Cdc15 integrates Tem1 GTPase-mediated spatial signals with Polo kinase-mediated temporal cues to activate mitotic exit

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Cdc15 Integrates Tem1 GTPase-mediated Spatial Signals with Polo kinase-mediated Temporal Cues to Activate Mitotic Exit

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Abstract

In budding yeast, a Ras-like GTPase signaling cascade known as the Mitotic Exit Network (MEN) promotes exit from mitosis. To ensure the accurate execution of mitosis, MEN activity is coordinated with other cellular events and restricted to anaphase. The MEN GTPase Tem1 has been assumed to be the central switch in MEN regulation. We show here that during an unperturbed cell cycle, restricting MEN activity to anaphase can occur in a Tem1 GTPase-independent manner. We find that the anaphase-specific activation of the MEN in the absence of Tem1 is controlled by the Polo kinase Cdc5. We further show that both Tem1 and Cdc5 are required to recruit the MEN kinase Cdc15 to spindle pole bodies, which is both necessary and sufficient to induce MEN signaling. Thus, Cdc15 functions as a coincidence detector of two essential cell cycle oscillators: the Polo kinase Cdc5 synthesis/degradation cycle and the Tem1 G-protein cycle. The Cdc15-dependent integration of these temporal (Cdc5 and Tem1 activity) and spatial (Tem1 activity) signals ensures that exit from mitosis occurs only after proper genome partitioning.
Introduction

The creation of a daughter cell requires the faithful duplication and segregation of the genome. The success of this process necessitates the temporal and spatial coordination of genome segregation with the final cell cycle transition, exit from mitosis, when the mitotic spindle is disassembled, nuclei are reformed, and cytokinesis splits the cell into two. In the absence of such coordination, significant genetic and epigenetic changes occur. Thus, as might be expected, the inability to coordinate genome segregation with exit from mitosis is strongly associated with cancer (Kops et al. 2005; Gonzalez 2007).

In budding yeast, exit from mitosis is controlled by the essential protein phosphatase Cdc14. Cdc14 antagonizes mitotic cyclin dependent kinases (Clb-CDKs), the inactivation of which is essential for exit from mitosis (Jaspersen et al. 1998; Visintin et al. 1998; Zachariae et al. 1998). Cdc14 activity is tightly regulated. In cell cycle stages prior to anaphase, Cdc14 is sequestered within the nucleolus as a result of its association with its nucleolar-localized inhibitor Cfi1/Net1 (Shou et al. 1999; Visintin et al. 1999). Upon anaphase entry, Cdc14 is released from the nucleolus and spreads throughout the nucleus and, to a significantly lesser extent, the cytoplasm. This early anaphase release of Cdc14 is mediated by the FEAR network and results in a pulse of Cdc14 activity (Pereira et al. 2002; Stegmeier et al. 2002; Yoshida et al. 2002). While not essential, FEAR network-mediated release of Cdc14 from the nucleolus is crucial for the accurate execution of anaphase (Rock and Amon 2009). Cdc14 release from the nucleolus during late anaphase is promoted by the Mitotic Exit Network (MEN), which drives the sustained release of Cdc14 in both the nucleus and the cytoplasm and results in exit from mitosis (Stegmeier and Amon 2004).

The MEN is a Ras-like GTPase signal transduction cascade (see Figure 7B for a pathway diagram, reviewed in (Stegmeier and Amon 2004). As in other G-protein
signaling pathways, the GTPase Tem1 is thought to be the central switch regulating MEN activity (Cooper and Nelson 2006; Wang and Ng 2006; Geymonat et al. 2009; Chan and Amon 2010). Tem1 is negatively regulated by its two-component GTPase activating protein (GAP) Bub2-Bfa1. The Bub2-Bfa1 complex is regulated by two protein kinases. The Polo kinase Cdc5 phosphorylates Bfa1, which reduces Bub2-Bfa1 GAP activity. The protein kinase Kin4 functions in opposition to Cdc5, phosphorylating Bfa1 and thus rendering the GAP insensitive to Cdc5-dependent inhibitory phosphorylation (Maekawa et al. 2007). Tem1 is positively regulated by Lte1, which inhibits Kin4 in the bud (Bertazzi et al. 2011; Falk et al. 2011).

During late anaphase, Tem1-GTP is thought to bind to and activate the protein kinase Cdc15, which then activates the downstream kinase Dbf2 associated with its activating subunit Mob1. Based on binding data and homology to known scaffolds, Nud1 is thought to function as a scaffold for the core MEN components Tem1, Bub2-Bfa1, Cdc15, and Dbf2-Mob1 at spindle pole bodies (SPBs) (Gruneberg et al. 2000; Valerio-Santiago and Monje-Casas 2011). Tem1 SPB localization is essential for MEN activation and it is thought that SPB localization of Cdc15, Dbf2, and Mob1 is also essential (Valerio-Santiago and Monje-Casas 2011). Activation of Dbf2-Mob1 results, at least in part, in the phosphorylation of Cdc14’s nuclear localization sequence, and causes the retention of Cdc14 in the cytoplasm where it can act on its substrates (Mohl et al. 2009). Activation of the MEN in late anaphase is essential for the sustained release of Cdc14 from the nucleolus, which ultimately promotes exit from mitosis.

The mechanisms by which MEN activity and exit from mitosis are temporally and spatially coordinated with genome segregation are beginning to be understood. MEN activity is controlled by spindle position. When the anaphase spindle is not correctly aligned along the mother – daughter cell axis, MEN signaling is inhibited (Yeh et al. 1995). This spindle position control of MEN signaling is accomplished by a system.
composed of a MEN-inhibitory and a MEN-activating zone, and a sensor that moves between them. The MEN inhibitor Kin4 is located in the mother cell, the MEN activator Lte1 in the bud, and the MEN GTPase Tem1 is localized to the SPB (Bardin et al. 2000; Pereira et al. 2000; D’Aquino et al. 2005; Maekawa et al. 2007). Only when the MEN-bearing SPB escapes the MEN inhibitor Kin4 in the mother cell and moves into the bud where the MEN activator Lte1 resides can exit from mitosis occur. In this manner, spatial information is sensed and translated to regulate MEN activity.

Spindle position cannot be the only event controlling MEN activity as exit from mitosis occurs at the appropriate time in \textit{lte1}\textDelta or \textit{kin4}\textDelta cells with correctly positioned spindles. Here, we describe the identification of a novel role for Cdc5 in regulating the timing of MEN activation. Interestingly, this essential Cdc5-dependent MEN activating signal does not regulate the GTPase Tem1, but rather the Tem1-effector Cdc15. We find that Cdc5 is essential for the anaphase-specific recruitment of Cdc15 to SPBs. Furthermore, the artificial targeting of Cdc15 to the SPB bypasses the requirement for both Tem1 and Cdc5 in MEN activation. Our results indicate that multiple signals converge on the MEN effector kinase Cdc15 to integrate spatial (spindle position) and temporal (Cdc5 activation) cues with mitotic exit. Thus, Cdc15 functions as a coincidence detector, integrating spatial and temporal signals to ensure that exit from mitosis only occurs after proper genome partitioning.

Results

\textit{LTE1} and \textit{KIN4}-Independent Activation of the MEN in Anaphase

\textit{LTE1} and \textit{KIN4} are the central mediators of MEN regulation by spindle position (Bardin et al. 2000; Pereira et al. 2000; Castillon et al. 2003; D’Aquino et al. 2005; Pereira and Schiebel 2005; Geymonat et al. 2009; Bertazzi et al. 2011; Falk et al. 2011). The subcellular partitioning of these two proteins ensures that cells that have a mis-
positioned anaphase spindle do not prematurely activate the MEN. It is unclear, however, whether LTE1 and KIN4 are also important for regulating the proper timing of MEN activation in cells where spindles are correctly aligned along the mother-bud axis. To address this question, we examined the consequences of deleting KIN4 and LTE1 on MEN activity. Cells were arrested in G1 using pheromone and then released to allow them to progress through the cell cycle in a synchronous manner. MEN activity was monitored by measuring the kinase activity of the most downstream MEN kinase Dbf2-Mob1. Dbf2 kinase activity was restricted to anaphase in wild-type cells (Toyn and Johnston 1994), Figure 1A,B). Similar results were obtained in lte1Δ kin4Δ cells (Figure 1A,B). Thus, there must exist Kin4 and Lte1-independent mechanisms that restrict MEN activity to anaphase in cells with correctly positioned spindles.

