogenous promoter does not localize to SPBs in teml-3 cells arrested in telophase at 37°C (Visintin and Amon, 2001). While these data show that TEM1 is required for the association of Cdc15 with SPBs, several lines of evidence indicate that Tem1 is not the only anchor for Cdc15 at SPBs. First, Tem1 primarily localizes to the SPB entering the daughter cell while Cdc15 is localized symmetrically to both SPBs (Bardin et al., 2000; Pereira et al., 2000; Xu et al., 2000; Visintin and Amon, 2001). Second, overproduced GFP-Cdc15 [1-974, full length] localizes to SPBs in temlΔ cells (Cenamor et al., 1999, this study). Finally, aa 276 – 362 within Cdc15, which are sufficient for Tem1 interaction (Asakawa et al., 2001) are not sufficient for SPB association (Table 1). We speculate that TEM1 is required to induce binding of Cdc15 to the SPB and that this induction may require an initial binding of Tem1 to Cdc15.

Cdc15 self-associates.

Studies in recent years have shown that homo-dimerization plays an important role in the regulation of many protein kinases (Schlessinger, 2000; Dan et al., 2001; Parrini et al., 2002). We have found that Cdc15 also self-associates, mediated by aa 360-702. Removal of either regions aa 362-464 or aa 465-550 severely reduces the ability of the mutant protein to associate with full length Cdc15 but did not affect the ability of the protein to interact with Tem1. Furthermore, overexpression of CDC15 lacking either region caused a dominant negative cell
cycle arrest in late anaphase/telophase, excluding the possibility that the proteins are grossly misfolded (data not shown).

It is not yet known whether self-association of Cdc15 itself is essential for CDC15 function or whether deletion of this region causes loss of function by interfering with other aspects of Cdc15. GFP-Cdc15 [Δ362-464] and GFP-Cdc15 [Δ465-550] also led to loss of SPB association (A. B., unpublished observations) raising the possibility that it is the inability of these constructs to bind to SPBs that causes this loss in CDC15 function. However, it is possible that self-association and SPB localization are interdependent. Point mutants that disrupt self-association specifically will be necessary to determine this. However, the fact that Cdc15 multimers possess kinase activity at least indicates that multimerization is not inhibitory. The exact number of molecules involved in self-association also remains to be determined. However, the fact that the kinase activity associated with Cdc15-3HA in cells overexpressing GFP-CDC15 is increased by 2.0 fold may suggest that Cdc15 forms dimers.

Overexpression of CDC15 lacking the kinase domain cause cell cycle arrest in telophase.

The overexpression of a large number of CDC15 truncations caused arrest in telophase despite endogenous full length CDC15 being expressed. Further examination revealed two classes of truncations that caused such a phenotype. We believe that the telophase arrest caused by high expression levels of class 1 truncations is due to the ability of these truncations to sequester proteins important for mitotic exit into a complex which lacks kinase activity and is thus incapable of promoting mitotic exit. Consistent with this idea, is the fact that all class 1 truncations no longer cause a telophase arrest when the kinase domain is present. In fact, these
constructs with the kinase domain, when overexpressed, support cell viability even in the absence of full length \textit{CDC15} (data not shown). Tem1, whose interaction with Cdc15 is mediated through aa 276 – 362 (Asakawa et al., 2001) could be one of the proteins sequestered by class 1 truncations. Indeed, the class 1 group of truncations retains the ability to bind Tem1 as judged by co-immunoprecipitation (A. B., unpublished observations). It is also possible that SPB-anchoring proteins or other components of MEN, such as Dbf2 or Mob1, are rendered inactive by these \textit{CDC15} truncations.

In contrast to class 1 truncations, class 2 truncations caused telophase arrest irrespective of whether the kinase domain was present. \textit{GFP-CDC15 [1-550]} does not disrupt progression through mitosis, whereas \textit{GFP-CDC15 [1-429]} causes cell cycle arrest in telophase. It seems, therefore, that amino acids 430 – 550 cause the difference in phenotype. It is possible that aa 430 – 550 are important for Cdc15 kinase activity \textit{in vivo} although it is not required for Cdc15 kinase activity \textit{in vitro}. GFP-CDC15 [1-376] does have a reduced specific \textit{in vitro} kinase activity compared to full length GFP-Cdc15 (Figure 6B). We favor the idea that class 2 truncations have kinase activity but are incapable of interacting with a subset of proteins, whose association with Cdc15 is important for exit from mitosis and depends on aa 430-550. It seems that these truncations can, however, bind and titrate other important MEN components from the endogenous Cdc15 resulting in a dominant negative telophase arrest. Class 2 truncations interact with Tem1 (Figure 3C, A. B., unpublished observations) but the truncations are no longer able to bind to SPBs or self-associate which could prevent MEN activation. Alternatively, Dbf2 – Mob1 binding to Cdc15 could be affected. We have not been able to test whether Dbf2 or Mob1 bind to aa 430 – 550 as we were unable to reliably immunoprecipitate Dbf2 with Cdc15.
Figure 8: Model for domains on Cdc15

The N-terminal kinase domain (1-273aa) is shown in purple. The SPB minimal localization domain is in red (267-702aa). A domain necessary and sufficient to facilitate self-association is shown in yellow (361-702), and the putative inhibitory C-terminus is in green (751-974aa).
An inhibitory domain in the C-terminus of Cdc15.

Removal of 224 amino acids from the C-terminus of GFP-CDC15 expressed from the GAL1 promoter results in hyperactivation of MEN as judged by the following four criteria: (1) GFP-Cdc15 [1-750] was found to have a higher specific activity towards MBP than full length Cdc15; (2) Dbf2 kinase activity was dramatically increased in cells overexpressing GFP-CDC15 [1-750]; (3) GFP-CDC15 [1-750] causes release of Cdc14 from the nucleolus in nocodazole-arrested cells; (4) Overexpression of GFP-CDC15 [1-750] led to a delay in entry into mitosis, which is a characteristic of cells with overactive Cdc14 (Visintin et al., 1999).

If the C-terminus of Cdc15 indeed harbors an inhibitory region we might expect that overexpression of this region causes arrest in telophase. However, expression of the C-terminus of CDC15 (GFP-CDC15 [751-974]) did not result in a detectable phenotype. It is possible that GFP interferes with the inhibitory effect or that the protein is not folded properly. Alternatively, inhibition may be intra-molecular in nature. The observation that CDC15 [1–750] expressed from its endogenous promoter was non-functional rather than hyperactive was also surprising. We believe that this is due to the protein being produced at lower levels or being more unstable than full length Cdc15, as protein levels of GFP-Cdc15 [1-750] are lower than those of GFP-Cdc15 [full length] when overexpressed. Furthermore, GFP-Cdc15 [1-750] exhibited a reduced ability to associate with SPBs and formed aggregates in cells, which may also contribute to the loss-of-function phenotype associated with this truncation when expressed at endogenous levels.
Auto-inhibitory regions of both Raf and PAK kinases function to prevent kinase activation until binding of GTP-bound GTPase occurs (Morrison and Cutler, 1997; Kerkhoff and Rapp, 2001; Parrini et al., 2002). How might the inhibitory region within Cdc15 function? Our data are consistent with the following two models: Region 751 – 974 could make contacts either with a region of the same protein or a second molecule of Cdc15, and cause inhibition of the kinase activity, similar to that seen in Raf and PAK. Alternatively, an inhibitor of MEN signaling could bind to this region of Cdc15 and negatively regulate Cdc15 kinase activity or interaction of Cdc15 with other proteins.

**Multiple mechanisms control Cdc15.**

Our data and that of others lend support to the idea that MEN signaling is regulated in part through Cdc15 (Jaspersen and Morgan, 2000; Xu et al., 2000; Menssen et al., 2001; Stegmeier et al., 2002). Dephosphorylation stimulates kinase activity and is brought about by Cdc14 during anaphase (Jaspersen and Morgan, 2000). Self-association is not a consequence of dephosphorylation of CDK consensus sites in Cdc15, as mutating the phosphorylation sites (Cdc15-7A (Jaspersen and Morgan, 2000)) in Cdc15 to amino acids that can no longer be phosphorylated does not affect self-association of Cdc15 (A. B., unpublished observations). Self-association may, thus, be another mechanism involved in regulating Cdc15. Our data also identified a region within Cdc15 that in some way functions in an inhibitory manner. Identifying how this region is involved in regulating MEN activity and whether it is itself a target of regulation will be of great interest. Finally, we note that our analysis of just one component of MEN has revealed two additional modes of controlling MEN activity. It, thus, appears that a
great amount of complexity is built into MEN signaling, emphasizing the importance of regulating exit from mitosis.

**Materials and Methods**

**Growth conditions and yeast strains**

All strains are derivatives of strain W303 (A2587). To construct *CDC15* truncation strains, the starting diploid strain A4267 (W303 diploid, *TEM1-3MYC*) was used. The *GAL1* promoter and the DNA sequence encoding GFP was inserted either upstream of, or at positions internal to one genomic copy of *CDC15* using a PCR based method (Longtine et al., 1998). C-terminal truncations were then created using the diploid strains with *GAL1* driven *CDC15* constructs and inserting a stop codon at positions internal to *CDC15*. To obtain haploid derivatives used in synchronous alpha factor release experiments, spores from diploid strains were sonicated and mated to A3932 (*cdc15::HIS3, leu2:: CDC15-HA3-LEU2*, SLJ511 a gift of D. Morgan), appropriate diploids selected, and sporulated to give strains with the *GAL1 CDC15* constructs at the *CDC15* locus and *CDC15-3HA* at the *LEU2* locus. Further details of strains and strain construction can be obtained upon request. Growth conditions for individual experiments are described in figure legends. Where growth media is unspecified, cells were grown in YEP + 2% glucose.

Strains containing *CDC15* with internal deletions were constructed by inserting plasmids containing the deletion construct into the *LEU2* locus. Plasmids lacking amino acids 362-464 (pCdc15 [Δ 362-464] -3HA) or amino acids 465-550 (pCdc15 [Δ 465-550] -3HA) were constructed by standard cloning techniques using pRS305-CDC15-3HA plasmid (a gift of D. Morgan). This plasmid contains *CDC15* expressed from the endogenous promoter C-terminally
tagged with the 3HA epitope. GAL1 driven versions of these constructs were created by inserted a PCR derived GAL1-GFP upstream of start codon of the integrated plasmids (Longtine et al., 1998).

**Immunofluorescence and fluorescence imaging**

Cells expressing GFP fusion proteins were fixed for 10 minutes in 3.7% formaldehyde, 0.1M potassium phosphate, pH 6.4, followed by 1 minute fixation in 70% EtOH. The cell pellet was resuspended in 1ng/ml DAPI in 0.1M potassium phosphate, pH 7.5. Cells were stored at 4°C until analysis. Fixation and immunofluorescence conditions to analyse mitotic spindles were performed as previously described (Visintin et al., 1999).

**Co-immunoprecipitation techniques, immunoblot analysis, Dbf2 kinase assays, and Cdc15 kinase assays**

For co-immunoprecipitation, 50 ml of mid-log phase cells were harvested and washed with 10mM Tris, pH 7.5. Pellets were resuspended in 200μl of NP40 lysis buffer (150mM NaCl, 50mM Tris pH 7.5, 1% NP40, 1mM DTT, 1mM Pefaflock (Roche), 10μg/ml TLCK (Sigma), 10μg/ml TPCK (Sigma), 10μg/ml Pepstatin A (Sigma), 10μg/ml Leupeptin (Sigma), 40μg/ml Aprotinin (Sigma)). Glass beads (Sigma) were added and samples were vortexed for 10 minutes on a VibraMix vortexer. Extracts were then centrifuged at 13,000 rpm 3X 8’. 1mg of pre-cleared extract in 200μl NP40 lysis buffer was used for immunoprecipitation. 4μl of antibody were added and incubated for 1 hour, 4°C (either mouse monoclonal anti-GFP (Chemicon), mouse monoclonal anti-HA.11 (Covance), or mouse monoclonal anti-MYC 9E10 (Covance) were used). 25μl of protein G sepharose beads (Pierce) (for GFP, and HA immunoprecipitations) or
25ml of protein A sepharose (Amersham) (for MYC immunoprecipitations) and incubated at 4°C, for 2 hours. Supernatant was removed and beads were then washed 5X with NP40 lysis buffer. Sample buffer was added, samples were boiled, run on SDS-PAGE gels followed by transfer to nitrocellulose membrane (VWR).

Western blot analysis was conducted using the following antibodies and dilutions in PBST: 1° 1/1000 polyclonal rabbit anti-GFP (AbCam), 1° 1/1000 polyclonal rabbit anti-MYC (Gramsch), 1° 1/1000 mouse monoclonal anti-HA.11 (Covance), 1° 1/1000 rabbit polyclonal anti-Clb2, and 1° 1/1000 polyclonal anti-Cdc28, 2° 1/2000 HRP conjugated anti-rabbit Ig (Amersham), and 2° 1/2000 HRP conjugated anti-mouse Ig (Amersham). Membranes were blocked in PBST + 5% milk for 1 hour, then incubated with primary antibodies diluted in PBST + 2% BSA and incubated overnight at 4°C. Washes in PBST were followed by incubation with secondary antibodies diluted in PBST + 1% BSA, 1% milk for 5 hours at room temperature. Supersignal West Pico Chemiluminescence substrate from Pierce was used to detect HRP.

Immunoprecipitates for kinase assays were obtained as described above except 60mM β-glycerophosphate, 0.1mM NaVO₃, and 15mM para-nitro-phenylphosphate were also added to NP40 lysis buffer and protease inhibitors. Cdc15 kinase assays were performed as described in (Jaspersen and Morgan, 2000; Asakawa, et al., 2001). Dbf2 kinase assays were performed as described in (Visintin and Amon, 2001).
Literature Cited


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Chapter IV: Discussion and Future Directions
Conclusions

Great progress over the past ten years has been made towards understanding how exit from mitosis is achieved in budding yeast. Temperature sensitive mutants known to arrest in late stages of the cell cycle have existed since Lee Hartwell’s seminal screen to identify cell division cycle mutants (“cdc” genes) in 1970 however, an understanding of their role in the cell cycle was not elucidated until the 1990s (Hartwell et al., 1970). The finding that high levels of mitotic CDK activity in both Xenopus and budding yeast caused a cell cycle arrest in telophase was the first indication that inactivation of mitotic CDK activity was a critical aspect of exiting from mitosis (Holloway, 1993; Surana et al., 1993). Importantly, Surana et al. determined that a temperature sensitive allele of CDC15 arrested in telophase with high levels of mitotic CDK activity (Surana et al., 1993). This finding was the first to link the defect of mutants that could not exit mitosis with the inability to inactivation mitotic CDK activity.

Only in the late 1990s were Tem1, Cdc15, Dbf2, Cdc5 and Cdc14 found to act together in a regulatory network (MEN; mitotic exit network) governing the inactivation of mitotic CDKs (Jaspersen et al., 1998). Work in our lab by Rosella Visintin demonstrated that Cdc14 played a key role in allowing mitotic CDK inactivation (Visintin et al., 1998). Cdc14 was shown to act on three targets Cdh1/Hct1, Sic1, and Swi5, which cause both degradation of the cyclin Clb2, and stabilization of the mitotic CDK inhibitor, Sic1 (Jaspersen et al., 1999; Visintin et al., 1998). Cdc14 was the only MEN component found to be both necessary and sufficient for CDK inactivation suggesting that it was the most downstream component.
To understand how mitotic exit was accomplished, much work in the field focused on the key question of how Cdc14 was regulated. An important Cdc14 inhibitory factor, Cfi1/Net1, was simultaneously found in four different studies: as a two-hybrid interactor with Cdc14 (Visintin et al., 1999); a two-hybrid interactor with the silencing factor, Sir2 (Straight et al., 1999); a suppressor of tem1Δ lethality (Shou et al., 1999); and as a high copy suppressor of the lethality of overexpression of CDC14 (de Almeida et al., 1999). Cfi1/Net1 was found to be a nucleolar protein tethering Cdc14 in the nucleolus throughout early stages of the cell cycle (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). The action of MEN allows sustained Cdc14 release and activity, thereby promoting exit from mitosis (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Regulation of mitotic exit was not well understood, although the concerted activity of Tem1, Cdc15, Dbf2, and Cdc5 was known to be essential for the sustained release of Cdc14. What signals allow the correct timing of activation of this network? What are the ways in which these proteins are regulated and how do they allow exit from mitosis? To begin to address these questions, I have investigated the regulation of two upstream components of the mitotic exit network: Tem1 and Cdc15. The work presented here elucidates mechanisms by which activation of the mitotic exit network (MEN) and propagation of the MEN signal occur.

