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

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 *lte1Δ* or *kin4Δ* 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

LTE1 and KIN4-Independent Activation of the MEN in Anaphase

LTE1 and *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 *BUB2* or *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 *bub2Δ* cells, *tem1Δ 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.

The FEAR Network is Not Required for MEN Activity in tem1Δ 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 *tem1Δ CDC15-UP* cells, we examined the consequences of deleting FEAR network components in this strain. *SPO12*, its close homolog *BNS1*, and *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 *tem1Δ 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 *tem1Δ 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 *tem1Δ 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 *tem1Δ CDC15-UP* cells.

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 *tem1Δ CDC15-UP* cells. The fact that

MEN activation occurred with similar kinetics in *tem1Δ CDC15-UP* and *tem1Δ CDC15-

UP 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 *tem1Δ CDC15-UP* cells.

To determine whether other aspects of anaphase entry were necessary for MEN

activation, we arrested *tem1Δ CDC15-UP* cells in metaphase. Entry into anaphase is

triggered by the activation of a ubiquitin ligase known as APC/C^{Cdc20}. Activation of the

spindle assembly checkpoint by microtubule depolymerization results in the inhibition of

APC/C^{Cdc20} and arrests cells in metaphase (Musacchio and Salmon 2007). We found

that entry into anaphase was not required for Dbf2-Mob1 activation in *tem1Δ CDC15-UP*

cells. *tem1Δ 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 *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 *tem1Δ 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^{Cdc20} 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^{Cdh1} (Charles et al. 1998; Cheng et al. 1998; Shirayama et al. 1998). If Cdc5 was limiting for MEN activation in a *tem1Δ CDC15-UP* 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 *MET25* promoter in *tem1Δ CDC15-UP* cells. We found that the premature accumulation of Cdc5 results in the premature activation of Dbf2-Mob1 in a *tem1Δ CDC15-UP* 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.

Cdc5 Promotes Localization of Cdc15 to SPBs

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 *TEM1*, 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 *tem1Δ CDC15-UP* strain (these cells harbor two overexpression constructs: *GAL-CDC15* and *GPD-CDC15*), 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

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 *tem1Δ CDC15-eGFP-UP* cells

containing an inhibitor-sensitive allele of *CDC5* (*cdc5-as1*). In the presence of the

inhibitor, Cdc15 is no longer able to localize to SPBs in the *tem1Δ CDC15-eGFP-UP*

cdc5-as1 cells (Figure 4B). As *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 *CDC5Δdb* allele led to the premature recruitment of Cdc15 to

SPBs in metaphase (Figure 4C). Taken together, these data indicate that *CDC5*

functions in parallel to *TEM1* to promote the association of Cdc15 with SPBs.

Cdc15 Functions as a Coincidence Detector of Tem1 and Cdc5 Activity

 Our data suggest that both *CDC5* and *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 *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). *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 *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-

15

dependent Dbf2-Mob1 activation. Although we do not yet know why Cdc15 must associate with SPBs, we have some understanding of how this association occurs. Tem1 recruits Cdc15 to SPBs via a region in Cdc15 immediately adjacent to its kinase domain (Asakawa et al. 2001). How Cdc5 promotes Cdc15 SPB localization is unknown. Preliminary data suggest that Cdc15 is not a Cdc5 substrate. ^{32}P incorporation into Cdc15 *in vivo* was not affected by modulating *CDC5* activity (J. M. R., unpublished observations). In addition, mutation of Cdc5 consensus binding sites (SSP to AAP) in Cdc15 did not abrogate Cdc15-dependent MEN activation (J. M. R., unpublished observations). These results and several other observations raise the possibility that the putative SPB anchor for Cdc15, Nud1, might be Cdc5's essential MEN activating target: (1) Nud1 is thought to bind to and recruit Cdc15 to the SPB (Stegmeier and Amon 2004); (2) *nud1* temperature sensitive mutants arrest in late anaphase with an inactive MEN (Gruneberg et al. 2000; Visintin and Amon 2001); (3) Nud1 is a substrate of Cdc5 *in vivo* and *in vitro* (Maekawa et al. 2007; Park et al. 2008); and (4) Nud1 hyper-phosphorylation coincides with Cdc15 recruitment to SPBs (Visintin et al. 2003; Maekawa et al. 2007; Park et al. 2008). Cdc5 could phosphorylate Nud1 in mitosis, thereby creating a phospho-dependent SPB binding site for Cdc15. As Nud1 is the most extensively phosphorylated SPB component (>50 phosphosites, (Keck et al. 2011), testing this hypothesis will be extremely challenging. It is important to note, however, that the *CDC15-SPB* fusion does not suppress the temperature sensitive lethality of cells harboring the *nud1-44* allele as the sole source of *NUD1* (J. M. R., unpublished observations). Thus, unlike *TEM1* and *CDC5, NUD1* has essential roles in MEN signaling in addition to recruiting Cdc15 to SPBs.