Anaphase-Specific Activation of the MEN in the Absence of TEM1

Our data indicate that regulatory mechanisms other than spindle position control MEN activity. To identify these signals we first asked whether, as in other GTPase signaling cascades, all critical MEN regulation is mediated by the GTPase Tem1. To this end, we measured Dbf2 kinase activity in cells lacking the essential MEN GTPase Tem1 but kept alive by overexpression of CDC15 (henceforth tem1Δ CDC15-UP; Pereira et al. 2000)). Surprisingly, growth of tem1Δ CDC15-UP cells was indistinguishable from that of wild-type cells (Figure 1C) and cell cycle progression occurred with near wild-type kinetics (Figure 1D). Even more remarkable was the observation that Dbf2 kinase activity remained restricted to anaphase in tem1Δ CDC15-UP cells, although activation was slightly delayed (Figure 1E,F).

Control of MEN activity by spindle position was, however, lost in the tem1Δ CDC15-UP strain. Cells lacking cytoplasmic dynein (dyn1Δ cells) frequently mis-position their spindles at low temperature and arrest in anaphase because the MEN GTPase
Tem1 is inhibited by Bub2-Bfa1 (reviewed in (Fraschini et al. 2008)). When the GAP is inactivated by deleting \textit{BUB2} or \textit{BFA1}, cells with mis-positioned spindles will not arrest in anaphase but rather exit mitosis to produce anucleate and multinucleate cells (Bardin et al. 2000; Bloecher et al. 2000; Pereira et al. 2000); Supplemental Figure 1). As in \textit{bub2}\textit{Δ} cells, \textit{tem1}\textit{Δ} \textit{CDC15-UP} cells did not arrest in anaphase in response to spindle mis-position (Supplemental Figure 1). Our data confirm that spindle position control of the MEN is mediated by Tem1. Our data also indicate that the Tem1 GTPase is not the sole switch controlling MEN activity and that there must exist GTPase-independent mechanisms of MEN regulation that restrict Dbf2-Mob1 kinase activity to anaphase in an unperturbed cell cycle.

\textit{The FEAR Network is Not Required for MEN Activity in tem1\textit{Δ} CDC15-UP Cells}

The phosphatase Cdc14 is an activator of the MEN (Jaspersen and Morgan 2000; Stegmeier et al. 2002; Konig et al. 2010). Cdc14 activated by the FEAR network dephosphorylates Cdc15 and Mob1 and thereby promotes their activity (see Figure 7B). Though not essential for MEN activation, inactivation of the FEAR network leads to a delay in MEN activation as judged by Dbf2 kinase activity (Stegmeier et al. 2002). To determine whether the FEAR network was also required for MEN activity in \textit{tem1}\textit{Δ} \textit{CDC15-UP} cells, we examined the consequences of deleting FEAR network components in this strain. \textit{SPO12}, its close homolog \textit{BNS1}, and \textit{SLK19} are components of the FEAR network; loss of function mutations in these genes inactivate the FEAR network and greatly reduce the release of Cdc14 from the nucleolus in early anaphase (Stegmeier et al. 2002; Visintin et al. 2003). Deletion of these FEAR network components did not affect the growth of \textit{tem1}\textit{Δ} \textit{CDC15-UP} cells (Figure 2A). More importantly, inactivation of the essential FEAR network component Separase (Esp1) or the ultimate FEAR network effector Cdc14 had a similar effect on the kinetics of Dbf2
activation in \textit{tem1\Delta CDC15-UP} cells as in wild-type cells. Dbf2 kinase activation was delayed by approximately 10 minutes (Figure 2B-D, Supplemental Figure 2). Our results indicate that the FEAR network regulates MEN activity in \textit{tem1\Delta CDC15-UP} and wild-type cells in a similar manner. Thus, the FEAR network promotes but is not essential for the anaphase-specific activation of the MEN in \textit{tem1\Delta CDC15-UP} cells.

\textbf{Anaphase Entry is Not Required for MEN Activity in the Absence of TEM1}

We next sought to determine the mechanism underlying the GTPase-independent activation of the MEN in anaphase. We first asked whether entry into anaphase was a prerequisite for MEN activation in \textit{tem1\Delta CDC15-UP} cells. The fact that MEN activation occurred with similar kinetics in \textit{tem1\Delta CDC15-UP} and \textit{tem1\Delta CDC15-UP \textit{esp1-1}} cells (Supplemental Figure 2), which cannot undergo anaphase spindle elongation due to an inability to eliminate sister chromatid cohesion, already suggested that spindle elongation was not essential for MEN activation in \textit{tem1\Delta CDC15-UP} cells.

To determine whether other aspects of anaphase entry were necessary for MEN activation, we arrested \textit{tem1\Delta CDC15-UP} cells in metaphase. Entry into anaphase is triggered by the activation of a ubiquitin ligase known as APC/C\textsuperscript{Cdc20}. Activation of the spindle assembly checkpoint by microtubule depolymerization results in the inhibition of APC/C\textsuperscript{Cdc20} and arrests cells in metaphase (Musacchio and Salmon 2007). We found that entry into anaphase was not required for Dbf2-Mob1 activation in \textit{tem1\Delta CDC15-UP} cells. \textit{tem1\Delta CDC15-UP} cells activated Dbf2-Mob1 with nearly identical kinetics in the presence or absence of the microtubule depolymerizing drug nocodazole (Supplemental Figure 3). Similar results were obtained when anaphase entry was blocked by depletion of the APC/C coactivator \textit{CDC20} (J. M. R., unpublished observations). Thus, although MEN activity is restricted to anaphase in an unperturbed cell cycle, anaphase entry is not a prerequisite for MEN activation in \textit{tem1\Delta CDC15-UP} cells. In contrast, anaphase
entry is required to activate the MEN in cells with a wild-type MEN. Dbf2 activation is greatly delayed in cdc23-1 mutants, which are defective in APC/C activity (Visintin and Amon 2001). We conclude that the dependence of MEN activation on anaphase entry is mediated by the MEN GTPase Tem1. However, the observation that MEN activation occurs 70 minutes after pheromone release irrespective of whether tem1Δ CDC15-UP cells enter anaphase (Supplemental Figure 3) indicates that a Tem1 GTPase-independent MEN regulatory timing mechanism must exist. Furthermore, this timing mechanism must be independent of Separase and APC/C<sup>Cdc20</sup> activation.

*Polo kinase Cdc5 Controls MEN Activity in the Absence of TEM1*

The Polo kinase Cdc5 is a key regulator of exit from mitosis (Lee et al. 2005). As a component of the FEAR network, Cdc5 promotes the release of Cdc14 from the nucleolus during early anaphase, which then promotes MEN activity (Stegmeier et al. 2002; Visintin et al. 2003). Cdc5 also regulates the MEN GAP Bub2-Bfa1. Cdc5 phosphorylates Bfa1, which reduces Bub2-Bfa1 GAP activity (Hu et al. 2001; Geymonat et al. 2003). Could Cdc5 have additional roles in regulating the MEN and confer MEN activation in tem1Δ CDC15-UP cells? If Cdc5 was required for MEN activity in tem1Δ CDC15-UP cells, then inactivating CDC5 should abrogate MEN activation. Consistent with this hypothesis, we find that the tem1Δ CDC15-UP allele combination exhibits synthetic lethality with the temperature sensitive cdc5-1 and cdc5-2 alleles at the permissive temperature (data not shown). However, we were able to construct a tem1Δ CDC15-UP cdc5-7 strain. We found that inactivation of CDC5 abolishes the ability of the tem1Δ CDC15-UP strain to activate Dbf2-Mob1 (Figure 3A, B). We conclude that the Polo kinase Cdc5 is essential to activate the MEN in the absence of TEM1.