MEN consists of a GTPase, Tem1; a two-component GAP (GTPase activating protein) Bub2-Bfa1; a GEF (guanine nucleotide exchange factor), Lte1; the protein kinases Cdc15, Dbf2, Cdc5; a Dbf2-associated factor, Mob1; and the SPB anchor protein, Nud1. The functional order of components of this pathway has been modeled by the work of many groups using genetic, cell biological, and biochemical means as well as homology to an analogous pathway in S. pombe (Shirayama et al., 1994; Bardin et al., 2000; Furge et al., 1998; Jaspersen et al., 1998; Jwa
Song, 1998; Schmidt et al., 1997; Sohrmann et al., 1998; Sparks et al., 1999; Visintin et al., 1999) (Guertin et al., 2000; Hou et al., 2000; Pereira et al., 2000; Salimova et al., 2000) (Asakawa et al., 2001; Lee et al., 2001a; Visintin and Amon, 2001). It is thought that Tem1 GTPase activity is modulated by the GEF Lte1 and the two-component GAP, Bub2-Bfa1. Tem1 binds to and allows activation of Cdc15, which in turn phosphorylates and activates Dbf2 in a Mob1 dependent manner. One of the most important and unanswered questions in the field is how Dbf2 kinase activity promotes the release of Cdc14. The function of Cdc5 is also poorly understood and thought to be required at several steps in the pathway.

**Understanding Tem1 Regulation**

In an attempt to understand how this genetic network is controlled, I investigated the regulation of the GTPase Tem1 by the two-component GAP (GTPase activating protein) Bub2-Bfa1 and the GEF (guanine nucleotide exchange factor) Lte1. I determined that one mechanism that prevents early activation of Tem1 is the spatial segregation of Tem1 from its exchange factor until anaphase. In late S phase, concomitant with mitotic spindle formation, Tem1 localizes to the spindle pole body (SPB) that will enter the daughter cell and remains SPB associated until telophase. Lte1 is diffuse throughout the cell in G1, but is sequestered into the growing daughter bud during S phase. These observations lead to the following model for activation of the mitotic exit pathway. The spatial segregation of Tem1 and Lte1 in early stages of the cell cycle prevent Lte1 driven activation of Tem1. However, during anaphase, the two proteins are forced into the same compartment when the mitotic spindle elongates pushing the SPB containing Tem1 into the daughter bud.
The model predicts that the Tem1-containing SPB must enter the daughter bud prior to activation of the mitotic exit pathway. We tested this using two mutant strains: one that has difficulty positioning its mitotic spindle along the mother-bud axis (dyn1Δ), and one in which the entire nucleus either moves into the daughter cell, or remains in the mother cell (esp1-1). Cultures of dyn1Δ cells at cold temperatures contain a population of cells that undergo anaphase with the mitotic spindle in the mother cell as well as a population that undergo a normal anaphase with the spindle positioned along the mother-bud axis. Previous studies indicated that cells with mis-positioned spindles do not undergo cytokinesis with the two nuclei in the mother cell, but have a mechanism that prevents cytokinesis until the mitotic spindle can re-position itself along the mother-bud axis (Muhua et al., 1998; Yeh et al., 1995). This phenotype allowed me to investigate the requirements for activation of mitotic exit in these cells. I found that cells with mis-positioned spindles are, in fact, also delayed in the release of Cdc14 from the nucleolus, indicating that mitotic exit, as well as cytokinesis, is delayed. The over-expression of the exchange factor Lte1, which drives a portion of Lte1 into the mother cell, bypasses the mitotic exit delay. This indicates that the sequestration of Lte1 from Tem1 can prevent inappropriate activation of MEN, supporting the spatial segregation model. Deletion of the GAP component, BUB2 also bypasses this mitotic exit delay. Therefore, these studies also revealed a role for the GAP component BUB2 in preventing activation of MEN until the spindle is correctly positioned. The mechanism of how Bub2 activity responds to spindle position is unclear.
To further test this model, *esp1-1* mutant cells were examined. A majority of *esp1-1* mutant cells move the entire nucleus into the daughter cell, whereas a small percent of cells retain the entire nucleus in the mother cell. Our model predicts that cells would not be able to exit mitosis until the SPB containing Tem1 moves into the daughter cell and this should be dependent on the spatial separation of Tem1 and Lte1. We found that in a majority of the cells in which the nucleus remained in the mother cell, mitotic exit does not occur. The overexpression of Lte1 allows a majority of cells with the nucleus in the mother cell to exit from mitosis, thus indicating that spatial separation of Lte1 from Tem1 is one mechanism by which premature mitotic exit is prevented.

While this model would account for Tem1 regulation from S phase through telophase, it raises the question as to why Tem1 is not active in G1 when it is in the same compartment as Lte1. Tem1 protein is present in G1, although not yet loaded onto the SPB, Lte1 is diffuse in the cell, and the downstream kinase Cdc15 is also present although not yet loaded onto the SPB. In an attempt to understand whether Tem1 is active in G1, we examined the ability of Tem1 and Cdc15 to interact by co-immunoprecipitation. Interestingly, we found that although Tem1 and Cdc15 interact in cells which are in mitosis, in G1 arrested cells, Tem1 and Cdc15 do not co-immunoprecipitate. This indicates that the interaction between Tem1 and Cdc15 is somehow inhibited in G1 arrested cells. It is possible that SPB loading is essential for stable association. Therefore, a mechanism exists to prevent Tem1 and Cdc15 interaction in G1 cells and likely helps to prevent pathway activation during G1. It is not known whether the interaction between Tem1 and Cdc15 is regulated by Tem1 GTP status. Although all of the genetic and cell biology
data predict that Tem1 is promotes mitotic exit upon GTP binding, no one has yet conclusively demonstrated when Tem1 becomes GTP bound in vivo and how this influences mitotic exit.

**Investigation of multiple modes of Cdc15 control**

Regulation of Tem1-GTP status seems to be one way in which cells control mitotic exit, several lines of evidence indicate that other layers of MEN regulation must exist. Cells lacking the GAP components *BUB2* and *BFA1* are viable, however, they do exit mitosis inappropriately when arrested in metaphase for prolonged periods of time due to either spindle damage or DNA damage (reviewed in Bardin and Amon, 2001; McColllum and Gould, 2001). Additionally, neither the deletion nor the overexpression of the exchange factor, Lte1, has a dramatic effect on the cell cycle at room temperature. The deletion of *LTE1*, however, causes arrest in telophase at 14° C. Tem1 has been demonstrated to have an unusually high rate of GDP release, that is significantly slowed at 13° C which could account for the non-essential role of *LTE1* at high temperatures, but essential function at 14° C (Geymonat et al., 2002). Additionally, the overexpression of *TEM1* does not constitutively activate MEN, but does allow exit from mitosis to occur when cells are arrested in metaphase for an extended period of time due to spindle damage (Alexandru et al., 1999). These observations suggest that additional control mechanisms function to prevent Cdc14 release from the nucleolus prior to the onset of anaphase.

Cdc15 is thought to be immediately downstream of Tem1 in the MEN signaling cascade. I demonstrated that Cdc15 interacts with Tem1 in a co-immunoprecipitation assay (Bardin et al., 2000). Additional genetic, biochemical, and cell biological data suggest that Cdc15 acts directly downstream of Tem1 and directly upstream of the Dbf2 kinase (Jaspersen et al., 1998; Lee et al.,
2001a; Mah et al., 2001; Shirayama et al., 1994; Visintin and Amon, 2001). In an attempt to understand other regulatory mechanisms controlling MEN, I characterized functional domains within the protein kinase Cdc15. I have determined that, in addition to the N-terminal kinase domain, Cdc15 has at least three other functional domains: a SPB localization domain, a self-association domain, and a C-terminal domain which seems to function in an inhibitory manner.

Cdc15 localization to both SPBs is TEM1 dependent (Visintin and Amon, 2001). Cdc15, however, localizes to both SPB while Tem1 primarily localizes to the SPB migrating into the daughter cell. Furthermore, when overproduced, Cdc15 can localize to the SPB in the absence of TEM1 and bypass the essential function of TEM1 (Cenamor et al., 1999). Together, these data suggests that Tem1 does not simply recruit Cdc15 to the SPB. I have mapped a domain within Cdc15 (aa 267-702) that is both necessary and sufficient for localization to the SPB when overproduced. The ability of Cdc15 to localize to the SPBs independently of Tem1 protein strongly suggests that it can bind to a SPB protein other than Tem1, although the SPB binding partner is unknown at the present time. How recruitment of Cdc15 to both SPBs occurs and under what circumstances this might be regulated are not known.

The ability of numerous kinases to be regulated by dimerization and multimerization prompted me to investigate whether Cdc15 could also self-associate. Through co-immunoprecipitation analysis, I found that Cdc15 can self-associate and that aa 361-702 are necessary and sufficient for this interaction. Further analysis showed that the multimers possess kinase activity, which would argue against an inhibitory role for self-association. Removal of either aa 362-464 or aa 465-550 almost completely abolishes the ability of Cdc15 to self-
associate. Additionally, removal of aa 362-464 or aa 465-550 results in loss of Cdc15 function. Whether this is due to loss of multimerization or loss of SPB localization, also requiring this region, is not known. Dominant negative phenotype analysis of the overexpression of \textit{CDC15} constructs also indicate that aa 360-550 is an essential region.

I have demonstrated that region aa 360-702 participates in SPB localization, self-association, and an essential function. The relationship between these functions is intriguing, yet ill-defined. While loss of the ability to self-associate correlates both with loss of function and loss of localization, only a careful mutagenesis of this region to separate functions will determine if self-association, SPB localization, or another uncharacterized function is the essential function of this region.

A C-terminal domain of Cdc15 (aa751-974) was found to possibly confer an inhibitory function. Removal of this domain from Cdc15 results in hyperactivation of MEN when overproduced as determined by the following four phenotypes: 1. the \textit{in vitro} kinase activity associated with Cdc15 was increased; 2. expression of Cdc15 lacking the C-terminus resulted in defects in progression into mitosis similar to those seen when Cdc14 is constitutively active (Visintin et al., 1999); 3. the kinase activity of Dbf2, which is thought to be immediately downstream of Cdc15, was dramatically increased; 4. Cdc14 was ectopically released from the nucleolus in metaphase arrested cells. The expression of full length Cdc15 did not result in these phenotypes indicating that these phenotypes are specific to Cdc15 lacking the C-terminal 224 aa.
Summary

The work presented in this thesis characterizes two steps at which regulation of the mitotic exit network occurs. Regulation of Tem1 GTP/GDP status is likely to play an important role in linking segregation of genetic material to activation of MEN leading to exit from mitosis. Control of Tem1 GTP/GDP status seems to be important to ensure against inappropriate mitotic exit in the presence of spindle damage, DNA damage, or spindle mis-positioning (Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Pereira et al., 2000; Wang et al., 2000). Co-localization of the GTPase, Tem1, and its exchange factor, Lte1, only during anaphase is one signal leading to activation of MEN. Additional control mechanisms are in place that regulate MEN during the course of a normal cell cycle, one such factor could be Cdc15. While the exact details of the intricacies of Cdc15 regulation remain to be solved, my data indicates that Cdc15 SPB localization, self-association, and a C-terminal inhibitory domain could all be important points of regulation of MEN activity. This work also demonstrates that the over-activation of MEN is sufficient to allow release of Cdc14 from the nucleolus.
Unanswered Questions and Future Directions

The work presented in this thesis describes some of the signals that regulate MEN activity. Nuclear position is one important way that the cell ensures that mitotic exit does not occur until the nuclei have been properly distributed between the mother and daughter cell. Interestingly, at cold temperatures LTE1 is essential indicating perhaps an increased dependence of mitotic exit on spindle position under these conditions. This system may have evolved due to the difference in microtubule dynamics at cold temperatures and the inherent difficulty in positioning the mitotic spindle resulting from this. Inhibition of MEN is also required for blocking inappropriate mitotic exit as BUB2 becomes essential during DNA damage induced arrest, benomyl induced arrest, and in mutants arresting from inactivation of the anaphase promoting complex (Hoyt et al., 1991; Pereira et al., 2000; Wang et al., 2000). My work on Cdc15 indicates that Cdc15 is inhibited by a C-terminal domain, although the mechanism of this inhibition of this and signals leading to relief of inhibition are not known. The relative abundance of Cdc15 protein differs greatly with growth media and it is therefore possible that an auto-inhibitory mechanism to block Cdc15 activation becomes essential under various growth conditions (AB, unpublished data). It is possible that binding of activated Tem1 is sufficient to allow Cdc15 SPB localization and activation. Addressing how components of MEN are regulated in response to cell cycle cues, other cellular events, and environmental conditions will be vital in our understanding of exit from mitosis.
Regulation of Tem1 GTP/GDP status

A fundamental question yet unanswered is whether Tem1 is active in the GTP bound state and if so, when in the cell cycle does this occur. An abundance of evidence suggests that Tem1 is activated upon GTP binding (Shirayama et al., 1994; Alexandru et al., 1999; Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Pereira et al., 2000; Wang et al., 2000; Ro et al., 2002; Stegmeier et al., 2002). Work in S. pombe strongly suggest that the Tem1 homolog is activated upon GTP binding (Cerutti and Simanis, 1999; Li et al., 2000). Thus far, however, direct measurement of Tem1 binding to GTP in vivo has not been shown. I attempted to address this by locating a domain of Cdc15 that could specifically bind to Tem1-GTP and therefore might localize only to the Tem1-bound SPB. I examined the localization of numerous truncated constructs of CDC15 overexpressed from the GAL1 promoter. Instead of localizing to only Tem1-bound SPB, the overexpression of a minimal Tem1 interaction domain resulted in the titration of Tem1 from the SPB (data presented in Appendix B). This could be due to the high levels of overproduced protein. Possibly expression at endogenous levels of the minimal Tem1 interaction domain of CDC15 (aa267-376), might result in localization of Cdc15 only to the Tem1 localized SPB. This would provide an in vivo assay for the status of Tem1-GTP binding. One would have to use an epitope tag with multiple tandem copies of the epitope since Cdc15-3HA is exceedingly difficult to detect in S. cerevisiae, as is Cdc15-13MYC (Visintin and Amon, 2001, AB unpublished observations).
Potential negative regulators and inhibitory signals of Tem1 and MEN

*Benomyl sensitive mutants:*

The microtubule depolymerizing drug benomyl induces a metaphase arrest or delay upon addition to cells due to activation of the spindle checkpoint. Sensitivity to benomyl arises from chromosome mis-segregation due to the inability properly halt cell cycle progression. Two events are known to cause benomyl sensitivity. A lack of inhibition of Cdc20 and the anaphase promoting complex via *MAD1*, *MAD2*, *MAD3*, *BUB1*, *BUB3* can facilitate the metaphase-to-anaphase transition through degradation of Pds1 leading to sister chromatid separation (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). Benomyl sensitivity can also arise if Tem1 is not inhibited by Bub2-Bfa1 leading to inappropriate inactivation of mitotic cyclin dependent kinase activity and entry into G1. Therefore, the loss of negative regulators of mitotic exit would lead to benomyl sensitivity. By examination of the Proteome database for genes that are benomyl sensitive, several interesting candidate regulators of mitotic exit can be found.