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^{Cdc20}, 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 *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 *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 KPO_4 pH 7.5) and resuspended in KPO_4/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).

Acknowledgements

References

Asakawa, K., Yoshida, S., Otake, F., and Toh-e, A. 2001. A novel functional domain of Cdc15 kinase is required for its interaction with Tem1 GTPase in Saccharomyces cerevisiae. *Genetics* **157**(4): 1437-1450.

Bachmair, A., Finley, D., and Varshavsky, A. 1986. In vivo half-life of a protein is a function of its amino-terminal residue. *Science* **234**(4773): 179-186.

Bardin, A.J., Boselli, M.G., and Amon, A. 2003. Mitotic exit regulation through distinct domains within the protein kinase Cdc15. *Mol Cell Biol* **23**(14): 5018-5030.

Bardin, A.J., Visintin, R., and Amon, A. 2000. A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell* **102**(1): 21-31.

Bertazzi, D.T., Kurtulmus, B., and Pereira, G. 2011. The cortical protein Lte1 promotes mitotic exit by inhibiting the spindle position checkpoint kinase Kin4. *J Cell Biol* **193**(6): 1033-1048.

Bloecher, A., Venturi, G.M., and Tatchell, K. 2000. Anaphase spindle position is monitored by the BUB2 checkpoint. *Nat Cell Biol* **2**(8): 556-558.

Bothos, J., Tuttle, R.L., Ottey, M., Luca, F.C., and Halazonetis, T.D. 2005. Human LATS1 is a mitotic exit network kinase. *Cancer Res* **65**(15): 6568-6575.

Castillon, G.A., Adames, N.R., Rosello, C.H., Seidel, H.S., Longtine, M.S., Cooper, J.A., and Heil-Chapdelaine, R.A. 2003. Septins have a dual role in controlling mitotic exit in budding yeast. *Curr Biol* **13**(8): 654-658.

Chan, L.Y. and Amon, A. 2010. Spindle position is coordinated with cell-cycle progression through establishment of mitotic exit-activating and -inhibitory zones. *Mol Cell* **39**(3): 444-454.

Charles, J.F., Jaspersen, S.L., Tinker-Kulberg, R.L., Hwang, L., Szidon, A., and Morgan, D.O. 1998. The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in S. cerevisiae. *Curr Biol* **8**(9): 497-507.

Cheng, L., Hunke, L., and Hardy, C.F. 1998. Cell cycle regulation of the Saccharomyces cerevisiae polo-like kinase cdc5p. *Mol Cell Biol* **18**(12): 7360-7370.

Cooper, J.A. and Nelson, S.A. 2006. Checkpoint control of mitotic exit--do budding yeast mind the GAP? *J Cell Biol* **172**(3): 331-333.

D'Aquino, K.E., Monje-Casas, F., Paulson, J., Reiser, V., Charles, G.M., Lai, L., Shokat, K.M., and Amon, A. 2005. The protein kinase Kin4 inhibits exit from mitosis in response to spindle position defects. *Mol Cell* **19**(2): 223-234.

Falk, J.E., Chan, L.Y., and Amon, A. 2011. Lte1 promotes mitotic exit by controlling the localization of the spindle position checkpoint kinase Kin4. *Proc Natl Acad Sci U S A* **108**(31): 12584-12590.

Fraschini, R., Venturetti, M., Chiroli, E., and Piatti, S. 2008. The spindle position checkpoint: how to deal with spindle misalignment during asymmetric cell division in budding yeast. *Biochem Soc Trans* **36**(Pt 3): 416-420.

Geymonat, M., Spanos, A., de Bettignies, G., and Sedgwick, S.G. 2009. Lte1 contributes to Bfa1 localization rather than stimulating nucleotide exchange by Tem1. *J Cell Biol* **187**(4): 497-511.

Geymonat, M., Spanos, A., Walker, P.A., Johnston, L.H., and Sedgwick, S.G. 2003. In vitro regulation of budding yeast Bfa1/Bub2 GAP activity by Cdc5. *J Biol Chem* **278**(17): 14591-14594.