Is Cdc5 also sufficient for MEN activation in a tem1Δ CDC15-UP strain? Cdc5 protein levels are tightly regulated during the cell cycle. Cdc5 is absent during G1,
begins to accumulate late in S phase, and peaks at the metaphase to anaphase transition. During exit from mitosis, Cdc5 is rapidly degraded by the APC/C<sup>CDh1</sup> (Charles et al. 1998; Cheng et al. 1998; Shirayama et al. 1998). If Cdc5 was limiting for MEN activation in a <i>tem1Δ CDC15-UP</i> strain, then the premature expression of Cdc5 might result in the premature activation of the MEN. To test this hypothesis, we expressed a stable form of Cdc5 (Cdc5Δdb) from the conditional <i>MET25</i> promoter in <i>tem1Δ CDC15-UP</i> cells. We found that the premature accumulation of Cdc5 results in the premature activation of Dbf2-Mob1 in a <i>tem1Δ CDC15-UP</i> strain (Figure 3C, D). It should be noted that the premature activation of Dbf2-Mob1 upon Cdc5Δdb expression is likely due to the premature activation of both the FEAR network and the MEN. Our results demonstrate that Cdc5 is essential for MEN activation in the absence of Tem1 GTPase function. Moreover, Cdc5 is sufficient to stimulate MEN signaling in other stages of the cell cycle.

<i>Cdc5 Promotes Localization of Cdc15 to SPBs</i>

To determine how Cdc5 controls MEN activity in the absence of Tem1, we examined the consequences of modulating Cdc5 activity on Cdc15 localization. Cdc15 localization in wild-type cells is dynamic. During G1, S, G2, and metaphase, Cdc15 is localized diffusely throughout the cytoplasm. Shortly after the metaphase to anaphase transition, Cdc15 localizes to the SPB that is pulled into the daughter and, in late anaphase, is found on both SPBs (Hu et al. 2001; Visintin and Amon 2001; Molk et al. 2004; Konig et al. 2010). Because Cdc15 recruitment to SPBs coincides with MEN activation and depends on <i>TEM1</i>, it is thought that localization of Cdc15 to SPB(s) is essential for MEN function (Visintin and Amon 2001).

Although Cdc15 is highly overexpressed in the <i>tem1Δ CDC15-UP</i> strain (these cells harbor two overexpression constructs: <i>GAL-CDC15</i> and <i>GPD-CDC15</i>), Cdc15 did not localize to SPBs prematurely and association with this organelle remained largely
restricted to anaphase (Figure 4A). The anaphase-restricted Cdc15 SPB localization in \textit{tem1Δ CDC15-UP} cells suggests a simple possible mechanism by which Cdc5 activates the MEN in parallel to Tem1: Cdc5 functions to promote Cdc15 SPB localization. To test this prediction, we followed Cdc15 localization in \textit{tem1Δ CDC15-eGFP-UP} cells containing an inhibitor-sensitive allele of \textit{CDC5} (\textit{cdc5-as1}). In the presence of the inhibitor, Cdc15 is no longer able to localize to SPBs in the \textit{tem1Δ CDC15-eGFP-UP cdc5-as1} cells (Figure 4B). As \textit{CDC5} is sufficient to activate the MEN in the absence of Tem1 (Figure 3D), it might be expected that the premature expression of Cdc5 results in the premature loading of Cdc15 onto SPBs. Indeed, we found that the premature activation of Cdc5 with the \textit{CDC5Δdb} allele led to the premature recruitment of Cdc15 to SPBs in metaphase (Figure 4C). Taken together, these data indicate that \textit{CDC5} functions in parallel to \textit{TEM1} to promote the association of Cdc15 with SPBs.

\textit{Cdc15 Functions as a Coincidence Detector of Tem1 and Cdc5 Activity}

Our data suggest that both \textit{CDC5} and \textit{TEM1} function to promote Cdc15 SPB localization. If true, Cdc15 could function as a coincidence detector of Cdc5 and Tem1 activity. By this model, wild-type levels of Cdc15 might integrate essential inputs from Tem1 and Cdc5, both of which are required for MEN activation. A prediction of this hypothesis is that both Tem1 and Cdc5 should be essential for Cdc15 SPB localization and Dbf2-Mob1 activity in a wild-type cell. We first monitored Cdc15 localization in a strain depleted of Tem1 but wild-type for \textit{CDC5}. Consistent with previously published data, depletion of Tem1 abolishes the localization of Cdc15 to SPBs ((Johnson et al. 1992; Visintin and Amon 2001), Figure 5A). \textit{CDC5} was also essential for Cdc15 association with SPBs. Cdc15 did not localize to SPBs in anaphase cells depleted of Cdc5 (Figure 5B). Similar results were obtained in \textit{bub2Δ} cells depleted of Cdc5 (Figure 5B). Importantly, depletion of Cdc5 did not affect Tem1 localization to the SPB.
(Supplemental Figure 4). These findings exclude the possibility that Cdc5 affects Cdc15 SPB localization indirectly by inactivating the Bub2-Bfa1 GAP complex or perturbing Tem1 SPB localization.

To further validate an essential role for Cdc5 in activating the MEN in wild-type cells, we monitored Dbf2 kinase activity in a synchronous cell cycle in a strain depleted for Cdc5. To control for Cdc5’s role in activating the FEAR network and in inactivating Bub2-Bfa1, these experiments were performed in a *cdc14-3* *bub2Δ* background. The *BUB2* deletion eliminates the role of *CDC5* in MEN GAP down-regulation and the *cdc14-3* mutation eliminates Cdc5-dependent FEAR network activation. As expected, Dbf2 kinase activity peaked in anaphase in the *cdc14-3* *bub2Δ* strain (Figure 5C, D). Consistent with the Cdc15 localization observations, Dbf2-Mob1 was not activated in the *cdc14-3* *bub2Δ* strain depleted of Cdc5 (Figure 5C, D). We conclude that Cdc5 is essential for MEN activation and regulates this pathway at multiple steps. Cdc5 stimulates MEN activity through its role in the FEAR network, it partially inhibits the Tem1 GAP Bub2-Bfa1, and it promotes the localization of Cdc15 to SPBs. Our data further indicate that Cdc15 behaves like a coincidence detector, requiring inputs from both Tem1 and Cdc5 to localize to the SPB and thus activate the MEN.

**Targeting Cdc15 to the SPB Bypasses the Need for Both Tem1 and Cdc5 in MEN Activation**

Localization of Cdc15 to the SPB is thought to be essential for MEN activation (Stegmeier and Amon 2004). Our observations suggest that the essential MEN activating function of both Tem1 and Cdc5 is to promote Cdc15 SPB localization. To test this possibility, we asked whether artificially targeting Cdc15 to SPBs bypasses the need for Tem1 and Cdc5 in MEN activation. We fused the *CDC15-eGFP* open reading frame (ORF) to the ORF of the SPB outer plaque component *CNM67* to generate a Cdc15-
eGFP-Cnm67 fusion protein (hereafter referred to as Cdc15-SPB). Expression of the fusion protein from the *CDC15* promoter is lethal (data not shown). We therefore placed Cdc15-SPB under the transcriptional control of the low-strength conditional *MET3* promoter. Induction of the Cdc15-SPB fusion was toxic (data not shown), but the fusion protein was well tolerated when the *MET3* promoter was repressed. Under these conditions, the Cdc15-SPB fusion protein was detectable by fluorescence microscopy (Figure 6C) but was not detectable by Western blot analysis (Figure 6A, lane marked with asterisk). The fusion protein was nevertheless present at high enough levels under *MET3* repressive conditions to allow the necessary experimental manipulations to follow. We therefore performed all experiments involving this fusion protein under conditions where the *MET3* promoter was repressed.