The loss of genes previously demonstrated to function in Rho signaling result in sensitivity to benomyl indicating a possible role in either the metaphase-to-anaphase transition or in exit from mitosis. *BEM2*, a GAP for Rho1; *ROM2*, a GEF for Rho1 as well as *BN11* and *BNR1*, all thought to be downstream targets of Rho1 all result in benomyl sensitivity upon loss of function. It is possible that the GAP, Bem2, could have a redundant function with Bub2-Bfa1 or that Rho signaling could function to inhibit Cdc14 release. Alternatively, components of the Rho pathway could result in a general sensitivity to a metaphase arrest due to the weakened cell walls characteristic of Rho mutants. Genetic interactions between these Rho components and MEN
regulators can be tested. It would be interesting to determine whether these components are synthetically lethal with the deletion of the negative regulator of MEN, \textit{BUB2}. The deletion of \textit{BUB2} can bypass the synthetic lethality resulting from the loss of two positive regulators of mitotic exit, \textit{LTE1} and \textit{SPO12}. Determining whether the loss of components of Rho signaling can also bypass this lethality will indicate if they inhibit exit from mitosis. The recent finding that Cdc42 signaling functions upstream of the MEN pathway as well as in an uncharacterized pathway to promote mitotic exit, raises the interesting possibility that Rho signaling could play a similar role under different environmental conditions.

\textit{Bfa1-Bub2 regulation}

Two-hybrid analysis has recently identified a Bfa1-associated protein, Ib2, that seems to negatively regulate MEN and function upstream of or parallel to Bfa1 and Bub2 (Hwang and Song, 2002). Ib2 localizes asymmetrically to one SPB, the deletion is nocodazole sensitive and the overexpression results in mixed phenotype with 67\% arresting in metaphase and 33\% arresting in telophase. The overexpression of \textit{IBD2} does not trigger the spindle checkpoint as this phenotype is independent of \textit{MAD2}. The nocodazole sensitivity of \textit{ibd2Δ} cells is rescued by extra copies of \textit{BFA1}, \textit{BUB2}, and \textit{CDC5}, suggesting that these genes act downstream or parallel to \textit{IBD2}. Investigation of the whether Ib2 is required for Bub2, Bfa1, and Tem1 to localize to the SPB and to co-immunoprecipitate might might help to elucidate the role of Ib2 in regulation of these proteins. An \textit{in vitro} GAP activity assay for Bub2 and Bfa1 on Tem1 has recently been established (Geymonat et al., 2002). Investigation of the Bfa1-Bub2 GAP activity in the presence and absence of recombinant Ib2 might determine how this novel regulator functions in MEN.
Bub2 and Bfa1 are involved in preventing Tem1 activation when the mitotic spindle is mis-positioned and during prolonged metaphase arrests. A control mechanism must therefore exist to inactivate the Bub2-Bfa1 complex in response to correct spindle positioning and progression into anaphase. It has been proposed that the phosphorylation status of Bfa1 is important for its activity and is regulated by Cdc5 and Cdc14 (Hu et al., 2001; Lee et al., 2001b; Pereira et al., 2002). Phosphorylated Bfa1 has a weaker affinity for Tem1 as measured by co-immunoprecipitation from extracts and has therefore been proposed to represent the inactivated form (Hu et al., 2001). A mutant form of Bfa1 that cannot be phosphorylated (Bfa1-11A), however, does not have the predicted telophase arrest phenotype which would be expected of hyperactive Bfa1 (Hu et al., 2001). This could be due to positive regulation of MEN components. However, because Bfa1-11A does not behave in the predicted hyperactive manner, another control mechanism is likely to regulate Bfa1-Bub2 in response to spindle position, spindle length, or sister chromatid separation. It is possible that Ibd2 (discussed above) is responsible for Bfa1-Bub2 regulation, as its function is required for inhibition of MEN in both nocodazole and dyn1Δ cells (Hwang and Song, 2002).

The discovery of unidentified upstream regulators of Bub2-Bfa1 would lead to further understanding of how the timing of MEN activation is regulated. Good candidates to be negative regulators of Bub2 and Bfa1 are genes whose deletion would result in synthetically lethality with mutants that misposition their mitotic spindle (dyn1Δ at 14°C; kar9Δ act5-ts at 37°C). If MEN is overactive in cells with mis-positioned spindles, cell death occurs due to accumulation of multinucleate and anucleate cells. Regulators of Bub2-Bfa1 could also be found through a
screen using a sensitized background. The deletion of two positive regulators of mitotic exit, \(LTE1\), and \(SPO12\) results in synthetic lethality that is rescued by the deletion of \(BUB2\). An \(lte1\Delta, spo12\Delta, bub2\Delta, GAL-BUB2\) strain would, therefore, live on glucose when \(BUB2\) is shut off. The overexpression of a negative regulator of Bub2 could allow growth on galactose as would the deletion of a positive regulator. It is likely that other interesting mitotic exit regulators functioning downstream of TEM1 would be found in both a \(dyn1\Delta\) synthetic lethal screen and the sensitized background.

**Positive Regulators of Mitotic Exit**

The MEN components Lte1, Tem1, Cdc15, Dbf2, Cdc5 and Nud1 positively regulate exit from mitosis. Downstream of MEN, Cdh1-APC, Swi5, and Sic1 also promote exit from mitosis by directly inactivating the Clb-CDKs. Our lab and other labs have recently identified another pathway capable of positively regulating exit from mitosis. The FEAR network (cdc fourteen early anaphase release) promotes the release of Cdc14 from the nucleolus in early anaphase and is independent of MEN. The loss of FEAR results in delayed mitotic exit, whereas loss of MEN leads to arrest in telophase and prevents mitotic exit altogether (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002), review in (Bardin et al., 2000). Exit from mitosis has multiple levels of regulation in response to various cellular stresses and environmental situations and it is possible even more that await discovery.

**Positive Regulators of Lte1:**

A role in promoting mitotic exit has been found for the cell polarity proteins Cdc24, Cdc42, Kel1, Cla4 and Ste20, as well as the septins (Hofken and Schiebel, 2002; Jensen et al.,
2002; Seshan, et al., in press). Cdc24 activates Cdc42 which seems to work in two ways to promote mitotic exit. First, Cdc42 activates Cla4 that phosphorylates Lte1 and leads to bud cortex association dependent on both the septins and Kel1 (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan, et al., in press). Secondly, Cdc42 activates Ste20 that then promotes mitotic exit in an unknown manner (Hofken and Schiebel, 2002). CDC14 promotes the dephosphorylation and delocalization of Lte1 from the bud cortex at the end of mitosis (Jensen et al., 2002; Seshan, et al., in press). Does Lte1 need to localize to the bud to be active? Does Cla4 only allow localization or does it promote activation as well? How does dephosphorylation lead to delocalization? The analysis of alleles of Lte1 that can no longer be regulated by Cla4 phosphorylation, or can no longer bind to the bud cortex will be useful in revealing the exact role of phosphorylation and localization on Lte1 function.

**STE20 and a potential role for the HOG pathway in mitotic exit**

One of the most interesting unanswered questions in the field is the role of Ste20 in promoting mitotic exit. High levels of Ste20 are capable of bypassing the telophase arrest of lte1Δ cells at 14°C, demonstrating that Ste20 can positively regulate mitotic exit. Ste20 is involved in several signal transduction pathways in yeast, including the HOG pathway (high osmolarity and glycerol), the pheremone signaling cascade, and the filamentous growth pathways (Leberer et al., 1997). Several unexplained observations in the field might be a result of Ste20 mediated cross-talk between the MEN cascade and the HOG pathway. Conditions of high osmolarity (1.2M sorbitol), which leads to activation of the HOG pathway, can partially bypass cdc15-2, dbf2-2, and cdc14-1 mutants (Grandin et al., 1998). Additionally, osmotic shock, also capable of activating the HOG pathway, is characterized by a transient accumulation
of cells in G1 and G2 stages of the cell cycle accompanied by transient inhibition of Clb2-CDK activity (Alexander et al., 2001). This inhibition is not due to activation of the morphogenesis checkpoint or Cdc28 phosphorylation, but is dependent on HOG1. The HOG pathway is well defined and one could easily test whether the ability of 1.2M sorbitol to partially bypass the essential function of MEN components requires STE20 as well as SHO1, STE11, PBS2, and HOG1, components of the HOG signaling pathway. Ste20 functions in one branch of the HOG pathway, and it could be determined whether the other branch contributes to mitotic exit by examining the sorbitol effect in sbl1Δ cells. The ability of Ste20 and the HOG pathway to influence mitotic exit in response to osmotic shock would be of great interest. It would also be interesting to investigate whether the pheromone signaling cascade, which also utilizes Ste20 and results in inactivation of CDKs, might regulate MEN or mitotic exit. In order for G1 arrest, it is necessary to inhibit not only the Cln CDKs, but also the Clb CDKs, and perhaps Ste20 facilitates inactivation of the Clb CDKs in the pheromone response.

**Nutrient sensing and mitotic exit**

Interestingly, components of the Ras signaling pathway have numerous genetic interactions with MEN. The GEF, LTE1, was found as a multicopy suppressor of the Ras GAP IRA1. A deletion of all three Ras-like genes in *S. cerevisiae* results in a telophase arrest which can be bypassed by the overexpression of CDC5, CDC15, DBF2, TEM1, and SPO12 (Morishita et al., 1995). However, seemingly contradictorily, a deletion of RAS2, can bypass the *dbh2-2* telophase arrest as well as *cdc27-1* and *cdc23-1* metaphase arrests (Irmiger, 2000). Deletion of RAS2 was shown to cause the mislocalization of Cdc42 (Ho and Bretscher, 2001). *CDC42* is required for Lte1 localization and it is therefore possible that the lack of sequestered Lte1 might
upregulate the MEN and allow exit from mitosis (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan, et al., in press). The dependence of this suppression on \( CDC42 \) and \( LTE1 \) could easily be tested. Additionally, Yak1 (a kinase that opposes PKA function) was found as a multicopy suppressor of temperature sensitive alleles of most MEN components (Jaspersen et al., 1998). A more thorough characterization of the phenotype of a \( ras2\Delta \) strain might yield insight into how the Ras pathway functions in mitotic exit. Determining whether a \( ras2\Delta \) can bypass a \( cdc14-3 \) telophase arrest will determine if it function upstream or downstream of Cdc14 nucleolar release. If the Ras pathway does not function through promotion of Cdc14 nucleolar release, it could impinge on Sic1 or Cdh1, inactivation of mitotic CDKs, or even downstream targets. How exactly cAMP and Ras signaling acts on mitotic exit is not well defined and appears to have both positive and negative functions, however, the large amount of genetic interacts seems to suggest that cross-talk occurs.

**Regulation of Tem1**

**Tem1 interacting proteins:**

A two-hybrid screen performed in our lab (Nick Hausman, unpublished data, Table1) found a large amount of interacting proteins. While many might result from non-specific interaction, several interactors are worthy of further investigation. The Tem1 interactors Spa2 and Boi1 function in cell polarity. Cell polarity proteins such as Cdc42 and Ste20 have recently been found to influence mitotic exit (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et
<table>
<thead>
<tr>
<th>Gene</th>
<th>Times Isolated</th>
<th>Cellular Function</th>
</tr>
</thead>
<tbody>
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<td>CDC15</td>
<td>1</td>
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</tr>
<tr>
<td>NUD1</td>
<td>2</td>
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</tr>
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</tr>
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</tr>
<tr>
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<td>2</td>
<td>homolog to human protein</td>
</tr>
<tr>
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<tr>
<td>ENT1</td>
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al., in press). In light of these findings, further investigation of the two-hybrid interactors with Tem1 involved in cell polarity should be conducted. Spa2 interacts with the cell cortex protein Bni1 (Fujiwara et al., 1998). Boi1 interacts with both Cdc42 and Ste20 and overproduction arrests cells as large, unbudded cell with multiple nuclei.

The two-hybrid screen with Tem1 conducted by Nick Hausman, also identified Nud1, Spc42, Gip2 and Csm1 as interactors. Nud1 is a known anchor of MEN components and Spc42 is an additional SPB component. It is interesting to note that the Tem1 interacting region of Nud1 is the same region of Nud1 mapped to interact with Spc72 (Nick Hausman, unpublished data). It is therefore possible that SPB association is mediated through Spc72. Gip2 is a regulatory subunit of protein phosphatase I. Given that the MEN kinase cascade is regulated through phosphorylation events, Gip2 could play a role in MEN signaling. Csm1 was found in a screen for meiotic chromosome segregation mutants. The deletion of Csm1 causes cells to be benomyl sensitive, suggesting it could function as a negative regulator of MEN, and has impaired nuclear migration and reduced spore viability (Rieger et al., 1997). It also interacts with the FEAR component Spo12 in a large-scale two-hybrid screen (Uetz et al., 2000).

Analysis of protein-protein interaction through mass spectrometry has revealed a potentially interesting protein, Ybr281c, that is found in a complex containing Tem1 and Cdc15, and is the only additional component found in this complex upon addition of MMS and NOC. Ybr281c has 4 WD40 repeats and a metallopeptidase domain and the null mutant is viable. It is possible that Ybr281c regulates Tem1 or Cdc15 function and further investigation of this protein
might reveal a MEN regulatory role. This protein could affect Cdc15 and Tem1 interaction, or could otherwise modulate Cdc15 or Tem1 activity. Investigation of the ability of Tem1 and Cdc15 to co-immunoprecipitate in the absence of this protein will determine if Ybr281c somehow mediates the interaction of Tem1 and Cdc15. Additionally, determination of whether the deletion of YBR281C is synthetically lethal with either the GAP BUB2 or the GEF LTE1, could indicate a negative or positive role in MEN, respectively. The loss of a negative regulator of MEN should also lead to a nocodazole sensitivity similar to that of deletion of BUB2.

**Regulation of Tem1 localization**

Bfa1 and Bub2 associate with Tem1 and seem to anchor it to the SPB entering the daughter cell. In *bfa1Δ* or *bub2Δ* cells, localization of Tem1 to the SPB in S phase and early mitosis is lost, however, Tem1 does localize to both SPBs symmetrically in late mitosis (Pereira et al., 2001; AB unpublished observations). I have investigated the possibility that the *BUB2* and *BFA1* independent localization might be dependent on downstream MEN components. Tem1 can localize independently of *CDC15, DBF2*, and *CDC14* (Bardin et al., 2000), however, it was possible that late anaphase localization is dependent on these genes. I examined Tem1 localization in *bub2Δ, cdc15-1; bfa1Δ, cdc15-2; bub2Δ, dbf2-2; bfa1Δ, dbf2-2; bub2Δ, cdc14-3; bfa1Δ, cdc14-3* cells. I found that in all MEN mutants combined with either *bub2Δ* or *bfa1Δ*, Tem1 did not exhibit late SPB localization (AB, unpublished data). This could reflect either an arrest prior to the time of localization, or a requirement for these proteins to mediate *BUB2* and *BFA1* independent localization.
While investigating the localization of Bfa1, Pereira et al. (Pereira et al., 2001) demonstrated a novel principle of yeast cell biology: in a "normal" cell cycle, the old SPB always segregates into the daughter cell. They found that the use of the microtubule depolymerizing nocodazole can randomize SPB inheritance. Interestingly, Bfa1 always segregates into the daughter cell, irrespective of the age of the SPB (Pereira et al., 2001). Bfa1, Bub2 and Tem1 localization are indistinguishable. How asymmetric localization of these components is achieved and the purpose it serves are important unsolved questions. My attempts to understand this problem are summarized in Appendix A. It seems that cell polarity might, in some way be involved in the regulation of this process, but the exact mechanism remains elusive.