Gonzalez, C. 2007. Spindle orientation, asymmetric division and tumour suppression in Drosophila stem cells. *Nat Rev Genet* **8**(6): 462-472.

Gruneberg, U., Campbell, K., Simpson, C., Grindlay, J., and Schiebel, E. 2000. Nud1p links astral microtubule organization and the control of exit from mitosis. *EMBO J* **19**(23): 6475-6488.

Haase, S.B. and Reed, S.I. 2002. Improved flow cytometric analysis of the budding yeast cell cycle. *Cell Cycle* **1**(2): 132-136.

Habedanck, R., Stierhof, Y.D., Wilkinson, C.J., and Nigg, E.A. 2005. The Polo kinase Plk4 functions in centriole duplication. *Nat Cell Biol* **7**(11): 1140-1146.

Halder, G. and Johnson, R.L. 2011. Hippo signaling: growth control and beyond. *Development* **138**(1): 9-22.

Hergovich, A., Lamla, S., Nigg, E.A., and Hemmings, B.A. 2007. Centrosome-associated NDR kinase regulates centrosome duplication. *Mol Cell* **25**(4): 625-634.

Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J., and Elledge, S.J. 2001. Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. *Cell* **107**(5): 655-665.

Jaspersen, S.L., Charles, J.F., Tinker-Kulberg, R.L., and Morgan, D.O. 1998. A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae. *Mol Biol Cell* **9**(10): 2803-2817.

Jaspersen, S.L. and Morgan, D.O. 2000. Cdc14 activates cdc15 to promote mitotic exit in budding yeast. *Curr Biol* **10**(10): 615-618.

Johnson, E.S., Bartel, B., Seufert, W., and Varshavsky, A. 1992. Ubiquitin as a degradation signal. *EMBO J* **11**(2): 497-505.

Keck, J.M., Jones, M.H., Wong, C.C., Binkley, J., Chen, D., Jaspersen, S.L., Holinger, E.P., Xu, T., Niepel, M., Rout, M.P., Vogel, J., Sidow, A., Yates, J.R., 3rd, and Winey, M. 2011. A cell cycle phosphoproteome of the yeast centrosome. *Science* **332**(6037): 1557-1561.

Kilmartin, J.V. and Adams, A.E. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast Saccharomyces. *J Cell Biol* **98**(3): 922-933.

Konig, C., Maekawa, H., and Schiebel, E. 2010. Mutual regulation of cyclin-dependent kinase and the mitotic exit network. *J Cell Biol* **188**(3): 351-368.

Kops, G.J., Weaver, B.A., and Cleveland, D.W. 2005. On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat Rev Cancer* **5**(10): 773-785.

Lee, K.S., Park, J.E., Asano, S., and Park, C.J. 2005. Yeast polo-like kinases: functionally conserved multitask mitotic regulators. *Oncogene* **24**(2): 217-229.

Luca, F.C., Mody, M., Kurischko, C., Roof, D.M., Giddings, T.H., and Winey, M. 2001. Saccharomyces cerevisiae Mob1p is required for cytokinesis and mitotic exit. *Mol Cell Biol* **21**(20): 6972-6983.

Maekawa, H., Priest, C., Lechner, J., Pereira, G., and Schiebel, E. 2007. The yeast centrosome translates the positional information of the anaphase spindle into a cell cycle signal. *J Cell Biol* **179**(3): 423-436.

Manzoni, R., Montani, F., Visintin, C., Caudron, F., Ciliberto, A., and Visintin, R. 2010. Oscillations in Cdc14 release and sequestration reveal a circuit underlying mitotic exit. *J Cell Biol* **190**(2): 209-222.

Mehta, S. and Gould, K.L. 2006. Identification of functional domains within the septation initiation network kinase, Cdc7. *J Biol Chem* **281**(15): 9935-9941.

Mohl, D.A., Huddleston, M.J., Collingwood, T.S., Annan, R.S., and Deshaies, R.J. 2009. Dbf2-Mob1 drives relocalization of protein phosphatase Cdc14 to the cytoplasm during exit from mitosis. *J Cell Biol* **184**(4): 527-539.

Molk, J.N., Schuyler, S.C., Liu, J.Y., Evans, J.G., Salmon, E.D., Pellman, D., and Bloom, K. 2004. The differential roles of budding yeast Tem1p, Cdc15p, and Bub2p protein dynamics in mitotic exit. *Mol Biol Cell* **15**(4): 1519-1532.