First, we confirmed the functionality of the fusion. While we were not able to measure kinase activity associated with the Cdc15-SPB fusion protein (presumably because the Cdc15-SPB protein is tightly bound to the SPB and is thus not amenable to standard immunoprecipitation-kinase techniques), the *CDC15-SPB* fusion suppressed the temperature sensitive lethality of cells harboring the *cdc15-2* allele as the sole source of *CDC15* (Figure 6B). Thus, the Cdc15-SPB protein is active as a kinase and is capable of performing the essential function of Cdc15. The fusion protein also exhibited the expected localization pattern. Cdc15-SPB localizes to the SPB constitutively throughout the cell cycle (Figure 6C, Supplemental Figure 5). To determine whether the Cdc15-SPB fusion can support the essential functions of *TEM1* and *CDC5* in MEN activation, we constructed a *tem1Δ GAL-URL-3HA-CDC5 CDC15-SPB* strain in which *TEM1* was deleted and Cdc5 could be efficiently depleted (Bachmair et al. 1986). We found that *tem1Δ* cells are viable when they harbor the *CDC15-SPB* fusion (Figure 6D), thus the essential function of *TEM1* can be bypassed by the *CDC15-SPB* allele. To determine whether *CDC5* function in MEN activation was also bypassed by the Cdc15-
SPB fusion protein, we examined Dbf2 kinase activity in tem1Δ cells that were also depleted for Cdc5. Strikingly, provision of the CDC15-SPB allele in the tem1Δ GAL-URL-3HA-CDC5 strain suppressed the defect in Dbf2-Mob1 activation observed in cells that lack TEM1 or CDC5 (compare Figure 6E, F, G with Figures 5D; (Visintin and Amon 2001)). Moreover, Dbf2 kinase activity was both premature and hyperactive in this strain (Figure 6F, G). Similar results were obtained in wild-type cells expressing the Cdc15-SPB fusion (Supplemental Figure 6).

Our analysis of a C-terminally truncated CDC15 allele (GAL-GFP-CDC15(1-750)) is consistent with the idea that targeting Cdc15 to SPBs bypasses the requirement for both Tem1 and Cdc5 in MEN activation (Bardin et al. 2003). Like the Cdc15-SPB fusion, Cdc15(1-750) localized to the SPB throughout the cell cycle in a manner independent of Tem1 and Cdc5 (Supplemental Figure 7A). Consistent with these observations, we found that Dbf2 kinase was both premature and hyperactive upon overexpression of Cdc15(1-750). Moreover, the overexpression of Cdc15(1-750) was sufficient to activate Dbf2-Mob1 in the absence of Cdc5 kinase activity (Supplemental Figure 7B-G).

Interestingly, Dbf2 kinase activity still fluctuates during the cell cycle in cells in which Cdc15 localizes to SPBs constitutively (Supplemental Figure 6, 7, 8). Thus, Dbf2-Mob1 kinase activity must be regulated by mechanisms in addition to Cdc15 SPB recruitment (see Discussion). It should also be noted that, despite premature and hyperactive Dbf2 kinase activity in Cdc15-SPB expressing cells, Cdc14 release from the nucleolus remained restricted to anaphase (Supplemental Figure 6A, Supplemental Figure 8). This indicates that yet additional mechanisms control Cdc14 localization downstream of and/or in parallel to Dbf2-Mob1 (see Discussion). We conclude that the sole essential MEN activating function of both TEM1 and CDC5 is to target Cdc15 to SPBs.
Discussion

Multiple Signals Converge on Cdc15 to Integrate MEN Activity with Other Cellular Events

The MEN is essential for exit from mitosis. The MEN GTPase Tem1 has been assumed to be the central switch in MEN regulation. We show here that robust MEN regulation occurs in a GTPase-independent manner and identify the Tem1-effector Cdc15 as an integrator of cell cycle signals. Cdc15 behaves like a coincidence detector (Figure 7A), integrating inputs from two essential cell cycle oscillators: the Tem1 GTPase cycle and the Polo kinase Cdc5 synthesis/degradation cycle. The Cdc15-dependent integration of these temporal (Cdc5 and Tem1 activity) and spatial (Tem1 activity) signals ensures that exit from mitosis occurs only after proper genome partitioning. Indeed, reliance on the timing signal alone (tem1Δ CDC15-UP) results in the inability to coordinate MEN activity with spindle position and the inappropriate exit from mitosis in the presence of a mis-positioned anaphase spindle (Supplemental Figure 1).

Tem1 and Cdc5 activity are read by the ability of Cdc15 to associate with the SPB. Artificially targeting Cdc15 to SPBs by fusing Cdc15 to an integral SPB component (Cdc15-SPB) bypasses the requirement for both proteins in MEN activation. Thus, it appears that recruitment of Cdc15 to SPBs is the essential function of Cdc5 and Tem1 in MEN activation.

It is unclear why Cdc15 recruitment to SPBs is essential for MEN activity. Cdc15 kinase activity, at least as measured by in vitro immunoprecipitation-kinase assays, does not change during the cell cycle (Jaspersen et al. 1998). It is possible that Cdc15 could be activated by a SPB associated protein, but such activation may not be detectable using standard immunoprecipitation-kinase assay conditions. An alternative but not mutually exclusive possibility is that a SPB scaffold, such as Nud1, may be required to increase the efficiency of interaction between MEN components to promote Cdc15-
Novel Temporal Regulators of the MEN
Our data indicate that MEN activity is regulated by multiple inputs (Figure 7B). The dependence of MEN activity on CDC5 ensures that the MEN can only be activated during G2 and mitosis, when Cdc5 is active. Our data also indicate that restricting MEN activity to anaphase is mediated by the GTPase Tem1. In wild-type cells arrested in metaphase, Dbf2-Mob1 activity remains low. In tem1Δ CDC15-UP cells arrested in metaphase, however, Dbf2-Mob1 is activated. Thus, an unknown anaphase event, likely under the control of the APC/C<sup>Cdc20</sup>, must be responsible for activating Tem1 at anaphase onset or keeping Tem1 inactive in earlier cell cycle stages. While the FEAR network contributes to activating the MEN in anaphase, the subtle effects of inactivating the FEAR network on mitotic exit kinetics argues that alternative pathways must regulate Tem1 activity.

As elaborated in this work, Cdc5 regulates the cell-cycle dependent localization of Cdc15 to SPBs. Despite the importance of regulating Cdc15 recruitment to SPBs, it is clear that additional mechanisms function downstream of and/or in parallel to Cdc15 to regulate exit from mitosis. Our data suggest that Dbf2 kinase activity is controlled by mechanisms in addition to Cdc15 recruitment to SPBs. Even though Dbf2 is hyperactive and active well before metaphase in CDC15-SPB cells, Dbf2 kinase activity nevertheless fluctuates during the cell cycle, being low in G1 and peaking in early anaphase (Supplemental Figures 6, 7, 8). Thus, there must exist a signal that promotes Dbf2 kinase activity as cells progress through S phase and mitosis or inhibits Dbf2 kinase activity in G1. Given that Dbf2-Mob1 kinase activity mirrors Clb-CDK activity in CDC15-SPB and GAL-GFP-CDC15(1-750) cells, it is tempting to speculate that Clb-CDKs directly or indirectly control Dbf2 kinase activity in these cells.

Our data also indicate that Dbf2 kinase activation is necessary but not sufficient to promote Cdc14 release from the nucleolus. In CDC15-SPB cells, Dbf2 specific activity is more than five times that seen in wild-type cells and substantial Dbf2-Mob1 kinase
activity (equal to the peak seen in a wild-type cell cycle) is achieved well before metaphase in the *CDC15-SPB* strain. In *GAL-GFP-CDC15(1-750)* cells the difference is even more striking, with Dbf2 specific activity levels more than 43 times that seen in wild-type cells. The difference in Dbf2 specific activity in these strains is likely due, at least in part, to the much higher expression levels of the *GAL-GFP-CDC15(1-750)* construct as compared to the *MET3-CDC15-SPB* construct. Despite premature and hyperactive Dbf2 kinase activity, Cdc14 is not released prematurely in these strains (Supplemental Figure 6, 7, 8). The mechanisms that restrict Dbf2-Mob1-dependent Cdc14 release to anaphase are unknown. Given that the overexpression of Cdc5 in combination with the premature activation of the MEN is sufficient to drive Cdc14 out of the nucleolus in any cell cycle stage (Manzoni et al. 2010), we propose that Cdc5 plays yet an additional key role in regulating Cdc14 release downstream of and/or in parallel to Dbf2-Mob1.