Cdc15 Regulation

Cdc15 localization

Cdc15 localization to SPBs is dependent on TEM1. However, it is clear that another protein must bind Cdc15 to the SPB. A temperature sensitive allele of the SPB component, Nud1, nud1-44, arrests with Tem1, Cdc15, and Dbf2 delocalized (Bardin et al., 2000; Gruneberg et al., 2000; Visintin and Amon, 2001); a nud1-2 allele, however, does not cause delocalization of Cdc15 although Tem1 and Dbf2 are delocalized (Gruneberg et al., 2000). This suggests that Cdc15 likely has different requirements than Tem1 and Dbf2. Understanding how Cdc15 localizes to both SPBs might reveal an additional step by which MEN is regulated. A two-
hybrid screen using either full length Cdc15, or the minimal SPB domain aa 267-702 as a bait, might be a fruitful way to determine the SPB component to which Cdc15 binds.

It is interesting that the SPB localization domain requires a region that interacts with Tem1 (aa 267-376, Asakawa et al., 2001). Endogenous Cdc15 SPB localization is TEM1 dependent, suggesting that Tem1 function is needed to promote Cdc15 SPB loading. However, the ability of overproduced Cdc15 to localize in the absence of Tem1 indicates that Tem1 interaction is not an absolute requirement for SPB localization. The Tem1 interaction domain is still required in the overproduced protein however, suggesting a second important function of this region in SPB localization. Studies of the localization of endogenous Cdc15 and overproduced Cdc15 when mutantions specifically disrupting the Tem1-Cdc15 interaction have been introduced into either Tem1 or Cdc15 would be useful in determining the exact function of this domain and Tem1’s role in Cdc15 SPB loading.

\textit{A function of Cdc15 multimerization:}

In addition to the requirement of the Tem1 interaction region for SPB loading, a region involved in self-association is also essential for localization to SPBs. My studies indicate that disruption of either aa 362-464 or aa 465-550 results in the loss of self-association, SPB localization and Cdc15 function. Point mutations disrupting this interaction would allow one to determine the relationship between SPB localization, self-association, and Cdc15 function. My data indicate that SPB localization is not required for self-association although self-association might be required for localization. It is possible that the deletion of large regions of a dimerization interface are required to disrupt self-association and that point mutations would not
result in loss of self-association. Cdc15 might interact with different subsets of proteins depending on its oligomerization state and, in that manner, the self-association region could promote binding to regulatory proteins or downstream substrates. Interactors found in a two-hybrid screen using full length Cdc15 could be tested for the ability to bind to Cdc15 constructs that cannot self-associate. Additionally, proteins that only associate with wild-type Cdc15 and not Cdc15 (Δ362-464) or Cdc15 (Δ465-550) can be identified by immunoprecipitation with the given Cdc15 construct.

**Cdc15 C-terminal inhibition**

My work has demonstrated that the C-terminal domain of Cdc15 can function in some manner to inhibit Cdc15 function. The mechanism of this inhibition and how it is relieved are unknown. It is possible that the C-terminus itself could physically inhibit kinase activity of Cdc15, either intramolecularly or intermolecularly. Alternatively, the C-terminus could bind to another protein which inhibits Cdc15 function. The mechanism of inhibition has been difficult to test however. The deletion of the C-terminus from Cdc15 expressed from its endogenous promoter results in a null allele, possibly due to the truncation being unstable. Also, the overexpression of the C-terminus when fused to GFP has no obvious phenotype.

If the C-terminus bound to an inhibitor, the overexpression of this region might titrate the inhibitor from the wild-type Cdc15 present and result in the hyperactivation of the wild-type protein. Possibly hyperactivation of the endogenous levels of protein present do not result in an obvious phenotype and a more careful analysis could detect this. Dbf2 kinase activity could be measured in strains overexpressing the C-terminus of Cdc15. Dbf2 kinase activity is a direct and
sensitive readout of Cdc15 activity because Dbf2 is thought to be directly downstream and requires Cdc15 for activity (Mah et al., 2001). A decrease in Dbf2 kinase activity would suggest that this domain can bind to Cdc15 and inhibit function, whereas an increase in Dbf2 kinase activity would indicate that this region could titrate a negative inhibitor of Cdc15, leaving Cdc15 more active. A two-hybrid screen using this C-terminal domain, or full-length protein, might be useful in the elucidation of how this region functions and is regulated. Determining how the C-terminus acts to inhibit Cdc15 kinase activity, and if it is somehow regulated, will shed light on this step of MEN regulation.

**Possible chaperone regulation of Cdc15**

Cdc37 functions as molecular chaperone for numerous protein kinases such as Raf and Aurora B (Lange et al., 2002; Silverstein et al., 1998). In yeast, temperature sensitive alleles arrest at START (Reed, 1980) whereas in Drosophila, it is essential for chromosome segregation and cytokinesis (Lange et al., 2002). In yeast, Cdc37 has been found to interact genetically with *CDC15, CDC5, MPS1, CDC7, CAK1* and *CDC28* (Mort-Bontemps-Soret et al., 2002), to associate with Ste11 by co-immunoprecipitation (Abbas-Terki et al., 2000), and to be required for the association of Cdc28 with the cyclins (Gerber et al., 1995). The genetic interaction with Cdc15 raises the possibility that Cdc37 could regulate Cdc15 stability, folding, self-association, or activity. One could investigate whether any of these properties are altered in a *cdc37* mutant. It is possible that Cdc37 could, under some circumstance, regulate Cdc15 activity.
Downstream Regulation

The order of function of MEN components has been characterized over the past several years (reviewed in Bardin and Amon, 2001), although many important questions remain to be answered. The most intriguing and important question in field is how is the release of Cdc14 from the nucleolus is accomplished. Understanding Cdc14 release has been complicated by the fact that that Cdc14 release occurs through the concerted action of at least two, and possibly multiple pathways, including MEN and FEAR network. All of the data indicate that MEN is critical for a sustained release, however, whether Dbf2 acts directly to promote Cdc14 release from Cfl1/Net1 is still unknown. My data using an activated version of Cdc15 under the GALI promoter suggest that overactivation of MEN can result in Cdc14 release. My attempts to investigate the genetic requirements of this effect using temperature sensitive alleles were unsuccessful due to a decrease in efficiency of the GALI promoter at 37°C.

It is possible that genetic requirements of activated Cdc15 to promote mitotic exit could be determined using null alleles of MEN components that did not require high temperatures. Alternatively, activated Cdc15 could be expressed by a promoter that is more efficient at high temperatures or multiple copies could be integrated into the genome. If this could be accomplished, one could design a screen to find regulators of Cdc14 release downstream of Cdc15. One could screen for loss of function alleles that restore the phenotype of cells associated with overproduction of activated Cdc15 (these strains have very elongated buds and are quite sick, so loss of function downstream would restore this to a wild-type morphology). Similarly, one could select for alleles which could either suppress the benomyl sensitivity of a bub2Δ strain, or suppress the dyn1Δ bub2Δ lethality. These screens would identify known as well
as unknown positive regulators of mitotic exit. The generation of alleles of Cdc14 and Cfi1/Net1 that result in constitutive release or sequestration will also be very useful in understanding how exit from mitosis is accomplished.

**Concluding Remarks:**

I have discussed how additional regulators of components of MEN as well as parallels pathways controlling exit from mitosis might be investigated. I have also discussed how two other signal transduction cascades (the HOG pathway and the nutrient sensing Ras pathway) might contribute to regulation of exit from mitosis and cited published data in support of this hypothesis. A more detailed understanding of how exit from mitosis is regulated will be important in determining how a cell can accurately coordinate the segregation of its genetic material with cytokinesis, leading to two daughter cells.
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Appendix A:

Regulation of Bfa1, Bub2, and Tem1 association with daughter bound spindle pole body
Background

In budding yeast, spindle pole body duplication occurs throughout S phase concomitant with duplication of the genome. The budding yeast spindle pole body (SPB) is a large complex of at least 20 different types of proteins spanning the nuclear membrane creating an inner plaque on the nucleoplasmic face, a central plaque within the nuclear membrane, and an outer plaque on the cytoplasmic side of the nucleus. The inner and outer SPB plaques nucleate microtubules (MT) that utilize motor proteins to carry out their cellular functions as do the higher eukaryotic equivalent, the centrosome. Cytoplasmic MTs emanating from the outer SPB plaque position the nucleus near the bud neck at metaphase, and help to elongate the nucleus during anaphase. The inner SPB plaque extends nuclear MTs that force the SPBs to opposing sides of the nucleus during S phase and then separate sister chromatids during anaphase. The SPBs are thus critical both for nuclear division resulting in one nucleus per cell, and for proper chromosome segregation resulting in one complement of the genome in each nucleus. The SPBs also play a role in linking cell cycle events to nuclear events. Indeed, they anchor components of the mitotic exit network (MEN) signaling cascade which is essential for the mitosis to G1 transition.

In eukaryotes, to complete mitosis and progress into G1, mitotic cyclin dependent kinase activity (CDKs) must be inactivated. Budding yeast accomplish this task by activating the phosphatase Cdc14 during anaphase. Cdc14 reverses the phosphorylation status of critical mitotic CDK targets thereby leading to the inactivation of mitotic CDKs,
collapse of the mitotic spindle, and finally cytokinesis. Cdc14 activity is regulated by the MEN signal transduction cascade. MEN consists of the two-component GTPase activating proteins (GAP) Bub2-Bfa1; the guanine nucleotide exchange factor (GEF) Lte1; the GTPase Tem1, the protein kinases Cdc15, Cdc5, and Dbf2; the Dbf2 associated factor Mob1; a SPB anchor protein Nud1; and the phosphatase, Cdc14 (reviewed in (Bardin and Amon, 2001; McCollum and Gould, 2001)).

With the exception of the GEF Lte1, all MEN components localize to the SPBs during some phase of the cell cycle. The exact role that SPB localization plays in promoting mitotic exit is unclear. SPB tethering of Tem1 is thought to prevent its activation by the GEF, Lte1, until anaphase, when SPB movement into the bud forces Tem1 into a common compartment with Lte1 (Bardin et al., 2000; Pereira et al., 2000). Co-localization of Tem1 and Lte1 during anaphase is one signal leading to MEN activation. Additionally, nud1-44 temperature sensitive cells have a defective outer SPB plaque and arrest in telophase with Tem1, Cdc15, and Dbf2 mis-localized from the SPB, suggesting that SPB localization of these components is essential in some way for their function (Bardin et al., 2000; Gruneberg et al., 2000; Visintin and Amon, 2001).

Bfa1 and Bub2 are a two-component GAP that both anchor Tem1 to the SPB and inhibit Tem1 function (reviewed in (Bardin and Amon, 2001)). Interestingly, Bfa1, Bub2 and Tem1 localize asymmetrically to the SPB destined to move into the daughter cell and not on the SPB remaining in the mother cell (Bardin et al., 2000; Pereira et al., 2000). The MEN kinases (Cdc15, Dbf2, and Cdc5) localize to both SPBs symmetrically, though
localization depends on TEM1 (reviewed in (Bardin and Amon, 2001)). There is no obvious reason for the asymmetric nature of Bfa1, Bub2 and Tem1 localization since segregation from Lte1 could also be attained through localization to both SPBs. Work in Elmar Schiebel’s lab has elegantly demonstrated that, during an unperturbed cell cycle, the SPB that migrates into the daughter bud is the “old” SPB from the previous cell cycle, whereas the newly replicated SPB remains in the mother cell (Pereira et al., 2001). Age related SPB segregation is dependent on intact MTs: if the MT depolymerizing drug nocodazole (NOC) is added, nuclear orientation is lost, and upon removal of the drug, SPB inheritance is randomized. Remarkably, however, Bfa1 always segregates to the SPB moving into the daughter cell, independent of the age of the SPB (Pereira et al., 2001). Thus, Bfa1 localization is not generated through temporal incorporation of an SPB anchor protein such that Bfa1 loads onto the “older” of the two SPBs.

The study conducted by Pereira et al. (2001) raises several questions about the purpose and mechanism of Bfa1, Bub2, and Tem1 asymmetric localization. Does asymmetric localization serve a regulatory function of these proteins? In S. pombe the Bfa1 and Bub2 homologs are asymmetrically localized whereas the Tem1 homolog is symmetric. This results in the Tem1 homolog being asymmetrically active as detected by the ability to recruit the downstream kinase (Sorhmann, et al., 1998; Cerutti and Simanis, 1999; Li et al., 2000). The conservation of asymmetrically active Tem1 in both budding yeast and fission yeast, could be indicative of an important function for the asymmetry. In S. cerevisiae, depolymerization of MTs results in symmetric Bfa1 localization, but removal of drug quickly allows the re-establishment of Bfa1 asymmetry (Pereira et al.,
Asymmetric Bfa1 localization is, therefore, a MT dependent process and occurs very rapidly upon MT polymerization. By what mechanism is this asymmetry achieved? Is it important for faithful transmission of the genome? Equally intriguingly, why, in an unperturbed cell cycle, is the older SPB always inherited by the daughter cell? Does it serve an important function or is it simply a consequence of the timing of SPB maturation?

Several possible mechanisms could facilitate Bfa1, Bub2 and Tem1 loading specifically onto the SPB moving into the daughter cell. 1. Microtubule motor proteins might facilitate loading asymmetrically on the SPB. 2. Bud neck proteins, such as the septins, could allow the loading of Bfa1, Bub2, Tem1 onto the SPB nearest the bud neck. 3. Microtubule generated tension could be “sensed” by Bfa1, Bub2, and Tem1 and facilitate SPB loading. 4. Other asymmetric cues in the mother or daughter cell could provide spatial information for Bfa1, Bub2, and Tem1 loading.

We have investigated several possible mechanisms of asymmetric Bfa1 localization. The work presented here does not conclusively determine how asymmetric SPB localization is achieved, but does rule out several possible mechanisms. We have determined that deletion of the MT motors *DYNI*, *CIN8* and *KIP2* has no effect on Bfa1 localization. Bfa1 localization is also unaffected in the septin mutants *cdc11-6* and *cdc12-6*, as well as in a temperature sensitive allele of kinetochore component, Ipl1. SPB localization of Bfa1 seems to be dynamic in metaphase. Often, during metaphase, Bfa1 is on the SPB away from the bud or on both SPBs, whereas by anaphase, it is 90%
asymmetric on the daughter bound SPB. The establishment of asymmetric localization is MT dependent; however, we find that maintenance of the asymmetric state in a telophase arrest is MT independent. We also examined whether cell polarity regulators facilitate Bfa1 asymmetry. Bfa1 can still localize asymmetrically in a myo2-66, bni1Δ, and cdc42-1, although subtle differences in localization cannot be ruled out. Finally, we find that Bfa1 is symmetrically localized in a large number of cells that do not bud due to high levels of mitotic cyclin. Whether this is due to loss of polarity of a protein, or high levels of phosphorylation is not known.

**Results:**

**Investigating motor proteins:**

To investigate whether motor proteins allow asymmetric loading of Bfa1, Bub2, and Tem1, the localization of Bfa1 was examined in dyn1Δ, cin8Δ, and kip2Δ strains. Because Tem1 and Bub2 are dependent on BFA1 for asymmetric localization, immunofluorescence of Bfa1-3HA was used to investigate localization in each mutant. I found that Bfa1 localization appeared normal in each of the single mutant strain (Figure 1). It is possible that the other MT motors Kip1, Kip3, Kar3 or Smy1 could mediate asymmetric localization of Bfa1 or that these motors have redundant functions. However, it is clear that loss of DYN1, CIN8, or KIP2 does not disrupt asymmetric localization of Bfa1.
**Effect of bud-neck proteins**

The septins are required for the localization of numerous proteins to the bud neck as well as for the anchoring of many proteins within the bud. Additionally, MT from the SPB entering the daughter cell contact the septins prior to entry of the nucleus into the bud (reviewed in (Segal and Bloom, 2001)). In order to examine whether the septins play a role in asymmetric localization of Bfa1, Bub2, and Tem1, I examined immunofluorescence of Bfa1-3HA in the strains with temperature sensitive alleles of the septins, *cdc11-6* and *cdc12-6*. Bfa1 remained asymmetric in these mutants, suggesting that disruption of the septin ring does not alter asymmetric loading of Bfa1 (data not shown).