Mulvihill, D.P., Petersen, J., Ohkura, H., Glover, D.M., and Hagan, I.M. 1999. Plo1 kinase recruitment to the spindle pole body and its role in cell division in Schizosaccharomyces pombe. *Mol Biol Cell* **10**(8): 2771-2785.

Musacchio, A. and Salmon, E.D. 2007. The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* **8**(5): 379-393.

Park, C.J., Park, J.E., Karpova, T.S., Soung, N.K., Yu, L.R., Song, S., Lee, K.H., Xia, X., Kang, E., Dabanoglu, I., Oh, D.Y., Zhang, J.Y., Kang, Y.H., Wincovitch, S., Huffaker, T.C., Veenstra, T.D., McNally, J.G., and Lee, K.S. 2008. Requirement for the budding yeast polo kinase Cdc5 in proper microtubule growth and dynamics. *Eukaryot Cell* **7**(3): 444-453.

Pereira, G., Hofken, T., Grindlay, J., Manson, C., and Schiebel, E. 2000. The Bub2p spindle checkpoint links nuclear migration with mitotic exit. *Mol Cell* **6**(1): 1-10.

Pereira, G., Manson, C., Grindlay, J., and Schiebel, E. 2002. Regulation of the Bfa1p-Bub2p complex at spindle pole bodies by the cell cycle phosphatase Cdc14p. *J Cell Biol* **157**(3): 367-379.

Pereira, G. and Schiebel, E. 2005. Kin4 kinase delays mitotic exit in response to spindle alignment defects. *Mol Cell* **19**(2): 209-221.

Rock, J.M. and Amon, A. 2009. The FEAR network. *Curr Biol* **19**(23): R1063-1068.

Rose, M.D., Misra, L.M., and Vogel, J.P. 1989. KAR2, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell* **57**(7): 1211-1221.

Schmidt, S., Sohrmann, M., Hofmann, K., Woollard, A., and Simanis, V. 1997. The Spg1p GTPase is an essential, dosage-dependent inducer of septum formation in Schizosaccharomyces pombe. *Genes Dev* **11**(12): 1519-1534.

Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. 1998. The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae. *EMBO J* **17**(5): 1336-1349.

Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Charbonneau, H., and Deshaies, R.J. 1999. Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* **97**(2): 233-244.

Sohrmann, M., Schmidt, S., Hagan, I., and Simanis, V. 1998. Asymmetric segregation on spindle poles of the Schizosaccharomyces pombe septum-inducing protein kinase Cdc7p. *Genes Dev* **12**(1): 84-94.

Stegmeier, F. and Amon, A. 2004. Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. *Annu Rev Genet* **38**: 203-232.

Stegmeier, F., Visintin, R., and Amon, A. 2002. Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. *Cell* **108**(2): 207-220.

Tanaka, K., Petersen, J., MacIver, F., Mulvihill, D.P., Glover, D.M., and Hagan, I.M. 2001. The role of Plo1 kinase in mitotic commitment and septation in Schizosaccharomyces pombe. *EMBO J* **20**(6): 1259-1270.

Toyn, J.H. and Johnston, L.H. 1994. The Dbf2 and Dbf20 protein kinases of budding yeast are activated after the metaphase to anaphase cell cycle transition. *EMBO J* **13**(5): 1103-1113.

Valerio-Santiago, M. and Monje-Casas, F. 2011. Tem1 localization to the spindle pole bodies is essential for mitotic exit and impairs spindle checkpoint function. *J Cell Biol* **192**(4): 599-614.

Visintin, R. and Amon, A. 2001. Regulation of the mitotic exit protein kinases Cdc15 and Dbf2. *Mol Biol Cell* **12**(10): 2961-2974.

Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M., and Amon, A. 1998. The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol Cell* **2**(6): 709-718.

Visintin, R., Hwang, E.S., and Amon, A. 1999. Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature* **398**(6730): 818-823.

Visintin, R., Stegmeier, F., and Amon, A. 2003. The role of the polo kinase Cdc5 in controlling Cdc14 localization. *Mol Biol Cell* **14**(11): 4486-4498.

Wang, Y. and Ng, T.Y. 2006. Phosphatase 2A negatively regulates mitotic exit in Saccharomyces cerevisiae. *Mol Biol Cell* **17**(1): 80-89.

Wilmeth, L.J., Shrestha, S., Montano, G., Rashe, J., and Shuster, C.B. 2010. Mutual dependence of Mob1 and the chromosomal passenger complex for localization during mitosis. *Mol Biol Cell* **21**(3): 380-392.