**Logic of MEN Activation**

Our results and those of previous studies suggest the following model for how MEN activity is restricted to anaphase and coupled to accurate spindle position by the integration of multiple spatial and temporal cues (Figure 7B). As cells approach the metaphase to anaphase transition and Cdc5 kinase reaches high levels of activity, Cdc5 phosphorylates an as yet unidentified target, which primes the MEN for activation by creating conditions that promote the association of Cdc15 with the SPB. Cdc5 also phosphorylates Bub2-Bfa1, thereby lowering its GAP activity. At the metaphase to anaphase transition, Cdc14 activated by the FEAR network dephosphorylates Cdc15 and Mob1, thereby stimulating MEN activity. This couples full MEN activation with the onset of chromosome segregation as components of the FEAR network are not only MEN activators but are also essential for inducing chromosome segregation. Additional
unknown signals regulate Tem1 and Dbf2-Mob1 to restrict their activity to anaphase. Finally, spindle position is integrated with MEN regulation via Tem1. As the spindle elongates along the mother - daughter axis, the Tem1-bearing SPB leaves the MEN inhibitory zone in the mother cell (defined by Kin4) and enters the MEN activating zone in the bud (defined by Lte1). This allows for the activation of Tem1 and recruitment of Cdc15 to SPBs. Additional signals functioning downstream of and/or in parallel to Dbf2-Mob1, and perhaps regulated by Cdc5, are needed to release Cdc14 from the nucleolus in anaphase in a sustained manner. While much remains to be learned about MEN regulation, it is clear that Cdc15 integrates both temporal (Cdc5 and Tem1) and spatial (Tem1) signals to mediate the robust and timely activation of the MEN in late anaphase.

**MEN-like Signaling Pathways in Other Eukaryotes**

The MEN is conserved in fission yeast where it is called the Septation Initiation Network (SIN) and regulates cytokinesis. Does Plo1 (Cdc5 homolog) regulate the SIN in a manner similar to the way Cdc5 regulates the MEN? plo1+ has been shown genetically to act as an activator of the SIN and placed to function upstream of spg1+ (Tem1 homolog; (Tanaka et al. 2001)). That said, the strong similarities between the MEN and SIN, and particularly between *S. cerevisiae* Cdc15 and its homolog in *S. pombe* Cdc7, suggest that Plo1 may also regulate the association of Cdc7 with SPBs. Cdc7 localizes to SPBs in mitosis and this localization is regulated by both Spg1 and Plo1 (Sohrmann et al. 1998; Mulvihill et al. 1999). Both Cdc15 and Cdc7 can associate with SPBs in at least two ways: one is mediated by a GTPase interaction domain and the other by an independent SPB localization domain (Asakawa et al. 2001; Bardin et al. 2003; Mehta and Gould 2006). Consistent with both modes of SPB localization being cell cycle regulated, localization of Cdc7 to SPBs is restricted to mitosis even when Cdc7 is overexpressed. Finally, while the Cdc7-Spg1 interaction is essential for SIN activation in
wild-type cells, overexpression of \( cdc7^+ \) can suppress the lethality of a strain deleted for \( spg1^+ \) (Schmidt et al. 1997). Thus, just as is the case for the MEN, there must exist GTPase-independent mechanism(s) of SIN activation, and these mechanism(s) might be mediated by Polo kinase.

The core MEN signaling module consisting of Cdc15, Dbf2, Mob1, and Nud1 also exists in higher eukaryotes. In higher eukaryotes, these proteins are known as Mammalian Sterile-20 related kinases (MSTs; Cdc15 homolog), Nuclear Dbf2 Related kinases (NDRs; Dbf2 homolog), Mob1 coactivators, and scaffolding (Nud1 homolog) families. While there are few known roles for these proteins in regulating mitotic exit (Bothos et al. 2005), they are essential components of signaling pathways that regulate a multitude of other cellular processes. As part of the Hippo pathway, this signaling module is essential for the proper regulation of organ growth in \textit{Drosophila} and vertebrates (Halder and Johnson 2011). Like their fungal counterparts, human NDR kinases and their Mob1 coactivators localize to centrosomes, the mammalian equivalent of SPBs (Hergovich et al. 2007; Wilmeth et al. 2010). Intriguingly, as is the case in \textit{S. cerevisiae} (J. M. R. unpublished observations; Luca et al. 2001), the localization of Mob1 isoforms to the centrosome is dependent on Polo-like kinase 1 activity (Wilmeth et al. 2010). Finally, we note that overexpression of human NDR1 results in centrosome overduplication as does overexpression of Polo-like kinase 4 (Plk4) (Habedanck et al. 2005; Hergovich et al. 2007). This raises the possibility that Plk4 plays a role in activating the MST/NDR1 signaling cascade. It will be interesting to explore whether or not Polo kinase activates NDR kinase signaling in higher eukaryotes.

**Materials and Methods**

**Yeast Strains and Growth Conditions**
All strains are derivatives of W303 (A2587) and are listed in Table S1. Growth conditions are described in the figure legends.

**Plasmid Construction**

All plasmids used in this study are listed in Table S2. Specifics of plasmid construction are detailed in the Supplemental Materials and Methods.

**Immunoblot Analysis**

For immunoblot analysis of Cdc15-eGFP, Cdc15-eGFP-Cnm67, GFP-Cdc15, GFP-Cdc15(1-750), Pgk1, and Kar2, cells were incubated for a minimum of 10 min in 5% trichloroacetic acid. The acid was washed away with acetone and cells were pulverized with glass beads in 166 μL of lysis buffer (50 mM Tris-Cl at pH 7.5, 1 mM EDTA, 2.75 mM DTT, complete protease inhibitor cocktail [Roche]) using a bead mill. Sample buffer was added and the cell homogenates were boiled. Cdc15-eGFP, Cdc15-eGFP-Cnm67, GFP-Cdc15, and GFP-Cdc15(1-750) were detected using an anti-GFP antibody (Clontech, JL-8) at a 1:1000 dilution. Pgk1 was detected using an anti-Pgk1 antibody (Invitrogen) at 1:5000 dilution. Kar2 was detected using a rabbit anti-Kar2 antiserum (Rose et al. 1989) at a 1:200,000 dilution.

**Dbf2 Kinase Assays**

Dbf2 kinase assays were performed as described previously (Visintin and Amon 2001) with the following modifications: approximately 1.5 mg of total protein was used per immunoprecipitation and kinase reactions were incubated for 45 minutes with gentle mixing. Histone H1 phosphorylation was quantified using the PhosphorImaging System. Western blots were quantified using ECL Plus (GE Healthcare) and fluorescence imaging. Quantifications were performed using NIH Image Quant software.
**Fluorescence Microscopy**

Indirect *in situ* immunofluorescence methods to detect Tub1 were performed as previously described (Kilmartin and Adams 1984). For imaging of Cdc15-eGFP and Cdc15-eGFP-Cnm67, cells were fixed for 2 minutes in 4% paraformaldehyde (in 3.4% sucrose solution). Cells were washed once in KPO4/sorbitol (1.2 M sorbitol, 0.1 M KPO4 pH 7.5) and resuspended in KPO4/sorbitol supplemented with 1% Triton. Prior to imaging, cells were stained with Prolong Gold Antifade Reagent (Invitrogen, P36935). Cells were imaged within 24 hours on a Zeiss Axioplan 2 microscope and a Hamamatsu OCRA-ER digital camera.

**FACS**

Flow cytometric DNA quantitation was performed as described by (Haase and Reed 2002).

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**References**


Figure Legends

**Figure 1: Anaphase-specific Activation of the MEN in the Absence of TEM1**

(A, B) Wild-type (A2747) and *lte1A kin4A* (A26379) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEP medium containing glucose (YEPD). When the arrest was complete (after 150 minutes), cells were released into pheromone free YEPD medium. After 80 minutes, α-factor pheromone (10 μg/ml) was re-added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, A), anaphase spindles (closed circles, A), 3HA-Cdc14 released from the nucleolus (open circles, A) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, B) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B) was determined at the indicated times.