**Microtubule tension**

Loss of Bfa1 asymmetry occurs when the microtubule depolymerizing drug nocodazole (NOC) is added to cells (Pereira et al., 2000). When the drug is washed out and cells resume the cell cycle, Bfa1 quickly regains its asymmetry to the daughter bound SPB. One possibility is that Bfa1 somehow can “sense” the SPB that is being pulled with the most force. A greater force is presumably generated at the daughter bound SPB since the nucleus undergoes a larger movement at this SPB. In NOC, no tension is generated, and Bfa1 localizes to both SPBs equally. If tension were somehow being sensed by Bfa1, one might expect to see differences between metaphase and anaphase in Bfa1 localization. During metaphase, the nucleus is positioning near bud neck through forces on both SPBs. However, during early anaphase, the SPB entering the daughter bud is
undergoing a constant force as the spindle is extending. In a synchronous release from G1, 60% of metaphase cells have Bfa1 only on the SPB facing the daughter cell, whereas in 24% of cells, Bfa1 is on the SPB away from the daughter. 16% of metaphase cells have Bfa1 symmetrically on both SPB. In contrast, in early anaphase, 90% of the cells have Bfa1 on the SPB in the daughter cell and 10% have Bfa1 symmetrically distributed (Figure 2A). These data could be consistent with Bfa1 localization responding to MT generated tension.

Mutants in the kinetochore protein, Ipl1, are defective in generating microtubule-kinetochore tension. Whether disruption of tension at the SPB inside the nucleus would disrupt tension at outer plaque is uncertain. In an attempt to test the effect of loss of tension, at least of the kinetochore, Bfa1-3HA localization in ipl1-32I mutants was examined. No obvious defect in Bfa1 localization was found (Figure 2B).

While intact MTs seem to be necessary to establish asymmetric Bfa1, it is possible that the maintenance of the asymmetric state could be accomplished independently of microtubules. In order to test this, I examined the effect of adding NOC to cells arrested in telophase with a temperature sensitive allele of CDC14; in these cells, Bfa1 is already localized asymmetrically to the daughter SPB (Figure 2C). Bfa1 localization remains asymmetrically distributed upon addition of NOC. This result suggests that once bound to the daughter SPB, MTs are not necessary to anchor it there. Alternatively, Bfa1 is no longer competent to load onto the other SPB. It would be interesting to determine whether this is particular to a cdc14-3 arrest, or if it would also
occur in another MEN mutant such as cdc15-2 or a metaphase arrest such as cdc23-1. If both MEN mutants, but not the cdc23-1, allowed Bfa1 maintenance, then likely something specific about being in telophase is important for Bfa1 SPB localization.

**Cell polarity**

We investigated whether cell polarity cues were necessary for the establishment of asymmetric Bfa1 localization. Myo2/She3 is an actin motor that is responsible for the transport of cargo along actin cables into the bud. A myo2-66 mutant is severely defective in the establishment of cell polarity and continues to divide without formation of a bud. Bfa1-3HA localization appeared asymmetric in temperature sensitive myo2-66 cells and, in fact, often was asymmetric on two spindles within the same cell body (Figure 3A). Thus Myo2 does not play an essential role in the establishment of Bfa1 asymmetry. Another protein involved in polarity establishment is Bni1/She5. Bni1 is a formin required to maintain polarized growth, mitotic spindle orientation and localization of numerous proteins to the bud. Bfa1 localization was examined in bni1Δ cells. Bfa1 localization was indistinguishable from wild-type cells in bni1Δ (Figure 3B).

Cdc42 is a GTPase involved in the generation of cell polarity. Cdc42 becomes activated by Cln CDKs after START and promotes cell polarity both through the coordination of actin polarity and by acting on other effectors important for cell polarity. Loss of Cdc42 function results in round, unbudded cells that are predominantly unpolarized. However, some proteins, such as components of the exocyst complex, are known to retain localization to the former bud scar in CDC42 mutants (Pruyne and
Bretscher, 2000). The effect of loss of Cdc42 function was examined using a cdc42-17 temperature sensitive allele. Bfa1 could still localize asymmetrically in these cells (data not shown). However, it is difficult to rule out the possibility that Cdc42 dependent asymmetry of molecules from the previous cell cycle could promote Bfa1 asymmetric localization.

Another way to depolarize the cell is through the use of artificially high levels of mitotic cyclins. Low mitotic CDK activity is required for bud formation, so cells overproducing a non-degradable Clb2 (Clb2Δ) often do not form a bud. In wild-type cells, Bfa1 is completely asymmetric on the daughter SPB in 80-90% of telophase cells. When cells overexpressing Clb2Δ were examined, in 80% of budded telophase cells containing a properly aligned spindle (extending between the mother and daughter cell) Bfa1-3HA was asymmetric. Whereas, of unbudded cells in telophase, 47% had Bfa1 localized symmetrically on the both SPBs, 27% had Bfa1 only on one SPB, 20% had one SPB brighter than the other, and 6% Bfa1 did was not seen on SPBs (Figure 4). The overproduction of Clb2Δ, however, does cause a partial defect in the asymmetric localization of Bfa1. This experiment does not distinguish whether high levels of mitotic CDKs cause a partial loss of asymmetry because of loss of polarity or due to artificially high levels of phosphorylation of a target protein.
Discussion and Conclusion

Pereira, et al., (2001) showed that any difference in protein composition of mother and daughter SPB does not affect the ability of Bfa1 to load onto the SPB moving into the daughter cell. The mechanism of generating asymmetry must, therefore, be inherent in either the bud, bud neck, or general cell polarity machinery. The studies presented here do not conclusively reveal a mechanism for sensing the daughter bound SPB. I have, however, ruled out a number of possible mechanisms and demonstrated that maintenance of the asymmetric state can occur without MT. I have shown that two of the major cytoplasmic microtubule motors, Dyn1, and Kip2 are not, at least solely, responsible for asymmetric localization of Bfa1. It is possible that the other two cytoplasmic motors, Kar3, Kip3 could play redundant functions to allow asymmetric localization of Bfa1 to the SPB.

Microtubule tension on the SPBs could play a role in Bfa1 asymmetric loading. Consistent with this is the finding that in 24% of metaphase cells, Bfa1 actually localized away from the bud neck; though this does not occur once anaphase has commenced. My data show that during metaphase, Bfa1 SPB localization appears more dynamic than during anaphase. This could be a consequence of rapid nuclear movements during metaphase and live cell analysis is needed investigate this finding further. To further test the idea that tension is somehow “sensed” by Bfa1, one could use yeast strains developed by Hoyt and co-workers that allow the manipulation of tension on the SPBs and force the nucleus back into the mother cell (Cottingham et al., 1999). If tension is sensed, one
would predict that Bfa1 would localize to the opposite SPB being pulled to the mother cell wall in these strains.

Cell polarity directed by polymerized actin, Myo2, Cdc42, and Bni1, does not seem to influence Bfa1 asymmetry. However, in cells expressing high levels of mitotic CDKs, a decrease in asymmetry was seen in cells without a bud as compared budded cells of the same genotype. This effect could be due to a loss in polarity of some molecules since high levels of mitotic CDKs inhibit budding, and thus some aspects of cell polarity. Alternatively, phosphorylation of a CDK target could allow loss of asymmetry specifically in those cells with the highest mitotic CDK levels, which are likely those that also could not form a bud.

The purpose of age-based segregation of SPBs is not understood. Possibly it is related to the longer G1 phase experienced by the daughter cell due to time necessary to obtain the appropriate cell size. One possibility is that it is evolutionarily beneficial to allow the older SPB a longer G1 phase so that any older, damaged components can be replenished prior to S phase and chromosome segregation. This could be tested by comparing the viability of mother and daughter cells receiving either a "new" or "old" SPB after release from a NOC arrest. Alternatively, age-based inheritance of SPBs might be simply a consequence of the ability of the old SPB to anchor to the future bud site, prior to duplication of the second SPB. This could account for the faithful segregation of the old SPB to the daughter cell during every cell cycle.
Literature Cited


Figure 1: Bfa1-3HA localization in Microtubule Motor Mutants

wild-type

dyn1Δ

cin8Δ

kip2Δ

Figure 1:
Bfa1-3HA localization was examined in wild-type (A4378), dyn1Δ (A5079) cin8Δ (A5080) and kip2Δ (A5398) strains. Cells were grown at room temperature to mid-log phase and then immunofluorescence samples were processed according to Appendix C.
Figure 2A: Distribution of Bfa1 on SPBs

<table>
<thead>
<tr>
<th>Metaphase Cells</th>
<th>Anaphase Cells</th>
</tr>
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<tbody>
<tr>
<td>60%</td>
<td>90%</td>
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<tr>
<td>24%</td>
<td>10%</td>
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<tr>
<td>16%</td>
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Figure 2B: Bfa1-3HA localization in ipl1-321 mutants

Figure 2C: Bfa1-3HA in cdc14-3 +/- nocodazole

- nocodazole
- +nocodazole

2A. Cells containing Bfa1-3HA (A4378) were synchronously released from G1 alpha factor arrest into YPD at room temperature. Cells from the 105’ timepoint were quantified. 2B. BFA1-3HA, ipl1-321 cells (A5081) were grown at midlog phase at 37°C for 2.5 hours. 2C. BFA1-3HA, cdc14-3 cells (A3727) were arrested at non-permissive temperature of 37°C for 2 hours. The culture was split and 15μg/ml NOC was added to one sample. All procedures were performed as described in Appendix C.
**Figure 3A:** Bfa1-3HA localization in *myo2-66* cells

![Image of Bfa1-3HA localization in myo2-66 cells]

**Figure 3B:** Bfa1-3HA localization in *bni1Δ* cells

![Image of Bfa1-3HA localization in bni1Δ cells]

**3A.** *myo2-66, BFA1-3HA* (A4444) cells were arrested in G1 with alpha factor and synchronously released into YPD at 37°C. Samples were processed for immunofluorescence, pictures are representative of cells 240' after release.

**3B.** Bfa1-3HA localization was examined in *bni1Δ* cells (A5087). Techniques performed as described in Appendix C.
4A. Bfa1-3HA localization in *GAL-CLB2dbΔ* cells

![Images showing localization of Bfa1-3HA and DAPI in unbudded and budded cells]

red=Bfa1-3HA, blue=DAPI

4B. Quantitation of Bfa1 localization

<table>
<thead>
<tr>
<th></th>
<th>1 SPB</th>
<th>2 SPBs</th>
<th>0 SPBs</th>
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<tbody>
<tr>
<td><strong>wild-type</strong></td>
<td><img src="wild-type_1SPB" alt="Diagram" /> <img src="wild-type_2SPBs" alt="Diagram" /> <img src="wild-type_0SPBs" alt="Diagram" /></td>
<td>86% 5% 4% 5%</td>
<td></td>
</tr>
<tr>
<td><strong>GAL-CLB2dbΔ</strong></td>
<td><img src="GAL-CLB2db%CE%94_1SPB" alt="Diagram" /> <img src="GAL-CLB2db%CE%94_2SPBs" alt="Diagram" /> <img src="GAL-CLB2db%CE%94_0SPBs" alt="Diagram" /></td>
<td>80% 7% 6% 7%</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>1 SPB</th>
<th>2 SPBs</th>
<th>0 SPBs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAL-CLB2dbΔ</strong></td>
<td><img src="GAL-CLB2db%CE%94_1SPB" alt="Diagram" /> <img src="GAL-CLB2db%CE%94_2SPBs" alt="Diagram" /> <img src="GAL-CLB2db%CE%94_0SPBs" alt="Diagram" /></td>
<td>27% 47% 20% 6%</td>
<td></td>
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</table>

4A. Cells expressing *CLB2dbΔ* from the *GAL1-10* promoter (A4379) were induced with 2% galactose for 2 hours. Bfa1-3HA localization, tubulin staining, and cell morphology were examined. 4B. Bfa1-3HA asymmetric localization in wild-type (A4378) and budded and unbudded *GAL-CLB2dbΔ* cells (A4379) were compared.
Appendix B: Tem1-Cdc15 Interaction
Background

The GTPase, Tem1, is at the top of the MEN regulatory cascade which governs inactivation of mitotic CDKs at the end of mitosis. Physical interaction, epistasis analysis, and localization studies indicate that the MEN protein kinase, Cdc15, is immediately downstream of Tem1 (Bardin et al., 2000; Jaspersen et al., 1998; Shirayama et al., 1994; Lee et al., 2001; Mah et al., 2001; Visintin and Amon, 2001). At endogenous levels, the recruitment of Cdc15 to the SPBs is dependent on TEM1, suggesting that Tem1 facilitates loading on both spindle pole bodies (SPB) of Cdc15 (Visintin and Amon, 2001). Localization of Cdc15 to SPBs seems to be important for localization and activation of the protein kinase, Dbf2, and its associated factor, Mob1, onto the SPBs (Lee et al., 2001; Visintin and Amon, 2001; Yoshida, 2001). Determining how the interaction between Tem1 and Cdc15 is regulated, is therefore important in order to understand MEN signaling.

In most small GTPase signaling cascades, interaction of GTPase and downstream kinase is dependent on structural changes in the GTPase occurring in response to GTP binding. Whether Tem1-Cdc15 interaction is facilitated by Tem1 GTP binding is unknown. However, Tem1 from one extract can recruit Cdc15 from a separate extract, suggesting that co-immunoprecipitation is not due to bridging from a large SPB complex. Despite Tem1 and Cdc15 being present in the G1 phase of the cell cycle, interaction between Tem1 and Cdc15 does not appreciably occur in an alpha factor mediated G1 arrest as assayed by co-immunoprecipitation (Figure 1; Bardin et al., 2000). However, Tem1 and Cdc15 do interact when cells are arrested in metaphase with either nocodazole or temperature sensitive alleles of the APC component,
CDC23, or in telophase using cdc14-1 (Figure 1; Bardin et al., 2000). Interaction between Tem1 and Cdc15, as judged by co-immunoprecipitation from yeast extracts, is appears to be subject to regulation throughout the cell cycle. However, we have not ruled out the possibility that changes in interaction between Tem1 and Cdc15 could be due to the alpha factor mediated SPB changes which occur to prepare for karyogamy (Pereira, et al., 1999).

GTP binding is known to be important for the interaction of the Tem1 and Cdc15 homologs in S. pombe (Cerutti and Simanis, 1999; Li et al., 2000). Deletion of GAP components results in a greater amount of Cdc15 co-immunoprecipitating with Tem1 as does addition of a non-hydrolyzable GTP analog to extracts (Ro et al., 2002). While in vitro interaction of Tem1 and Cdc15 is detected in S phase in extracts, Cdc15 does not localize to the SPBs until anaphase suggesting, perhaps that in vivo regulation is more complicated. The immunoprecipiation is performed under conditions that result in soluble Tem1 and Cdc15 likely dissociating them from other SPB components. Seemingly contradictorily, Tem1 is predicted to be activated by its GEF during anaphase (Bardin et al., 2000; Pereira et al., 2000), later in the cell cycle than the interaction with Cdc15 is detected. It is possible that Cdc15 and Tem1 have multiple modes of interaction, one of which is GTP dependent. Conclusive determination of when in the cell cycle Tem1 becomes GTP bound and whether this influences interaction with Cdc15 is thus far unknown.

In the following study, we have used truncated versions of CDC15 to investigate the interaction with Tem1. Co-immunoprecipitation studies indicate that many regions of Cdc15 can interact with Tem1 in extracts. The physiological relevance of this is, however, unknown. We
have developed a second assay with which to address the *in vivo* interaction of Cdc15 and Tem1. We find that when full length Cdc15 is overproduced, Tem1 SPB associated staining is lost. One possibility is that the ability to do so reflects the *in vivo* interaction between the two proteins. Through this assay, we map a putative minimal domain for *in vivo* interaction. Additionally, we note that *in vivo* titration of Tem1 from the SPB might be the cause of the telophase phenotypes associated with class II overexpression constructs described in Chapter III.