Yeh, E., Skibbens, R.V., Cheng, J.W., Salmon, E.D., and Bloom, K. 1995. Spindle dynamics and cell cycle regulation of dynein in the budding yeast, Saccharomyces cerevisiae. *J Cell Biol* **130**(3): 687-700.

Yoshida, S., Asakawa, K., and Toh-e, A. 2002. Mitotic exit network controls the localization of Cdc14 to the spindle pole body in Saccharomyces cerevisiae. *Curr Biol* **12**(11): 944-950.

Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. 1998. Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. *Science* **282**(5394): 1721-1724.

Figure Legends

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

(A, B) Wild-type (A2747) and *lte1Δ kin4Δ* (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 *tem1Δ 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 *tem1Δ 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) *tem1Δ CDC15-UP* (A22670) and *tem1Δ cdc5-7 CDC15-UP* (A24305) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium at 30°C. 30 minutes prior to release the cells were shifted to 37°C. When the arrest was complete (after 3 hours), 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, 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) *tem1Δ CDC15-UP* (A22670) and *tem1Δ MET25-CDC5Δdb CDC15-UP* (A25175) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/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 Cdc5Δdb) supplemented with α-factor pheromone (5 μg/ml). When the arrest was complete (after 3 hours), cells were released into pheromone free -MetRG 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, 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) *tem1∆ 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) *tem1∆ CDC15-eGFP-UP* (A25630) and *tem1∆ 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) *tem1∆ CDC15-eGFP-UP* (A25744) and *tem1∆ CDC15-eGFP-UP MET25-*

CDC5∆N70 (*tem1∆ 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 Cdc5∆N70)