(C) Wild-type (A2747) and *tem1A CDC15-UP* (A22670) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were spotted on YEP plates containing raffinose and galactose (YEPRG) at 30°C. Approximately 3 X 10^4 cells were deposited in the first spot and each subsequent spot is a 10-fold serial dilution. The picture shown depicts 3 days of growth.
(D, E) Wild-type (A2747) and tem1Δ CDC15-UP (A22670) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 µg/ml) in YEPRG medium. When the arrest was complete (after 2 hours 50 minutes), cells were released into pheromone free YEPRG medium. After 60 minutes, α-factor pheromone (10 µg/ml) was added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, D), anaphase spindles (closed circles, D), 3HA-Cdc14 released from the nucleolus (open circles, D) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, E) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, E) was determined at the indicated times.

(F) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (E) was determined by quantitative autoradiography and quantitative Western blot, respectively. Shown is the specific Dbf2-associated kinase activity.

Figure 2: The FEAR Network is Not Required for Dbf2 activity in tem1Δ CDC15-UP Cells

(A) Wild-type (A2747), tem1Δ CDC15-UP (A22670), tem1Δ spo12Δ bns1Δ CDC15-UP (A23392), and tem1Δ slk19Δ CDC15-UP (A23387) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were spotted on YEPRG plates as in Figure 1C.

(B, C) tem1Δ CDC15-UP (A23782) and tem1Δ cdc14-3 CDC15-UP (A23712) cells containing a 3MYC-Dbf2 fusion protein were arrested in G1 with α-factor pheromone (5 µg/ml) in YEPRG medium at room temperature. 30 minutes prior to release the cells were shifted to 35°C. When the arrest was complete (after 3 hours 30 minutes), cells were released into pheromone free YEPRG medium at 35°C. After 65 minutes, α-factor pheromone (10 µg/ml) was re-added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, B), anaphase spindles
(closed circles, B) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, C) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, C) was determined at the indicated times. (D) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (C) was determined as in Figure 1F. Shown is the specific Dbf2-associated kinase activity. Note that the specific Dbf2-associated kinase activity continues to rise in the tem1\(\Delta\) cdc14-3 CDC15-UP strain as a result of a prolonged anaphase arrest.

**Figure 3: Polo-like kinase Cdc5 Controls MEN Activity in the Absence of TEM1**

(A, B) tem1\(\Delta\) CDC15-UP (A22670) and tem1\(\Delta\) cdc5-7 CDC15-UP (A24305) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with \(\alpha\)-factor pheromone (5 \(\mu\)g/ml) in YEPRG medium at 30\(^\circ\)C. 30 minutes prior to release the cells were shifted to 37\(^\circ\)C. When the arrest was complete (after 3 hours), cells were released into pheromone free YEPRG medium at 37\(^\circ\)C. After 65 minutes, \(\alpha\)-factor pheromone (10 \(\mu\)g/ml) was added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, A), anaphase spindles (closed circles, B) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, B) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B) was determined at the indicated times.

(C, D) tem1\(\Delta\) CDC15-UP (A22670) and tem1\(\Delta\) MET25-CDC5\(\Delta\)db CDC15-UP (A25175) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with \(\alpha\)-factor pheromone (5 \(\mu\)g/ml) in YEPRG medium. 90 minutes prior to release, the cells were transferred to -Met medium containing raffinose and galactose (-MetRG; to induce the expression of Cdc5\(\Delta\)db) supplemented with \(\alpha\)-factor pheromone (5 \(\mu\)g/ml). When the arrest was complete (after 3 hours), cells were released into pheromone free -MetRG medium. After 70 minutes, \(\alpha\)-factor pheromone (10 \(\mu\)g/ml) was re-added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed
squares, C), anaphase spindles (closed circles, C) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, D) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, D) was determined at the indicated times.

**Figure 4: Cdc5 Promotes Localization of Cdc15 to SPBs**

(A) *tem1Δ CDC15-eGFP-UP* (A25630) cells containing a mCherry-Tub1 fusion protein were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium. When the arrest was complete (after 2 hours 50 minutes), cells were released into pheromone free YEPRG medium and imaged after a brief paraformaldehyde fixation. Cell cycle stage was determined based on spindle morphology and correlated with Cdc15 localization at SPBs (n ≥ 100 cells for each cell cycle stage). Representative images of G1/S, metaphase, and anaphase cells are shown. Cdc15 is shown in green, microtubules in red and DNA in blue.

(B) *tem1Δ CDC15-eGFP-UP* (A25630) and *tem1Δ CDC15-eGFP-UP cdc5-as1* (A25633) cells containing a mCherry-Tub1 fusion protein were arrested in G1 as in Figure 4A. Cells were released into pheromone free YEPRG medium supplemented with 5mM CMK (cdc5-as1 inhibitor). Cells were scored as in Figure 4A. Representative images of anaphase cells are shown.

(C) *tem1Δ CDC15-eGFP-UP* (A25744) and *tem1Δ CDC15-eGFP-UP MET25-CDC5ΔN70* (*tem1Δ CDC15-eGFP-UP CDC5-UP*; A25983) cells containing a Spc42-mCherry fusion protein were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium supplemented with 8mM methionine. 90 minutes prior to release, the cells were transferred to -MetRG medium (to induce the expression of Cdc5ΔN70) supplemented with α-factor pheromone. When the arrest was complete (after 3 hours), cells were released into pheromone free -MetRG medium. Cells were imaged and
scored as in Figure 4A. Representative images of metaphase cells are shown. Cdc15 is shown in green, Spc42 in red, and DNA in blue.

**Figure 5: Cdc15 Functions as a Coincidence Detector of Tem1 and Cdc5 Activity**

(A) *CDC15*-eGFP (A26481) and *CDC15*-eGFP *GAL-UPL-TEM1* (A27055) cells containing a mCherry-Tub1 fusion protein were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium. UPL, which stands for ubiquitin-proline-LacZ, acts as a destabilizing module that permits rapid degradation of appended proteins. One hour prior to release, glucose was added to a final concentration of 2% (to repress expression of *GAL-UPL-TEM1*). When the arrest was complete (after 2 hours 40 minutes), cells were released into pheromone free YEPD medium. Cells were imaged and scored as in Figure 4A.

(B) *CDC15*-eGFP (A26481), *CDC15*-eGFP *bub2Δ* (A26480), *CDC15*-eGFP *GAL-URL-3HA-CDC5* (A26556), and *CDC15*-eGFP *bub2Δ GAL-URL-3HA-CDC5* (A26558) cells containing a mCherry-Tub1 fusion protein were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium. URL, which stands for ubiquitin-arginine-LacZ, acts as a destabilizing module that permits rapid degradation of appended proteins. Two hours prior to release, glucose was added to a final concentration of 2% (to repress expression of *GAL-URL-3HA-CDC5*). When the arrest was complete (after 2 hours 45 minutes), cells were released into pheromone free YEPD medium. Cells were imaged and scored as in Figure 4A.

(C, D) *bub2Δ cdc14-3* (A26844) and *bub2Δ cdc14-3 GAL-URL-3HA-CDC5* (A26842) cells containing a 3MYC-Dbf2 fusion protein were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium. Two hours prior to release, glucose was added to repress expression of *GAL-URL-3HA-CDC5*. When the arrest was complete (after 2
hours 45 minutes), cells were released into pheromone free YEPD medium. The percentage of cells with metaphase spindles (closed squares, C), anaphase spindles (closed circles, C) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, D) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, D) was determined at the indicated times.

**Figure 6: Targeting Cdc15 to SPBs Bypasses the Need for TEM1 and CDC5 in MEN Activation**

(A) **CDC15-eGFP** (CDC15; A20935), pMET3-CDC15-eGFP-CN67 (CDC15-SPB; A26417), and **CDC15-eGFP-UP** (CDC15-UP; A25515) cells were grown to log phase in either YEPRG+methionine (+ MET) or – Met medium to determine the amount of Cdc15-eGFP (α-GFP) in cells. Kar2 was used as a loading control in Western blots.

(B) Wild-type (A2587), cdc15-2 (A2597), pMET3-CDC15-eGFP-CN67 (CDC15-SPB; A26419), and pMET3-CDC15-eGFP-CN67 cdc15-2 (CDC15-SPB cdc15-2; A26413) cells were spotted on YEPRG plates supplemented with 8 mM methionine as in Figure 1C. The picture shown depicts 2 days of growth at 37°C and 3 days of growth at 23°C.