**Results and Discussion**

*Analysis of Cdc15 truncation interaction with Tem1 in vitro*

To gain insight into regions of Cdc15 important for interaction with Tem1, we examined the ability of various Cdc15 truncations to immunoprecipitate Tem1 from cell extracts. All Cdc15 truncations retained some ability to immunoprecipitate Tem1 from cell extracts (Figure 2A, Table 1). We do not know whether this lack in specific interaction between Tem1 and Cdc15 observed *in vitro* is due to a general affinity of these two proteins in extracts or due to co-immunoprecipitation of endogenous Cdc15 (Cdc15 forms at least dimers if not multimers, see Chapter III) which binds to Tem1. It is possible that all regions of Cdc15 do contain a weak affinity for Tem1. Additionally, we cannot rule out that GFP can bind non-specifically to Tem1-3MYC in our co-immunoprecipitations.
The observation that *GFP-CDC15* caused loss of Tem1 from SPBs, however, provided a potential *in vivo* assay to assess which regions in *CDC15* are important for Tem1 binding. We reasoned that overproduced Cdc15, also present in the cytoplasm, would lead to loss of MEN components from SPBs. This was indeed the case. Overexpression of *GFP-CDC15* leads to loss of Tem1 from SPBs (Figure 2A, Table 1). Furthermore, a large number of truncations caused when overexpressed loss of Tem1 from SPBs (Table 1). It is unlikely that these Cdc15 truncations compete with Tem1 for SPB binding as some of the constructs did not themselves localize to SPBs (Table 1). It is also unlikely that overproduction of Cdc15 is titrating the SPB anchor Nud1 because Dbf2 localization, which is *NUD1* dependent, appears wild-type when *GFP-CDC15* is overexpressed (AB, data not shown). Expression of truncations as small as *GFP-CDC15* [267–376] led to loss of Tem1 from SPBs suggesting that this region of Cdc15 might mediate interaction of Cdc15 with Tem1 *in vivo*, a notion that is in good agreement with previous findings showing that aa 276-362 of Cdc15 interact with Tem1 in a two-hybrid analysis (Asakawa et al., 2001).

Using truncated versions of *CDC15*, we previously examined the phenotypes associated with their overexpression. We determined that *CDC15* constructs that cause a dominant negative telophase arrest fell into two classes: the telophase arrest of Class 1 constructs results from loss of the kinase domain (Chapter III). Overexpression of these constructs can titrate out endogenous Cdc15 and presumably bind to important interactors, however, these constructs are incapable of signaling due to lack of kinase activity. Class 2 constructs also produce a telophase arrest or delay although it is independent on the presence or absence of the kinase domain. Class 2 induced telophase arrest depends on aa 430-550, such that cells expressing *GAL-CDC15* [1-
progress through the cell cycle with wild-type kinetics, though *GAL-CDC15 [1-429]* arrest in telophase. All Class 2 constructs contain the minimal Tem1 interaction region (aa 267-376) and overproduction results in loss of Tem1 from the SPBs (Figure 2B, Table 1). It is therefore possible that the phenotype associated with Class 2 overproduction is due to the titration of Tem1 by the Cdc15 construct away from another important interactor of Cdc15. In order for MEN signal propagation, perhaps Cdc15 needs to be associated with Tem1 to be activated, and a downstream effector which binds to aa 430-550 (Figure 3).

It is interesting to note that the overexpression of the Tem1 GAP component Bfa1, thought to tether Tem1 to the SPB, can result in a dominant negative telophase arrest. The telophase arrest induced by *GAL-BFA1* is independent of *BUB2*, the catalytic domain of the GAP (Li, 1999; Ro et al., 2002). This has previously been interpreted as a demonstration that Bfa1 has a separate inhibitory function towards Tem1. Given my data that SPB titration of Tem1 using a small region of Cdc15 causes a telophase arrest, I think it is very likely that *GAL-BFA1* is causing a telophase arrest through a similar, non-physiological mechanism.
Conclusion

This work has investigated the interaction with domains of Cdc15 and Tem1. We find that using co-immunoprecipitation, all of the GFP-Cdc15 truncated proteins have some ability to interact with Tem1. We used these constructs to examine the ability of Tem1 to be removed from the SPB in vivo. We found that regions aa 267-376 of Cdc15 are necessary and sufficient to inhibit Tem1 SPB localization, whereas aa 1-275 and aa 361-974 have no affect on the ability of Tem1 to localize to the SPB. The ability to titrate Tem1 from the SPB could reflect an in vivo interaction between Cdc15 and Tem1. If this is the case, aa267-376 represents the minimal in vivo interaction domain which is in good agreement with that found by Asakawa et al., 2001.
Literature Cited


Table 1: Tem1 Localization in cells expressing Cdc15 constructs

<table>
<thead>
<tr>
<th>CDC15</th>
<th>SPB localization?</th>
<th>Tem1 localization?</th>
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<tbody>
<tr>
<td>1-974</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>267-974</td>
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<td>No</td>
</tr>
<tr>
<td>1-750</td>
<td>Yes**</td>
<td>No</td>
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<tr>
<td>267-750</td>
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</tr>
<tr>
<td>1-702</td>
<td>Yes**</td>
<td>?</td>
</tr>
<tr>
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<tr>
<td>394-702</td>
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<tr>
<td>550-974</td>
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</tr>
<tr>
<td>751-974</td>
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</table>

** Indicates aggregates of GFP in the cell.
**Figure 1:** Tem1-Cdc15 interaction is impaired in G1

<table>
<thead>
<tr>
<th>Tem1-3MYC</th>
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<th>+</th>
<th>+</th>
<th>+</th>
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<tr>
<td>Cdc15-3HA</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>cell cycle stage: cyc</td>
<td>cyc</td>
<td>cyc</td>
<td>cyc</td>
<td>G1</td>
<td>meta</td>
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</table>

Total: Cdc15-3HA

Total: Tem1-3MYC

IP: Tem1-3MYC
W: Cdc15-3HA

IP: Tem1-3MYC
W: Tem1-3MYC

IP: Cdc15-3HA
W: Tem1-3MYC

IP: Cdc15-3HA
W: Cdc15-3HA

**Figure 1: Tem1-Cdc15 interaction is impaired in G1.**
Mid-log phase cultures were grown of wild-type (A2587), Tem1-3Myc (A1828), Cdc15-3HA (A1787), or Tem1-3Myc, Cdc15-3HA (A2193) in YPD at room temperature were diluted to OD600=0.2. Cells were arrested in G1 with 5mg/ml alpha factor, metaphase (meta) with 15mg/ml NOC, or grown asynchronously (cyc). Immuno-precipitation was performed as described in Appendix C.
Figure 2A: Co-immunoprecipitation of Cdc15 constructs with Tem1

<table>
<thead>
<tr>
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<td>-</td>
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<tr>
<td>input Tem1-3MYC</td>
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<td>+</td>
</tr>
<tr>
<td>IP'd Tem1-3MYC</td>
<td>light exposure</td>
<td></td>
</tr>
<tr>
<td>IP'd Tem1-3MYC</td>
<td>dark exposure</td>
<td></td>
</tr>
<tr>
<td>IP'd CDC15 construct</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative Tem1 IP'd: 1.00 0.23 1.73 13.50 1.18 1.75 0.10 1.45 1.00 0.90
Relative GFP-CDC15 construct IP'd: 1.00 5.56 12.04 205.6 17.59 2.00 32.41 37.04 0.78 2.39
Ratio (X100): 100 4.05 14.33 6.57 6.68 87.5 0.31 3.92 128.6 37.7

Figure 2A: Co-immunoprecipitation of Cdc15 Constructs with Tem1.
Cells were grown to mid-log phase in Yep +2% raffinose, and induced for 2.5 hours with 2% galactose, then prepared as described in Appendix C.
The following strains were used: wild-type (A2587); Tem1-3MYC (4267); GAL-GFP-Cdc15 (4368); GAL-GFP-Cdc15, Tem1-3MYC (A4441); GAL-GFP-Cdc15[1-273], Tem1-3MYC (A4528); GAL-GFP-Cdc15[1-273], Tem1-3MYC (A4528); GAL-GFP-Cdc15[1-376], Tem1-3MYC (A4527); GAL-GFP-Cdc15[267-376], Tem1-3MYC (A6373); GAL-GFP-Cdc15[267-429], Tem1-3MYC (A4542); GAL-GFP-Cdc15[550-974], Tem1-3MYC (A4442); GAL-GFP-Cdc15[751-974], Tem1-3MYC (A4443); GAL-GFP-Cdc15[266-974], Tem1-3MYC (A4376); GAL-GFP-Cdc15[361-974], Tem1-3MYC (A5254); GAL-GFP-Cdc15[394-974], Tem1-3MYC (A5255).
2B. Tem1 localization in strains expressing \( \text{GAL-CDC15} \) Constructs

<table>
<thead>
<tr>
<th></th>
<th>( \text{GAL-CDC15} ) 1-974</th>
<th>( \text{GAL-CDC15} ) (267-376)</th>
<th>( \text{GAL-CDC15} ) (1-273)</th>
<th>( \text{GAL-CDC15} ) (361-974)</th>
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<td>( \text{wt} )</td>
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<td><img src="image3" alt="Image" /></td>
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Figure 2B: Tem1 localization in strains expressing GAL-CDC15 constructs. Cells from Figure 2A experiment were used and processed as described in Appendix C.
Figure 3. Model for Tem1 and Effector binding sites on Cdc15. Using data from in vivo titration of Tem1 from SPBs and phenotypic analysis of Cdc15 constructs, this model speculates on the Tem1 binding site, light green (267-376aa) and a putative effector binding site in blue (430-550aa). The N-terminal kinase domain (1-273aa) is shown in purple. The SPB minimal localization domain is in red (267-702aa). A domain necessary and sufficient to facilitate self-association is shown in yellow (361-702), and the putative inhibitory C-terminus is in green (751-974aa).
Appendix C: Protocols and Methods
1. Competent Cell Preparation (DH5alpha for electroporation)

1. 2 days before: Start 5 ml culture of cells in LB. Grow in 50 ml falcon at 37°C overnight from competent cell stock or frozen E. coli stock.
2. 1 day before: Inoculate 50 ml LB with 0.5-1 ml of first culture in 250 ml flask. Grow overnight at 37.
3. Day of: Inoculate 2L LB total with 10ml of second culture per liter LB. Use 2 4L flasks or 4 2L flasks. Grow at 37 until OD$_{600}$ is 0.5-0.8, 0.6 is ideal (takes ~3.5hrs).
4. After inoculating cultures, prechill in cold room all supplies: Centrifuge rotor, centrifuge bottles, 3L sterile water, 50 ml 10% glycerol, 1L graduated cylinder, 1 50 ml falcon tubes, ~100 eppendorf tubes, 3 eppendorf racks, and a 5 ml combitip.
5. When cultures are at the proper density, harvest cells by chilling on ice in cold room 15-30 min. After harvesting, all work must be done in cold room.
6. Balance culture among the centrifuge bottles and spin at 5000 rpm for 15 min at 4°C.
7. Decant, resuspend pellets in 2L cold sterile water total, and centrifuge at 5000 rpm for 15 min at 4°C.
8. Decant, resuspend pellets in 1L cold sterile water total, and centrifuge at 5000 rpm for 15 min at 4°C.
9. Decant, resuspend pellets in 40 ml 10% glycerol total, pooling cells into 1 50 ml falcon. Centrifuge at 5000 rpm for 15 min at 4°C.
10. Resuspend in final volume of 4-6 ml 10% glycerol. Cell concentration should be at least 3x10$^{10}$ cells/ml.
11. Aliquot cells, 100 µl/eppendorf with repeat pipettor. Drop in liquid nitrogen, then transfer to a box in -80. Should last ~6 months.
12. To test competency transform plasmid of known concentration and calculate transformants per µg input plasmid DNA. 10$^9$ is perfect, 10$^7$-10$^8$ is good.

For tester plasmid, transform 1 µl of 0.02 ng/ul, resuspend in 500 µl 2xLB, plate 100 ul. Competency = # colonies*270,500
Remember to transform a no DNA negative control to insure no contamination by Amp resistant E. coli.

2. E. coli Transformation by Electroporation

1. Thaw DH5 alpha competent cells on ice.
2. Put electroporation cuvettes on ice while cells is thawing.
3. Reaction
   i. 40 µl of cells
   ii. Less than 5 µl of DNA.
4. Put reaction mix in cuvette and make sure sample settle to bottom of cuvette.
5. Wipe moisture off of electroplates on cuvette.
6. Pulse cells at 2.5 volts. A beep will sound when done.
7. Transfer to glass tube with 1ml of 2x LB.
8. Incubate at 37°C for 1hr.
9. Spin down at 3rpm for 3 min. Remove supernatant.
10. Plate on selective medium with residual solution.

3. *E. coli* Mini prep:

1. Inoculate single colony into 2 ml culture overnight. Transfer to eppendorf tube.
3. Add 200μl of SOL2 and 100μl of KOAc = SOL3. Vortex briefly.
4. Spin down at max speed for 5 min. Transfer supernatant to 1ml of cold EtOH.
5. Spin down at max speed for 5 min. Discard supernatant.
6. Resuspend in 40μl of TE. Add 40μl of cold 5M LiCl. Put on ice for ~5 min.
7. Spin down at max speed for 5 min. Transfer supernatant to 200 μl of cold EtOH.
1. Spin down at max speed for 5 min. Remove supernatant. Air dry pellet for a couple of minutes and resuspend in 20μl of TE.

Solutions:

SOL 1: (GTE) 50mM glucose, 10mM EDTA, 25mM Tris pH 8.0 (keep at 4°C)
SOL 2: (SDS solution) 1% SDS, 0.2M NaOH
SOL 3: 250 g KOAc, 150g acetic acid per 1L dH₂O (keep at 4°C)
5M LiCl

4. *E. coli* Midi prep:

Start with 50 ml culture 12-18 hours 37°C.

1. Centrifuge 5 min. 3600 rpm
2. Resuspend pellet in 2.5 ml GTE
3. Add slowly 5ml SDS/NaOH (SOL2), mix carefully
4. Add 2.5ml SOL3 (cold!), vortex briefly
5. Centrifuge 5 min 3600 rpm
6. Pour supernatant through kimwipe to get rid of white stuff
7. Add 10ml isopropanol
8. Centrifuge 5 min 3600 rpm
9. Resuspend pellet in 750μl TE
10. Add 1ml 5M LiCl, put on ice (RNA will precipitate)
11. Centrifuge 5 min 3600 rpm
12. Take supernatant and add 3.5ml EtOH (2 vol.), [put at -20 C]
13. Centrifuge 5 min 3600 rpm
14. Dissolve pellet in 200µl TE
15. Precipitate with 2.5 vol. EtOH/0.3M NaAc [put at -20°C]
16. Dissolve pellet in 100-200µl TE

Solutions:

SOL 1: (GTE) 50mM glucose, 10mM EDTA, 25mM Tris pH 8.0 (keep at 4°C)
SOL 2: (SDS solution) 1% SDS, 0.2M NaOH
SOL 3: 250 g KOAc, 150 g acetic acid per 1L dH₂O (keep at 4°C)
5M LiCl

5. PCR

100µl
10µl 10X Takara ExTaq buffer
8µl 2.5mM dNTPs
5µl primer 1 (20 µM)
5µl primer 2 (20 µM)
DNA
1µl ExTaq

Cycles:

94°C 3’
Repeat the following 25X:
94°C 1’
55°C 1’
72°C 1’/1000bp

72°C 10’
4°C soak

6. Smash and Grab yeast DNA prep:

1. Dissolve a toothpick worth of cells in 500µl of TE.
2. Quick spin down and remove supernatant.
3. Add 200µl of DNA breakage buffer, 200µl of phenol and ~0.3g of glass beads.
4. Vortex on mulivortexer for 4 min.
5. Centrifuge at 14,000 rpm for 5 min. Transfer upper aqueous layer to 1ml of cold EtOH. ~100-130µl. DO NOT DISTURB THE INTERFACE.Recentrifuge if necessary. Mix by inversion
6. Centrifuge at 14,000 rpm for 5 min. Discard the supernatant.
7. Add 400μl of TE. 1.5μl of RNAase A 20mg/ml. Mix by inversion. Incubate at 37°C for 5 min.
8. Add 1ml of 0.3M NaAc:EtOH. Mix by inversion.
9. Centrifuge at 14,000 rpm for 5 min. Remove supernatant and air dry.
10. Resuspend in 50μl of TE.