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-CNM67* (*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-CNM67* (*CDC15-SPB*; A26419), and *pMET3-CDC15-eGFP-CNM67 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-CNM67* (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-CNM67* (*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-CNM67* (*tem1Δ GAL-URL-3HA-CDC5 CDC15-SPB*; A27051) cells containing 3HA-
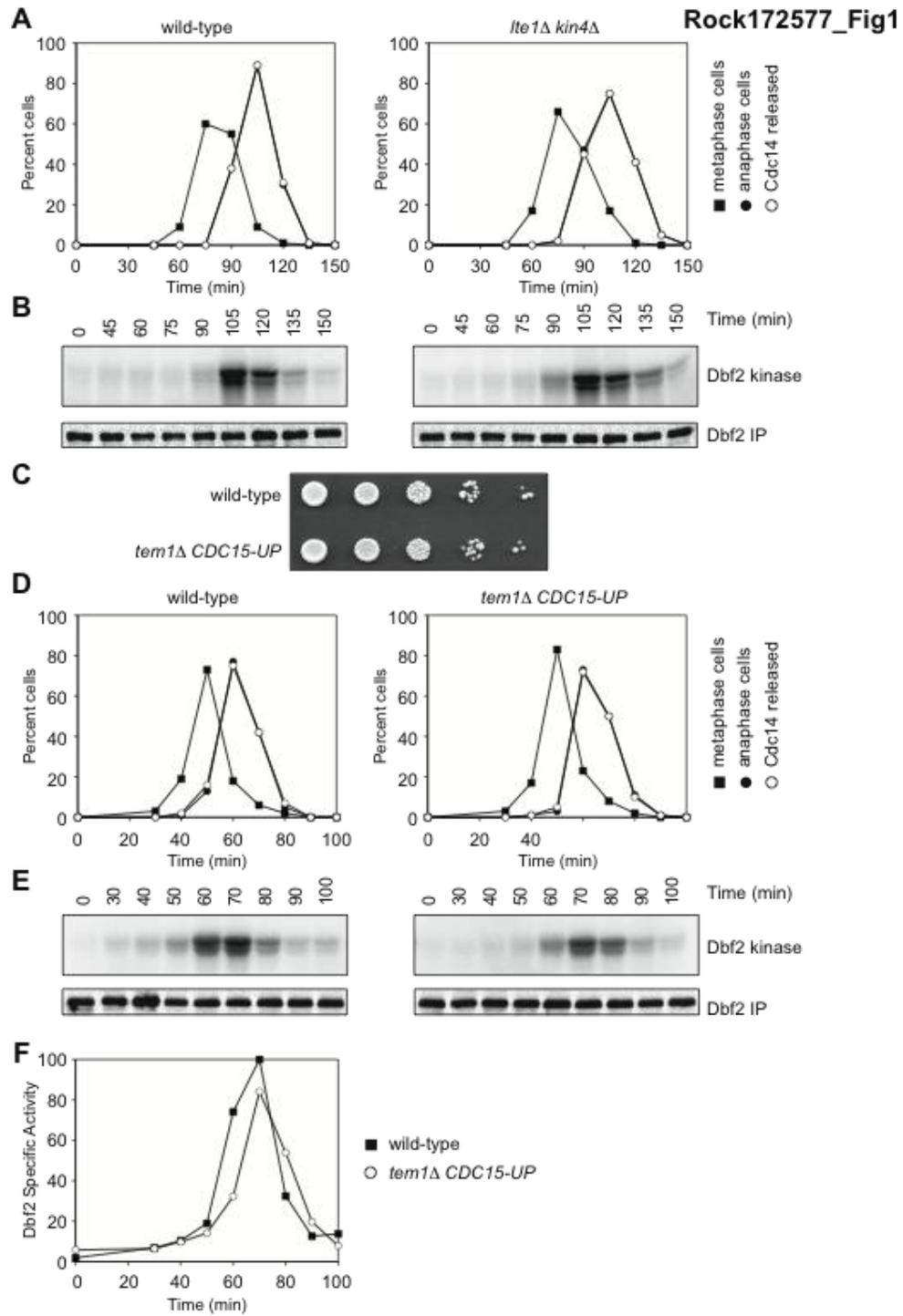
Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium supplemented with 8 mM methionine. Two hours prior to release, glucose was added (to repress expression of *GAL-URL-3HA-CDC5*). When the arrest was complete (after 2 hours 50 minutes), cells were 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
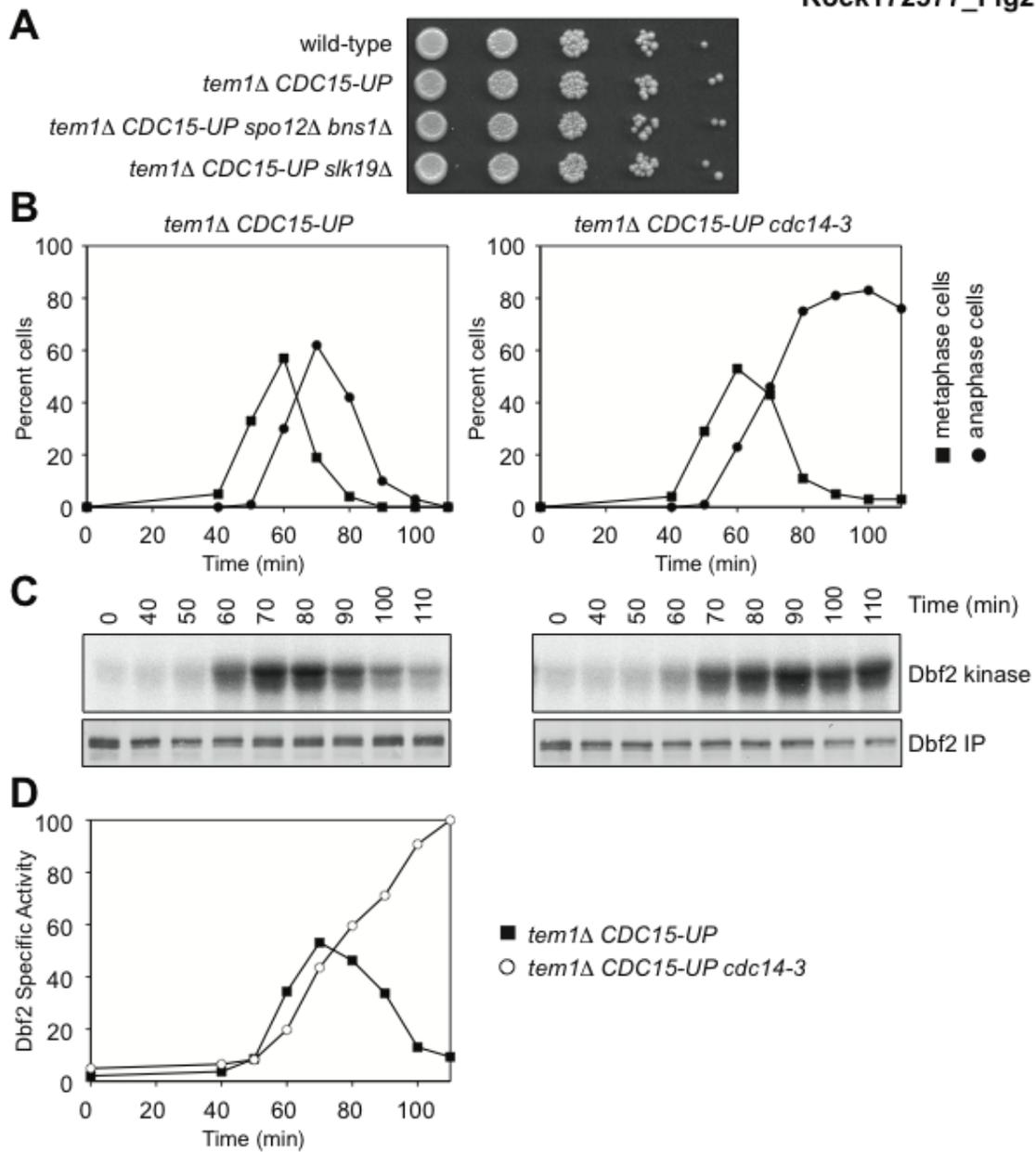
(A) Cdc15 functions as a coincidence detector of Tem1 and Cdc5 activity, both of which are required for the association of Cdc15 with SPBs. See text for details.