(C) pMET3-CDC15-eGFP-CN67 (CDC15-SPB; A26486) cells containing a mCherry-Tub1 fusion protein were grown to log phase in YEPRG medium supplemented with 8 mM methionine and imaged after a brief paraformaldehyde fixation. Representative images of G1/S, metaphase, and anaphase cells are shown.

(D) Wild-type (A2747), tem1Δ CDC15-UP (A22670), and tem1Δ pMET3-CDC15-eGFP-CN67 (tem1Δ CDC15-SPB; A26396) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were spotted on YEPRG plates supplemented with 8 mM methionine as in Figure 1C. The picture shown depicts 3 days of growth.

(E, F) Wild-type (A2747) and tem1Δ GAL-URL-3HA-CDC5 pMET3-CDC15-eGFP-CN67 (tem1Δ GAL-URL-3HA-CDC5 CDC15-SPB; A27051) cells containing 3HA-
Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium supplemented with 8 mM methionine. Two hours prior to release, glucose was added (to repress expression of GAL-URL-3HA-CDC5). When the arrest was complete (after 2 hours 50 minutes), cells were released into pheromone free YEPD medium supplemented with 8 mM methionine. After 65 minutes, α-factor pheromone (10 μg/ml) was added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, E), anaphase spindles (closed circles, E) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, F) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, F) was determined at the indicated times. (G) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (F) was determined as in Figure 1F. Shown is the specific Dbf2-associated kinase activity.

**Figure 7: A Model for the Coordination of Exit from Mitosis with Spatial and Temporal Cues**

(A) Cdc15 functions as a coincidence detector of Tem1 and Cdc5 activity, both of which are required for the association of Cdc15 with SPBs. See text for details.

(B) Multiple signals control MEN activity. The core MEN components are shown in blue, activators of the MEN shown in green, and inhibitors of the MEN shown in red. Experimentally validated interactions are shown with solid lines; more speculative interactions are shown with dashed lines. See text for details.
A

wild-type

$\text{tem1}^{\Delta} \text{CDC15-UP}$

$\text{tem1}^{\Delta} \text{CDC15-UP} \text{spo12}^{\Delta} \text{bns1}^{\Delta}$

$\text{tem1}^{\Delta} \text{CDC15-UP} \text{skl19}^{\Delta}$

B

$\text{tem1}^{\Delta} \text{CDC15-UP}$

$\text{tem1}^{\Delta} \text{CDC15-UP} \text{cdc14-3}$

C

D

$\text{tem1}^{\Delta} \text{CDC15-UP}$

$\text{tem1}^{\Delta} \text{CDC15-UP} \text{cdc14-3}$

Dbf2 kinase

Dbf2 IP

Dbf2 Specific Activity

Time (min)
Amon Rock 35

**Rock172577_Fig3**

A. *tem1Δ CDC15-UP*

- **Percent cells** vs **Time (min)**
  - Time: 0, 30, 60, 90, 120
  - Percent: 0, 20, 40, 60, 80, 100

B. *tem1Δ CDC15-UP cdc5-7*

- **Time (min)**:
  - 0, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130

C. *tem1Δ CDC15-UP MET26-CDC5Δdb*

- **Time (min)**:
  - 0, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130

**Images**:

- **Dbf2 kinase**
- **Dbf2 IP**

**Graphs**:

- **Metaphase cells**
- **Anaphase cells**
Rock172577_Fig4

A

\text{tem1}Δ \text{CDC15-UP}

\begin{itemize}
  \item G1/S
  \item metaphase
  \item anaphase
\end{itemize}

\begin{itemize}
  \item Cdc15 on SPB
  \item Cdc15 not on SPB
\end{itemize}

B

\text{tem1}Δ \text{CDC15-UP}

\begin{itemize}
  \item \text{tem1}Δ \text{CDC15-UP}
  \item \text{cdc5-as1}
\end{itemize}

\begin{itemize}
  \item Cdc15 on SPB
  \item Cdc15 not on SPB
\end{itemize}

C

\text{tem1}Δ \text{CDC15-UP}

\begin{itemize}
  \item \text{tem1}Δ \text{CDC15-UP}
  \item \text{cdc5-as1}
\end{itemize}

\begin{itemize}
  \item Cdc15 on SPB
  \item Cdc15 not on SPB
\end{itemize}
Supplemental Materials:

Supplemental Figures:

**Supplemental Figure 1: tem1Δ CDC15-UP Cells are Spindle Position Checkpoint Defective.**

dyn1Δ (A2444), bub2Δ dyn1Δ (A2270), and tem1Δ CDC15-UP dyn1Δ (A23657) cells were grown to mid-exponential phase at 30°C and then incubated for 24 h at 14°C. Cells were fixed and the number of nuclei in cells was determined. Cells that were anucleate, multinucleate, or multi-budded with two nuclei in the mother cell body were counted as “bypassed”. Single budded cells with two nuclei in the mother cell body were counted as “arrested”.

**Supplemental Figure 2: Separase is Not Required for Dbf2 activity in tem1Δ CDC15-UP Cells**

(A,B) tem1Δ CDC15-UP (A22670) and tem1Δ esp1-1 CDC15-UP (A23716) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium at room temperature. Thirty minutes prior to release the cells were shifted to 37°C. When the arrest was complete (after 3 hours 30 minutes), cells were released into pheromone free YEPRG medium at 37°C. After 65 minutes, α-factor pheromone (10 μg/ml) was added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, A), anaphase spindles (closed circles, A) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, B) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B) was determined at the indicated times.

(C) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (B) was determined by quantitative autoradiography and quantitative Western blot, respectively. Shown is the specific Dbf2-associated kinase activity.

**Supplemental Figure 3: Anaphase Entry is not Required for MEN Activity in the Absence of TEM1**

(A, B, C) tem1Δ CDC15-UP (A22670) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG
medium. When the arrest was complete (after 2 hours 45 minutes), cells were released into YEPRG medium supplemented with nocodazole (15 μg/ml; (+) nocodazole) or solvent control (DMSO; (-) nocodazole). After 65 minutes, α-factor pheromone (10 μg/ml) was added to prevent entry into the subsequent cell cycle. The percentage of budded cells (A), DNA content (as assayed by flow cytometry, B) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, C) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, C) was determined at the indicated times. (D) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (C) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.

**Supplemental Figure 4: Cdc5 is Not Required for Tem1 SPB Localization**

*TEM1-GFP* (A22556) and *TEM1-GFP GAL-URL-3HA-CDC5* (A28411) cells containing a mCherry-Tub1 fusion protein were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium. URL, which stands for ubiquitin-arginine-LacZ, acts as a destabilizing module that permits rapid degradation of appended proteins (Bachmair et al. 1986). Two hours prior to release, glucose was added to a final concentration of 2% (to repress expression of *GAL-URL-3HA-CDC5*). When the arrest was complete (after 2 hours 45 minutes), cells were released into pheromone free YEPD medium. Cell cycle stage was determined based on spindle morphology and correlated with Tem1 localization at SPBs (n ≥ 100 cells for each cell cycle stage). Representative images of anaphase cells are shown. Tem1 is shown in green, microtubules in red and DNA in blue.

**Supplemental Figure 5: Cdc15-SPB localizes to the SPB constitutively throughout the cell cycle**

*pMET3-CDC15-eGFP-CN67* (*CDC15-SPB*; A26486) cells containing a mCherry-Tub1 fusion protein were grown to log phase in YEPRG medium supplemented with 8 mM methionine and imaged after a brief paraformaldehyde fixation. Cell cycle stage was determined based on spindle morphology and correlated with Cdc15-SPB localization at SPBs (n ≥ 100 cells for each cell cycle stage).

**Supplemental Figure 6: Dbf2-Mob1 Kinase Activity is Not Sufficient for Cdc14 Release Prior to Anaphase**
(A, B) Wild-type (A2747) and pMET3-CDC15-eGFP-CN67 (CDC15-SPB; A26418) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium supplemented with 8 mM methionine. When the arrest was complete (after 2 hours 40 minutes), cells were released into pheromone free YEPD medium supplemented with 8 mM methionine. After 65 minutes, α-factor pheromone (10 μg/ml) was re-added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, A), anaphase spindles (closed circles, A), 3HA-Cdc14 released from the nucleolus (open circles, A) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, B) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B) was determined at the indicated times.