7. Genomic Yeast DNA Prep:

1. Inoculate cells into 10ml minimal or 5ml YPD O/N.
2. Spin cells down 3 min at 3000rpm.
3. Transfer to 1.5ml eppendorf tube with 1 ml of H₂O.
4. Spin down cells and pour off supernatant.
5. Resuspend cells in 200μl of SCE/zymolase/B-mercaptoethanol. Mix and incubate for 30-60 min at 37°C on rotating rack.
6. Check spheroplasting of cells by adding 3μl of cells to 3μl of 1% SDS. When spheroplasted, see no intact cells just crystals under microscope.
7. When spheroplasting is complete. Add 200μl of SDS solution. Mix and vortex briefly. Incubate at 65°C for 5 min.
8. Add 200μl of 5M KOAc. Mix and vortex briefly. Leave on ice for 20 min.
9. Spin down at max speed for 10 min.
10. Transfer 400-500μl of supernatant to 2ml tube using cut P1000 tip to avoid shearing.
11. Add 200μl of 5M NH₄OAc and 1ml of isopropanol.
12. Mix and spin down at 3000 rpm for 15-30sec. NOTE:DO NOT WANT A COMPACT PELLET.
13. Remove supernatant and resuspend in 200μl of TE.
14. Add 20μl of 5M NH₄OAc and 400μl of isopropanol.
15. Mix and spin down at 2500 rpm for 15-30 sec.
16. Remove supernatant and resuspend in 90 μl of TE.
17. Add 10μl of 5M NH₄OAc and 200μl of isopropanol.
18. Mix and spin down at 2500 rpm for 20 sec.
19. Remove supernatant and resuspend in 30μl of TE.

Solutions:

SCE: 1M Sorbitol, 0.1M Na Citrate, 0.06M EDTA, pH 7.0

SCE/zymolyase/βME: to 1ml of SCE, add 1-2 mg zymolyase T100 and 8μl of βME (A stock of this can be made and frozen at -20°C)

SDS solution: 2% SDS, 0.1M Tris/Cl pH 9.0, 0.05M EDTA.

5M NH₄OAc (filter sterilized)
5M KOAc.

1X TE: 10mM Tris pH 7.5, 1mM EDTA

8. Yeast Transformation:

1. Grow yeast ON to mid log phase in YPD; redilute if necessary.
2. Prepare fresh solutions from stocks below:
   - A. 1X TE, 1X LiAc
   - B. 1X TE, 1X LiAc, 40% PEG.
3. Centrifuge yeast cells at 3,000 rpm, wash with 50 ml dH2O, transfer pellet to eppendorf tube in dH2O, centrifuge at 3,000 rpm, rinse with 1 ml 1X TE, 1X LiAc. Resuspend in 250 μl of 1X TE, 1X LiAc.
4. Resuspend DNA for transformation in ~10 μl 1X TE.
   - for CEN plasmid use 0.1-1 μl of midi prepped plasmid
   - for transforming a linearized plasmid, use approximately 5 μl of a midi prep to cut, then precipitate.
   - for transforming Pringle PCR constructs use 500 μl of PCR product, precipitated.
5. Aliquot 300 μl of PEG solution per transformation
   Add to it:
   - 10μl 10mg/ml sssssDNA
   - Resuspended DNA
   - 50μl of prepared yeast cells.
   - mix by quick vortex
6. Incubate rotating at 30°C for 30’ (if temperature sensitive, use room temperature
8. Centrifuge at 3000 rpm. Resuspend gently in 200 μl 1X TE. Plate on selective media. If using kanMX, plate on YPD ON, then replica plate to G418 plates.

Solutions:

10mg/ml salmon sperm DNA
10X LiAc (1.0M, pH 7.0)
50% polyethylene glycol MW 3,350
10X TE (0.1M Tris pH 7.5, 10mM EDTA)
9. Pre-Absorbing Antibodies for Immunofluorescence

Note: this is to pre-absorb monoclonal antibodies for tags (MYC, HA, etc) as well as secondary antibodies.

I. Making Fixed Cells:

1. Grow 100 mls wild-type yeast (ie haploid W303) overnight, YPD, room temp.
2. Harvest mid-log phase cells (OD$_{600}$ around 0.6-1.0) in 2X 50ml Falcon tubes, bench top rotor 3-4,000rpm.
3. Resuspend cells in 2X 50 mls 3.7% formaldehyde, O.1M Kphos
4. Fix overnight at 4 degrees, rotating to prevent cells from settling.
5. Wash cells 3X with 2X 50 ml 0.1M Kphos buffer, 1X 1.2M Sorbitol buffer
6. Resuspend cells in 2X 10 ml 1.2M Sorbitol buffer.
7. Spheroplast cells by adding 2 X 100 ul 10mg/ml zymolyase, 2 X 1ml glusulase
8. Digest for 1 hour at 30°C. Check after by adding 3ul of 1% SDS to 3ul cells on a slide and check to make sure cells lyse. If not, digest longer.
9. Note: After cells are spheroplasted only centrifuge at low speed (1,000rpm).
10. Wash cells 1X with 2X 50 ml 1.2M Sorbitol buffer. Centrifuge at 1000rpm for 5minutes.
11. Resuspend prepared cells gently in approximately 5-10 mls 1.2 M Sorbitol buffer
   (Note: this part is rather qualitative, you want 200ul of cells to have a pellet about the size of 2mls of OD$_{600}$ 0.6)
12. Freeze in 1ml aliquots, -20 degrees

II. Pre-absorption:

1. Thaw antibody and aliquot in 200µl aliquots in eppendorf tubes.
2. Thaw fixed, spheroplasted cells.
3. Add 200µl of fixed, well resuspended cells to 200µl antibody.
4. Incubate rotation at room temp for 20 min.
5. Centrifuge antibody + cell mixture, transfer supernatant to new eppendorf tube.
6. Add 200ul more cells and repeat for a total of 5 times. (that is a 6 fold dilution of antibody).
7. After pre-absorbing antibody, mix the supernatant of all aliquots and add Na azide.
8. Aliquot, and freeze pre-absorbed antibody (label as “pre-absorbed, 1/6 dilution”).
Solutions:

Note: it is important to filter solutions for IF to get rid of particulate matter

0.1M Kphosphate buffer pH 6.4:
First make 1M solutions of Kphosphate dibasic and monobasic,
Mix 27.8ml 1M $K_2HPO_4$ + 72.2ml 1M $KH_2PO_4$ and 900ml $H_2O$.
Filter sterilize.

3.7% formaldehyde, 0.1M Kphosphate, pH 6.4:
Mix 9 parts 0.1M Kphosphate and 1 part 37% formaldehyde
Mix just prior to use, this goes bad within a couple days

1.2M Sorbitol, 0.1M phos-cit pH5.9:
1.2 M Sorbitol in 0.1M phos-cit
0.1M phos-cit : 2.28g $K_2HPO_4.3H_2O$, 0.7g citric acid per 100ml.
Filter sterilize

10. Immunofluorescence:

1. Collect at least 1ml of cells at OD$_{600}$ ~ 0.4.
2. Spin down, remove sup and resuspend in 1ml of 3.7% formaldehyde (37% formaldehyde in 0.1M KPhos pH 6.4.). Fix O/N at 4° C.
3. Wash 3X in 1ml of 0.1M KPhos pH 6.4.
4. Wash 1X in 1ml of 1.2M sorbitol-citrate.
5. Resuspend in 200µl of 1.2M sorbitol-citrate Store at -20.
6. Add 20µl glusulase, 2µl zymolyase to each 200µl. Rotate at 30° C for 30 min.
7. Prepare slide chamber by wrapping a petri dish in aluminum foil and put a wet paper towel inside.
8. Slides: use 30 well slides from Erie Scientific (ER-212W). Scrub slides with scratchy sponge to remove red background. Prepare slide by placing 5µl of 0.1% polylysine on each well and let it sit for 5 min
9. Wash slide under distilled water and air dry completely.
10. Check digestion by mixing equal amount of cells and 1% SDS. If done, should see crystals and no cells under light microscope.
11. Centrifuge 3000 rpm. Remove sup and wash with 1ml of sorbitol soln by inversion. DO NOT vortex for pipette mix.
13. Fully resuspend the cells by tapping and add 5µl to each well. DO NOT touch well with tip. Let cells sit for 10 min.
14. Remove sup from each well by placing vacuum tip to the side of the well. DO NOT touch the well.
15. Check cell density under light microscope. Add more cell if necessary. See step 12 & 13.
16. Put slide in -20°C MeOH bath for 3 min, followed by 10 sec in -20°C acetone bath. Let it dry completely.
17. Add 5μl of primary antibody to each well. Incubate in wet chamber for 1-2 hr.
18. Remove antibody and wash 5X in PBS/BSA. Lay down a drop and remove if with vacuum line. DO NOT touch well.
19. Add 5μl of secondary antibody, for 1-2 hours.
20. Remove sup and wash 5X in PBS/BSA.
21. Remove slide from wet chamber and add 3μl of DAPI-MOUNT to each sample.
22. Put cover slip on and lightly wipe the coverslip with kimwipe to press out bubbles.
23. Paint with nail polish around the edges of the cover slip and let it dry for 10 min.

Antibody Conditions:

(mix α-tubulin with others for co-staining)
Tubulin: 1° 1/200 α-tubulin rat primary (2° 1/100 α-rat FITC donkey) (if doing co-staining with Cdc14, use 1/100)
Tem1-3MYC: 1° 1/750 pre-absorbed α-mouse MYC (9E10) (2° 1/1000 α-mouse Cy3)
*NOTE: Tem1 staining works best if use 1 hour incubations for both 1° and 2°
Bfa1-3HA: 1° 1/100 pre-absorbed α-mouse HA (HA.11) (2° 1/250 α–mouse Cy3)
Lte1-3HA: 1° 1/300 pre-absorbed α-mouse HA (HA.11) (2° 1/2000 α–mouse Cy3)
Dbf2-3MYC: 1° 1/1000 pre-absorbed α-mouse MYC (9E10) (2° 1/1000 α-mouse Cy3)
Cdc14-3HA: 1° 1/150 pre-absorbed α-mouse HA (HA.11) (2° 1/1000 α–mouse Cy3)

Antibodies:
1° YOL1/34 Oxford Biotechnology MAS 078p purified 0.5mg/ml
1° anti-HA.11 Covance
1° anti-MYC 9E10 Covance
2° Donkey Anti-Rat IgG FITC-conjugated minimal cross reaction, Jackson 712-095-153
2° Anti- Mouse IgG (min X rat ) FITC Jackson ImmunoResearch 115-095-100
2° Donkey Anti-mouse IgG(min X mouse) Cy3 conjugated Jackson Immuno Research 715-165-151
Solutions:

0.1M KPhos pH 6.4 (27.8ml K$_2$HPO4 + 72.2ml KH$_2$PO4 + 900ml dH$_2$O)

1.2M Sorbitol-citrate (1.2M Sorbitol+ 2.28g K$_2$HPO4.3H$_2$O + 0.7g citric acid per 100 ml)

PBS-BSA: 1% crude BSA, 0.04M K$_2$HPO4, 0.01M KH$_2$PO4, 0.15M NaCl, 0.1% NaN$_3$)

DAPI-mount: 100 mg p-phenylenediamine + 10ml PBS, adjust pH to 8.0 add 90ml glycerol and DAPI at 0.5µg/ml; store long term at -80°C, short term -20°C.

11. Western Blot:

1. Inoculate a 5-10 ml culture overnight.
2. Centrifuge cultures to harvest cells, in the cold room. Transfer to 2ml tube with 1ml of cold TE.
3. Pellet cells. Snap freeze in liquid nitrogen then store at -80.
4. Thaw samples on ice and add 100-200µl of protein breakage buffer.
   a. 1ml of TE (50mM Tris pH 7.5, 1mM EDTA)
   b. 50µl of DTT (1M solution.)
   c. Other protease inhibitors: 40µg/ml Aprotinin, 10µg/ml pepstatin A, 10µg/ml leupeptin, 10µg/ml TLCK, 10µg/ml TPCK, 1mM Pefablock.
5. Add 1 volume of glass beads. Put on multivortexer for 10 min.
6. Prepare marker by taking 10ul of rainbow marker and add 5ul of 3X SDS sample buffer. Punch hole in cap and store on ice. Turn on boiling water bath.
7. Protein concentration determination:
   a. 20µl of 50mM TRIS pH 7.5; 0.3M NaCl
   b. Add 10µl of extracts.
8. To remainder of extract add 50µl of 3X SDS sample buffer. Punch hole in cap and boil for 5 min along with rainbow marker.
9. Spin down for 5 min after boiling and transfer supernatant to fresh eppendorf tube. Store on ice.
10. Finishing protein concentration determination.
    a. Spin down for 3 min.
    b. Dilute Bradford protein dye 1+4 with water and aliquot 1ml to 2ml cuvettes.
    c. Add 3ul of extract to each sample.
    d. Take OD$_{595}$. (Determine slope of BSA titration curve for spectrophotometer used, for Amon lab protein concentration in mg/ml = OD$_{595}$/0.043.)
    e. Calculate volume for 50µg of protein to load on to SDS PAGE gel.
12. Transfer and Western: (Western done in PBST)
   i. Transfer for 2h15'. Use 10% MeOH + 1X running buffer (Note: this is what I use which has more SDS than the “transfer buffer”)
   ii. Cut out IgG below 60kD if possible.
   iii. Block in 5% dry milk for 1hr.
iv. Primary antibody 1hour-O/N 4°C-this is essential for good detection of Co-
Ipd band.
(α-HA-11 mouse (Covance) 1:1000, α-MYC mouse 1:500 (Covance), α-
MYC rabbit (Gramsch)1:500, α-GFP mouse (Chemicon) or rabbit (AbCam)
1:1000).
v. Secondary antibody 1hour-10hrs. (α-mouse HIRP or α-rabbit HRP1:2000-
5000).
vi. Note: I use 2% BSA for rabbit MYC and GFP antibodies.
vii.Use Supersignal West Pico Chemilumiscence from Pierce to detect

10 x PBS: NaCl 80gr
pH7 KCl 2gr
Na2HPO4 11.5gr
KH2PO4 2gr
to 1 liter with water

12. SDS PAGE gels

Separation Gel: (10% gel, for 30mls)
(#mls of acrylamide =percent, adjust with dH2O volume to make different
percents)

7.5 ml 4X separation buffer
10 ml acrylamide (Protogel, National Diagnostics, 30% acrylamide, 0.8% bis)
12.34 µl dH2O
300 µl 10% ammonium persulfate
15 µl TEMED
• cover with isopropanol

Stacking Gel: (for 15 mls)

7.5 mls 2X stacking gel
2 mls acrylamide
5.3 mls dH2O
150 µl 10% ammonium persulfate
10 µl TEMED

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**Solutions:**

4X Separation Buffer (2L):
- Trizma base (must use base!) 363.3g
- Dissolve in 1.2L dH₂O
- pH to 8.8 with glacial acetic acid
- 8g of SDS
- add dH₂O to 2L

2X Stacking Buffer (2L)
- Trizma base (must use base!) 60.55g
- Dissolve in 1.0 L dH₂O
- pH to 6.8 with glacial acetic acid
- 4g of SDS
- add dH₂O to 2L

**13. Immunoprecipitation and Co-immunoprecipitation:**

1. Grow 50ml culture O/N to an OD600 = 0.5-0.9

In Cold Room:

2. Centrifuge cells 3000 rpm for 5 min at 4°C. Resuspend in 1ml of 10mM Tris and transfer to 2ml tube.
3. Centrifuge and aspirate the supernatant. *Can be snap freeze in liquid N₂ for processing later.*
4. Resuspend pellet in 200µl of NP40 lysis buffer + inhibitors. Make sure pellet is fully resuspended and thawed. Add equal amount of beads.
5. Breaks cells on multivortexer for 7', 1'ice, 3'.
6. Spin down at max speed for 5 min. Transfer supernatant to new tube. Avoid debris on the bottom and lipids on surface.
7. Spin down at max speed for 10 min. Transfer supernatant to new eppendorf tube and leave 4°C.
   v. Bradford Assay (5µl extract + 20µl 50mM TRIS pH 7.5, 0.3M NaCl, add 5 µl to 1ml Bradford reagent)
   vi. Perform. IP 500µg-1mg of extract in 200µl volume if possible. Add lysis buffer so that all tubes are at the same volume. (For Co-IP, 1mg should be used!!)
8. Add primary antibody at a concentration of 1:50µl (4µl). Tap mix and leave at 4°C for 1hr.
9. Add 25µl of protein G bead slurry using cut tips. Tap mix and leave on rotating rack for 2 hr.
10. Centrifuge at 3000 rpm for 2.5 min. Remove ~150µl or as much as possible. (**This is important to do to reduce non-specific background as much as possible**)

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11. Add 1ml of NP40 buffer. Tap mix and spin down at 2500rpm for 2.5min. Aspirate the supernatant, leaving ~50μl of liquid above the beads. DO NOT DISTURB THE BEADS.