(C) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (B) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.

Supplemental Figure 7: Removal of the C-terminal 274 Amino Acids of Cdc15
Results in Constitutive Cdc15 SPB Targeting and Tem1 and Cdc5-Independent
Activation of the MEN

(A) tem1Δ GAL-GFP-CDC15 (A25662), tem1Δ GAL-GFP-CDC15 cdc5-as1 (A25661),
tem1Δ GAL-GFP-CDC15(1-750) (A25596), and tem1Δ GAL-GFP-CDC15(1-750) cdc5-
as1 (A25594) cells containing a mCherry-Tub1 fusion protein were arrested in G1 as in
Figure 4A. Cells were released into pheromone free YEPRG medium supplemented with
5 mM CMK (cdc5-as1 inhibitor) and imaged after a brief paraformaldehyde fixation. Cell
cycle stage was determined based on spindle morphology and correlated with Cdc15
localization at SPBs (n ≥ 100 cells for each cell cycle stage).

(B, C) GAL-GFP-CDC15 (A24698) and GAL-GFP-CDC15 cdc5-as1 (A24695) cells
containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor
pheromone (5 μg/ml) in YEPRG medium. When the arrest was complete (after 2 hours
50 minutes), cells were released into pheromone free YEPRG medium. After 70
minutes, α-factor pheromone (10 μg/ml) was re-added to prevent entry into the
subsequent cell cycle. The percentage of cells with metaphase spindles (closed
squares, B), anaphase spindles (closed circles, B) and the amount of Dbf2-associated
kinase activity (Dbf2 kinase, C) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, C) was
determined at the indicated times.
(D) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (C) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.

(E, F) GAL-GFP-CDC15(1-750) (A21924) and GAL-GFP-CDC15(1-750) cdc5-as1 (A24508) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were examined at the same time and in the same manner as strains described in Supplemental Figure 7B-D. The percentage of cells with metaphase spindles (closed squares, E), anaphase spindles (closed circles, E) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, F) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, F) was determined at the indicated times.

(G) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (F) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.

Supplemental Figure 8: Overexpression of Cdc15(1-750) hyperactivates Dbf2-Mob1 but does not Result in the Premature Release of Cdc14 from the Nucleolus

(A, B, C) GAL-GFP-CDC15 (A21922) and GAL-GFP-CDC15(1-750) (A21924) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPR medium. 45 minutes prior to release, galactose was added to induce expression of GAL-GFP-CDC15 and GAL-GFP-CDC15(1-750). When the arrest was complete (after 3 hours), cells were released into pheromone free YEPRG medium. After 85 minutes, α-factor pheromone (10 μg/ml) was re-added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, A), anaphase spindles (closed circles, A) 3HA-Cdc14 released from the nucleolus (open circles, A), the amount of Dbf2-associated kinase activity (Dbf2 kinase, B) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B), and the amounts of GFP-Cdc15 and GFP-Cdc15(1-750) (α-GFP, C) was determined at the indicated times. Pgk1 was used as a loading control in Western blots.

(D) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (B) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.
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A26486  MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, ura3::pRS306-mCherry-TUB1::URA3, PMET3-CDC15-eGFP-CNM67::LEU2
A26556  MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, CDC15-eGFP::KanMX6, cdc5::GAL-URL-3HA-CDC5::KanMX6, ura3::pRS306-mCherry-TUB1::URA3
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A26842  MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, cdc5::GAL-URL-3HA-CDC5::KanMX, bub2::HIS3, cdc14-3, DBF2-3MYC
A26844  MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, bub2::HIS3, cdc14-3, DBF2-3MYC
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Supplemental Experimental Procedures

Yeast Strains

All strains are derivatives of W303 (A2587) and are listed in Table S1. Cdc15-eGFP and PGPD-CDC15 were constructed by standard PCR-based methods (Longtine et al. 1998; Janke et al. 2004).

Plasmid Construction

All plasmids used in this study are listed in Table S2.

pA1813: CDC5Δdb (Charles et al. 1998; Shirayama et al. 1998) was cloned under the control of the MET25 promoter using the following strategy. Approximately 1 kb of the MET25 promoter was amplified with primers (5'-'aataAAGCTTCCGGATGCAAGGGTTCGAATC-3') and (5'-'aataCTGCAGGGATGGGTGAATGAATTG-3') from A2587 genomic DNA (PCR product 1); the N-terminally truncated (70 amino acids) CDC5 ORF was amplified with primers (5'-aataCTGCAGAAATGCCACCTTCATATATCAAACAG-3') and (5'-CATGGCAATTTTGAATAGATATAG-3') from A2587 genomic DNA (PCR product 2). PCR product 1 was digested with HindIII and PstI; PCR product 2 was digested with PstI and XbaI; plasmid YIplac211 was digested with HindIII and XbaI (Gietz and Sugino 1988). Fragments were three way ligated to yield: YIplac211-MET25-CDC5ΔN70.

pA1880: CDC15-eGFP-CN67 was cloned under the control of the MET3 promoter using the following strategy. PMET3 was amplified with primers (5'-'TTACGCCAGCTTGCATGCAGGACTCTAGAGGATGAAACTGAGTAAGATGCTCAGAATAC-3') and (5'-'GAGTCAAGTTGACTCTATCGGTATCGGCCATACCTGTTCCATCTAGGGTAAATTATACTTTATTCTTG-3') with a PMET3 containing plasmid as template; CDC15-eGFP was amplified with primers (5'-ATGAACAGTATGGCCGATACC-3') and (5'-GCCACCACCAGCGCCACCTCCACCAAAGCTCCACTCCACCACCTAGTGTGTACAAATTCATCAATACCAGT-3') with A20791 genomic DNA as PCR template; and CNM67 was amplified with primers (5'-'CTAGGTGGTTGGAGGTTCTGTTGAGGTTGGGTGGGATGACTGATTCTCATTAGTTATG-3') and (5'-'ATTTAATG-3').
TAAAACGACGGCCAGTGAATTGCAGCTCGGTACCCGGGGAACCCCTAAAAGCTCA
TAGTAGCAG-3') with A2587 genomic DNA as template. Plasmid YCplac22 was
digested with BamHI (Gietz and Sugino 1988). Approximately equimolar amounts of
BamHI-digested plasmid YCplac22 and each of the three PCR products above were
cotransformed into yeast strain A2587. Homologous recombination between YCplac22
and the three PCR fragments generates the PMET3-CDC15-eGFP-CN67 allele.
Plasmids were recovered from resulting Trp+ colonies and sequence confirmed to
contain mutation-free PMET3-CDC15-eGFP-CN67. PMET3-CDC15-eGFP-CN67
was then subcloned into the Sphl & KpnI sites of YIplac128 (Gietz and Sugino 1988).

Note that expression of the fusion protein shows cell-to-cell variability under non-
inducing conditions (as is evident by GFP signal intensity in fluorescence microscopy).
Even under these conditions, however, Cdc15-SPB protein levels remained high enough
in all or almost all cells to complement the temperature sensitive lethality of the cdc15-2
allele.

**Cell Cycle Staging by Spindle Morphology**
The stage of the cell cycle of individual cells was assessed by spindle morphology. G1
or S phase cells were defined as having unduplicated or newly duplicated spindle pole
bodies but lacking a spindle that spanned the DAPI-stained nucleus. Metaphase cells
were defined as having a thick, bar shaped spindles that spanned an undivided DAPI-
stained nucleus. Anaphase cells were defined as cells with separated DNA masses
connected by an elongated spindle.

**Spindle position checkpoint assay**
Cells were grown to mid-exponential phase at 30°C and then incubated for 24 h at 14°C.
Cells were fixed and the number of nuclei in cells was determined. Cells that were
anucleated, multinucleated, or multi-budded with two nuclei in the mother cell body were
counted as exhibiting a checkpoint bypass morphology. Single budded cells with two
nuclei in the mother cell body were counted as arrested.

**Supplemental References**


cdc15-SPB visible on SPB

cdc15-SPB not visible on SPB