12. Wash another 4 times in NP40 buffer as in step 12. (A total of 5X 1ml washes)

13. After removing ~900μl of last wash, centrifuge at 2500rpm for 2 min. Remove residual liquid with p200.

14. DO NOT DISTURB THE BEADS.

15. Transfer and Western: (Western done in PBST)

   i. Transfer for 2h15'. Use 10% MeOH + 1X SDS PAGE running buffer.
   ii. Cut out IgG below 60kD if possible.
   iii. Block in 5% dry milk for 1hr.
   iv. Primary antibody O/N 4°C-this is essential for seeing good CoIPd band.
       (α-HA-11 mouse (Covance)1:1000, α-MYC mouse1:500 (Covance), α-
       MYC rabbit (Grampsch)1:500, α-GFP mouse (Chemicon) or rabbit (AbCam)
       1:1000).
   v. Secondary antibody >5hr. (α-mouse HRP or α-rabbit HRP1:2000).
       Note: I use 2% BSA for rabbit MYC and GFP antibodies.
       Use Supersignal West Pico Chemilumiscence from Pierce to detect HRP conjugate.

Solutions:

NP40 Buffer: 150mM NaCl, 50mM Tris pH7.5, 1% NP40

NP40 Buffer + Inhibitors: 10ml NP40 + 40μg/ml Aprotinin, 1mM DTT, 10μg/ml pepstatin A, 10μg/ml leupeptin, 10μg/ml TLCK, 10μg/ml TPCK, 1mM Pefablock.

SDS PAGE running buffer: 0.1% SDS, 250mM glycine, 25mM Tris, pH 8.3 (make with Tris base!!)

Note: I do not use phosphatase inhibitors for IP/CoIP.
14. IP Phosphatase Treatment of Phosphorylated proteins:

1. Immunoprecipitate protein of interest as described above.
2. Substitute last two washes with: 1ml of 1x CIP (calf intestinal phosphatase) buffer (dilute in H₂O from stock).
3. Spin down again at 2500rpm for 2.5min. Remove residual with p200. DO NOT DISTURB THE BEADS.
4. Resuspend in 40μl of 1x cip buffer. Add 5μl of cip to sample. 5μl of dH₂O to control.
5. Incubate at 37°C for 1hr.
6. Wash 2X in 1ml of NP40 buffer.
7. Remove residual supernatant.
8. Resuspend in 30μl of 3X sample buffer. Boil for 3min.
9. Spin down max speed for 5min. Load on appropriate percentage of gel.

15. In Vitro Kinase Assay (Clb2, Dbf2-3MYC):

1. Grow 15-50ml culture O/N to an OD600 = 0.5-0.9

In Cold Room:

2. Spin down cells 3000 rpm for 5 min at 4°C. Resuspend in 1ml of 10mM Tris and transfer to 2ml tube.
3. Spin down and aspirate the supernatant. Can be snap freeze in liquid N2 for processing later.
4. Resuspend pellet in 100-200μl of NP40 lysis buffer + inhibitors. Make sure pellet is fully resuspended and thawed. Add equal amount of beads.
5. Breaks cells on multivortexer for 7', 1'ice, 3'.
6. Spin down at max speed for 5 min. Transfer supernatant to new eppendorf tube. Avoid debris on the bottom.
7. Spin down at max speed for 10 min. Transfer supernatant to new eppendorf tube and leave 4°C.
8. Bradford Assay (5μl extract + 20μl 50mM TRIS pH 7.5, 0.3M NaCl, add 5 μl to 1ml Bradford reagent)
9. Perform. IP (50-150)μg of extract in 50-100μl volume if possible. Add lysis buffer so that all tubes are at the same volume.
10. Add primary antibody at a conc. of 1:50μl (R98 for Clb2, αMYC 9E10 for Dbf2-3MYC). Tap mix and leave at 4°C for 1hr.
11. Add 15-20μl of IgA bead slurry for Clb2 using cut tips. Tap mix and leave on rotating rack for 2 hr.
12. Add 1ml of NP40 buffer. Tap mix and spin down at 2500rpm for 2.5min. Aspirate the supernatant, leaving ~50μl of liquid above the beads. DO NOT DISTURB THE BEADS.
13. Wash another 3 times in NP40 buffer as in step 12.

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14. Wash 2 times in 25mM MOPS as in step 12.
15. Spin down at 2500rpm for 2 min. Remove residual liquid with p200.
16. DO NOT DISTURB THE BEADS.
17. Add 6μl of HBII buffer and leave on the bench at RT for 15min. Prepare kinase cocktail.
18. Add 10μl of kinase reaction mix to beads. Tap mix every 5min for 15min.
19. Add 12μl of 3X sample buffer to stop reaction. Puncture hole on top of tube and boil for 3 min.
20. Spin down at max speed for 5 min. Can be store at -20°C for later processing.
   If samples are store at -20°C, spin down at max speed for 5min before loading.
21. Load on 15% gel and run at 65mA for 3-3.5 hr (3h12') until free ATP band is close to the end.
   DO NOT LET FREE ATP RUN OUT.
22. Cut out stacking gel and free ATP and discard in radioactive waster. Fix gel in 10% methanol, 10% acetic acid for 20-30min. Prepare gel dryer.
23. Dry gel for 1.5hr.
24. Expose in cassette with intensifier screen.

Solutions:

NP40 Buffer: 150mM NaCl, 50mM Tris pH7.5, 1% NP40

NP40 Buffer + Inhibitors: 9ml NP40 + 600μl β-glycerophosphate, 0.1mM Na Orthovanadate, 15mM p-Nitrophenylphosphate, 40μg/ml Aprotinin, 1mM DTT, 10μg/ml pepstatin A, 10μg/ml leupeptin, 10μg/ml TLCK, 10μg/ml TPCK, 1mM Pefablock.

MOPS: 25mM, 50mM pH 7.0

HBII Buffer: 60mM β-glycerolphosphate, 15mM MgCl2, 5mM EGTA, 1mM DTT, 1mM Pefablock, 20μg/ml leupeptin, 40μg/ml Aprotinin, 0.1mM Na Orthovanadate.

Kinase Cocktail: Mix equal amounts of SOL1 and 2.

SOL1: 1. 1.1μl of 100mM cold ATP
   2. 275μl of water.
   3. 3μl of 6000Ci/mmol gamma-ATP.
SOL2: 4mg/ml Histone H1 in 50mM MOPS.
16. *In Vitro* Cdc15 Kinase Assay

1. Grow 50ml culture O/N to an OD600 = 0.5-0.9

In Cold Room:

2. Centrifuge cells 3000 rpm for 5 min at 4°C. Resuspend in 1ml of 10 mM Tris and transfer to 2ml tube.
3. Spin down and aspirate the supernatant. *Can be snap freeze in liquid N2 for processing later.*
4. Resuspend pellet in 200μl of NP40 lysis buffer + inhibitors. Make sure pellet is fully resuspended and thawed. Add equal amount of beads.
5. Breaks cells on multivortexer for 7’, 1’ice, 3’.
6. Spin down at max speed for 5 min. Transfer sup to new eppendorf tube. Avoid debris on the bottom.
7. Spin down at max speed for 10 min. Transfer sup to new eppendorf tube and leave 4°C.
8. Bradford Assay (5μl extract + 20μl 50mM TRIS pH 7.5, 0.3M NaCl, add 5 μl to 1ml Bradford reagent)
9. Perform. IP 500μg-1mg of extract in 200μl volume if possible. Add lysis buffer so that all tubes are at the same volume.
10. Add primary antibody (use either anti-GFP (Chemicon) or anti-HA mouse) at a conc. of 1:50μl. Tap mix and leave at 4°C for 1hr.
11. Add 20μl of protein G bead slurry using cut tips. Tap mix and leave on rotating rack for 2 hr.
12. Add 1ml of NP40 buffer. Tap mix and spin down at 2500rpm for 2.5min. Aspirate the supernatant, leaving ~50μl of liquid above the beads. **DO NOT DISTURB THE BEADS.**
13. Wash another 3 times in NP40 buffer as in step 12.
14. Wash 2 times in 50mM HEPES pH 7.4 as in step 12.
15. Spin down at 2500rpm for 2 min. Remove residual liquid with p200.
16. **DO NOT DISTURB THE BEADS.**
17. Add 5μl of 15A buffer and leave on the bench at RT for 15min. Prepare kinase cocktail.
18. Add 10μl of kinase reaction mix to beads. Tap mix every 5min for 15min.
19. Add 12μl of 3X sample buffer to stop reaction. Puncture hole on top of tube and boil for 3 min.
20. Spin down at max speed for 5 min. *Can be store at −20°C for later processing.* . *If samples are store at −20°C, spin down at max speed for 5min before loading.*
21. Load on 15% gel and run at 65mA for 3-3.5 hr (3h12”) until free ATP band is close to the end.
**DO NOT LET FREE ATP RUN OUT.**
22. Cut out stacking gel and free ATP and discard in radioactive waste. Can cut gel at 30kDa in order to transfer (prepare transfer as normal) the top part as an IP
control. Can save gel after transfer to dry and look at remaining protein in gel or autophosphorylation. Fix (below 30kDa or whole gel) gel in 10% methanol, 10% acetic acid for 20-30min. Prepare gel dryer.

23. Dry gel for 1.5hr.
24. Expose in cassette with screen.

Solutions:

NP40 Buffer: 150mM NaCl, 50mM Tris pH7.5, 1% NP40

NP40 Buffer + Inhibitors: 9ml NP40 + 600μl β-glycerophosphate, 0.1mM Na Orthovanadate, 15mM p-Nitrophenylphosphate, 40μg/ml Aprotinin, 1mM DTT, 10μg/ml pepstatin A, 10μg/ml leupeptin, 10μg/ml TLCK, 10μg/ml TPCK, 1mM Pefablock.

15A Buffer (7X): 350mM HEPES pH 7.4, 60mM β-glycerolphosphate, 35mM MgCl2, 17.5mM MnCl2, 7mM DTT, 1mM Pefablock. (Should be added to 20μl beads, then 10μl of cocktail, so 1X final after cocktail)

Kinase Cocktail: Mix equal amounts of SOL1 and 2.

15-SOL1: 1. 1.1μl of 100mM cold ATP  
2. 195.4μm of 50mM HEPES pH 7.4  
3. 3μl of 6000Ci/mmol gamma-ATP.

15-SOL2: 20μl of 5mg/ml MBP + 230μl 50mM HEPES pH 7.4

17. BAR-test / alpha factor sensitivity

1. Resuspend cells (one streak through a patch) in 30μl of TE.
2. Boil 0.8% agar in YPD. (Use big bottle since it tends to boil over).
3. Let it cool down to until its warm to the touch. Mix 5ml of agar/YPD with 10μl of cells.
4. Shake to resuspend and pour into small petri dish.
5. Put petri dish on ice until it solidifies.
6. Use a toothpick to put a small dot of alpha tester strain in the center of dish.
7. BAR+ strains will produce a halo around central tester strain where they cannot grow.
18. Alpha Factor G1 Block and Release

Procedure:

1. Dilute exponentially growing cells to OD600=0.2.
2. Add 5μg/ml, α-factor. (If using bar1 cells instead add 0.5μg/ml [0.3 μM] α-factor).
3. Examine cells 60 minutes after pheromone addition to determine how many cells are arrested in G1 (cells are unbudded and form a mating projection). Repeat every 15 minutes until fully arrested. At 30°C in YEPD cells are usually arrested in G1 90 - 120 minutes after pheromone addition.
4. When more than 90% of cells are arrested in G1, filter cells through vacuum filter (Millipore or Nalgene makes large vacuum filters).
5. Wash with 5 culture volumes.
6. Place filter into medium and shake to remove cells.
7. Time points are taken at the desired times after release. Monitor synchrony of the release and cell cycle progression. In a good release more than 75 percent of cells should have small buds 45 minutes after transfer into fresh medium. Cells will initially be clumpy and this will stop once cells have released from G1 block.
8. Evaluation of cell cycle progression and cell synchrony
   •Budding index: Fix cell samples with 70% EtOH and count % cells with a bud at each timepoint.
   •DNA content analysis: Use flow cytometry (FACS) to analyze the proportion of cells with a 1C and 2C DNA content.
   •Mitotic spindle formation: Visualize the mitotic spindle using immunofluorescence with anti-tubulin antibody.

Reagents and equipment:

Purified, synthesized α-factor pheromone peptide (soluble in DMSO, stored at -20°C)
NH₃ - Trp - His - Trp - Leu - Gln - Leu - Lys - Pro - Gly - Gln - Pro - Met - Tyr - COOH
Dissolved to 5mg/ml
Filtration apparatus (Millipore)
Circular nitrocellulose filters
Microscope
19. Hydroxyurea and nocodazole block-release

Arrest in early S phase can be obtained using hydroxyurea which blocks ribonucleotide reductase. A metaphase arrest can be induced using nocodazole which depolymerizes microtubules leading to cell cycle arrest in metaphase. Removal of the drugs will cause cells to release from the block.

**Method:**

1. Dilute exponentially growing cells to OD₆₀₀=0.2 - 0.5 and add 10 mg/ml (130 mM) of solid hydroxyurea to cultures. To arrest cells with nocodazole, 15μg/ml nocodazole (50μM, dissolved in DMSO) is added to cultures. 60 minutes after addition of the drug cells are examined under the microscope to assess the percentage of arrested cells (arrested cells appear dumbbell shaped). This is repeated every 15 minutes thereafter. At 30°C in YEPD cells are usually arrested 90 - 120 min after addition of the drug.

2. When more than 90% of cells are arrested, remove medium by filtration. Cells are then washed with 5 culture volumes either by filtration or centrifugation.

**NOTE:** When releasing cells from a nocodazole arrest it is critical that the medium used for washing cells contains 1% DMSO.

3. Place the nitrocellulose filter containing cells in a flask with fresh medium (for nocodazole block - release the medium should contain 1% DMSO) and shaken vigorously to detach cells from the filter.

4. Time points are taken at the desired times after release. The synchrony of the release and cell cycle progression are monitored as described for α-factor block - release.

**Comments:**

The synchrony of cultures after release obtained with these methods is not nearly as good as that achieved with an α-factor block - release experiment or elutriation.

**Reagents and equipment:**

Hydroxyurea (Sigma) or nocodazole (Sigma, 1.5 mg/ml stock dissolved in DMSO, stored at -80°C)
DMSO (for nocodazole block – release only)
Filtration apparatus (Millipore)
Circular nitrocellulose filters
Microscope