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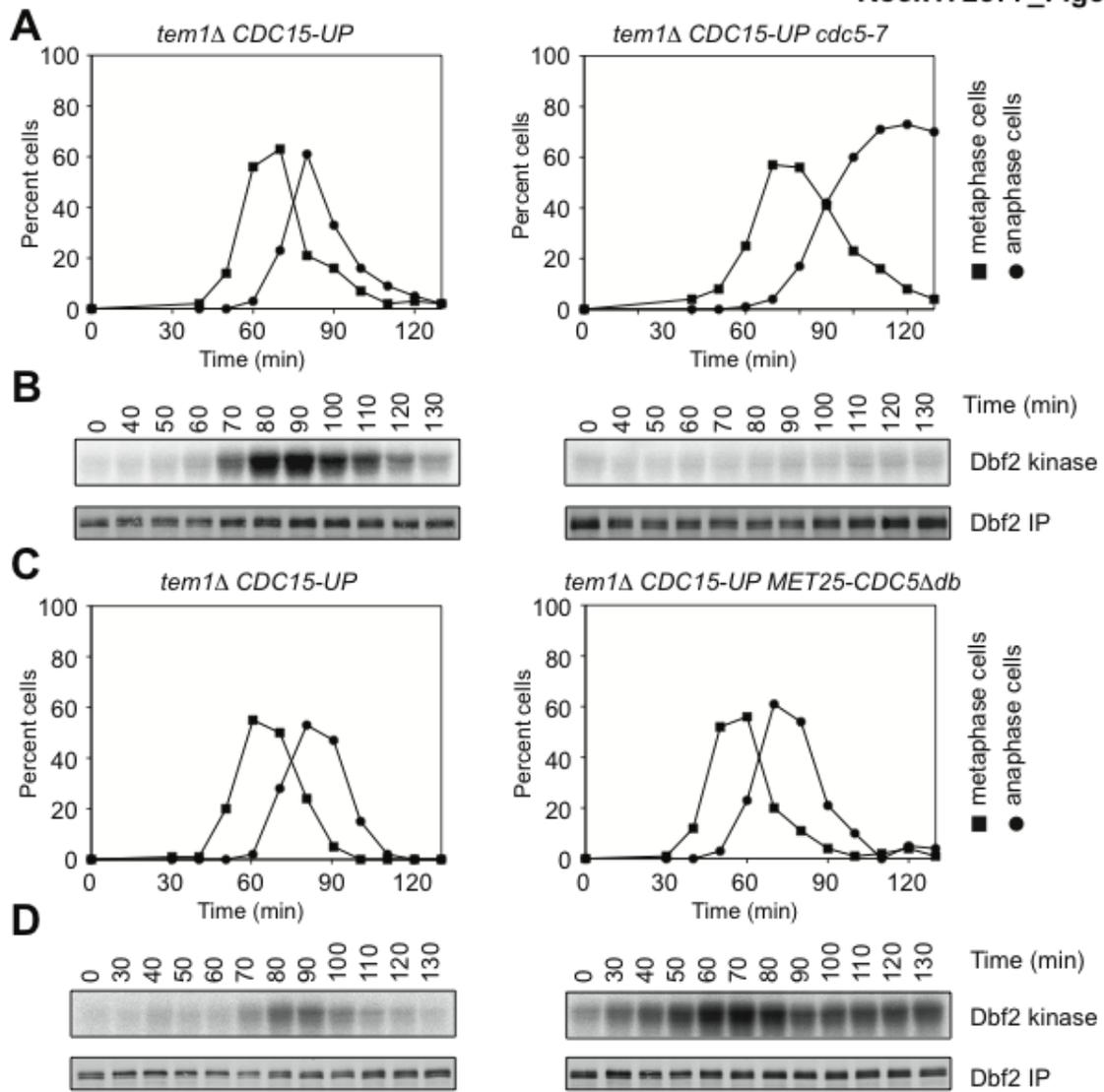 inhibitors of the MEN shown in red. Experimentally validated interactions are shown with solid lines; more speculative interactions are shown with dashed lines. See text for details.
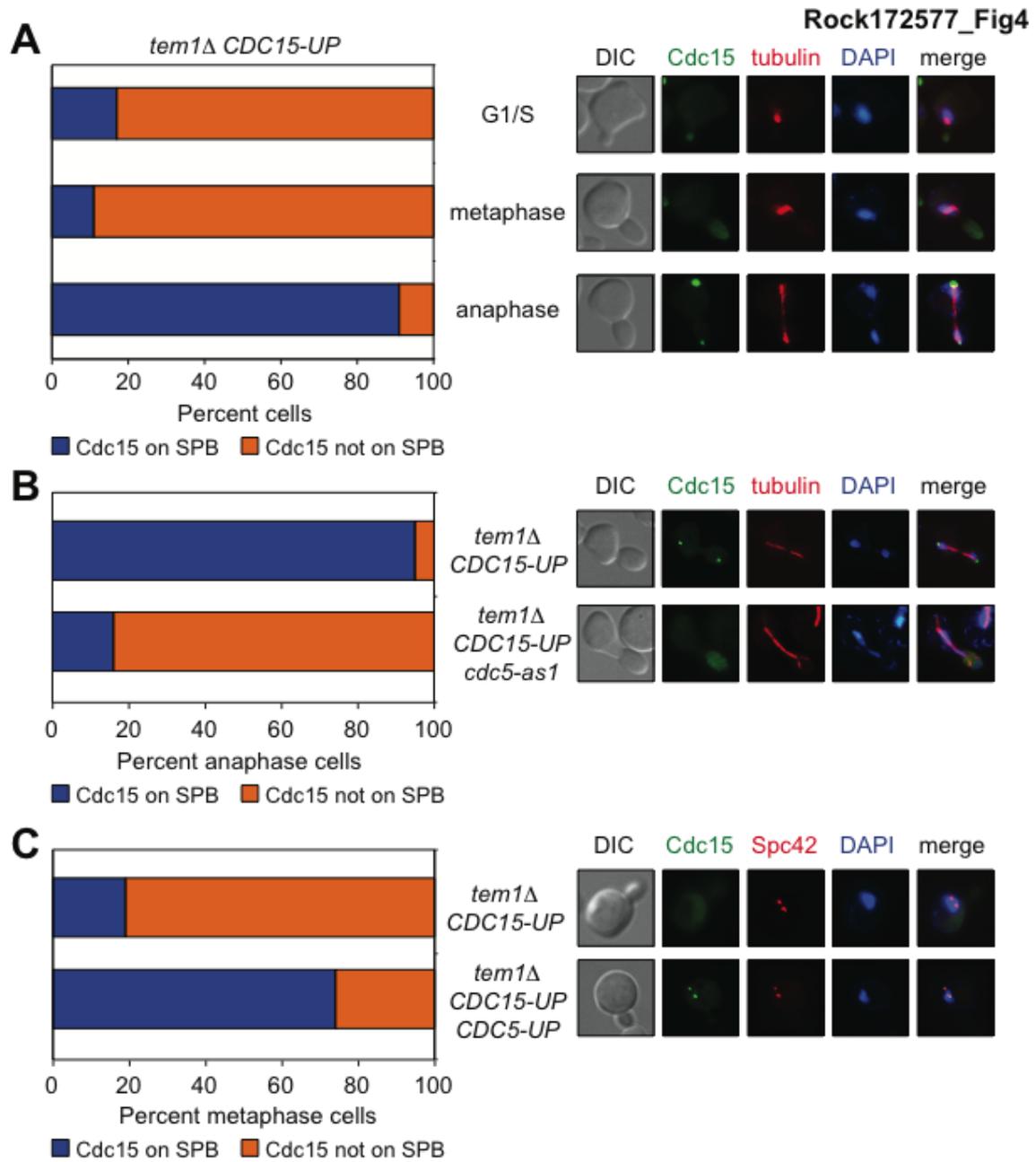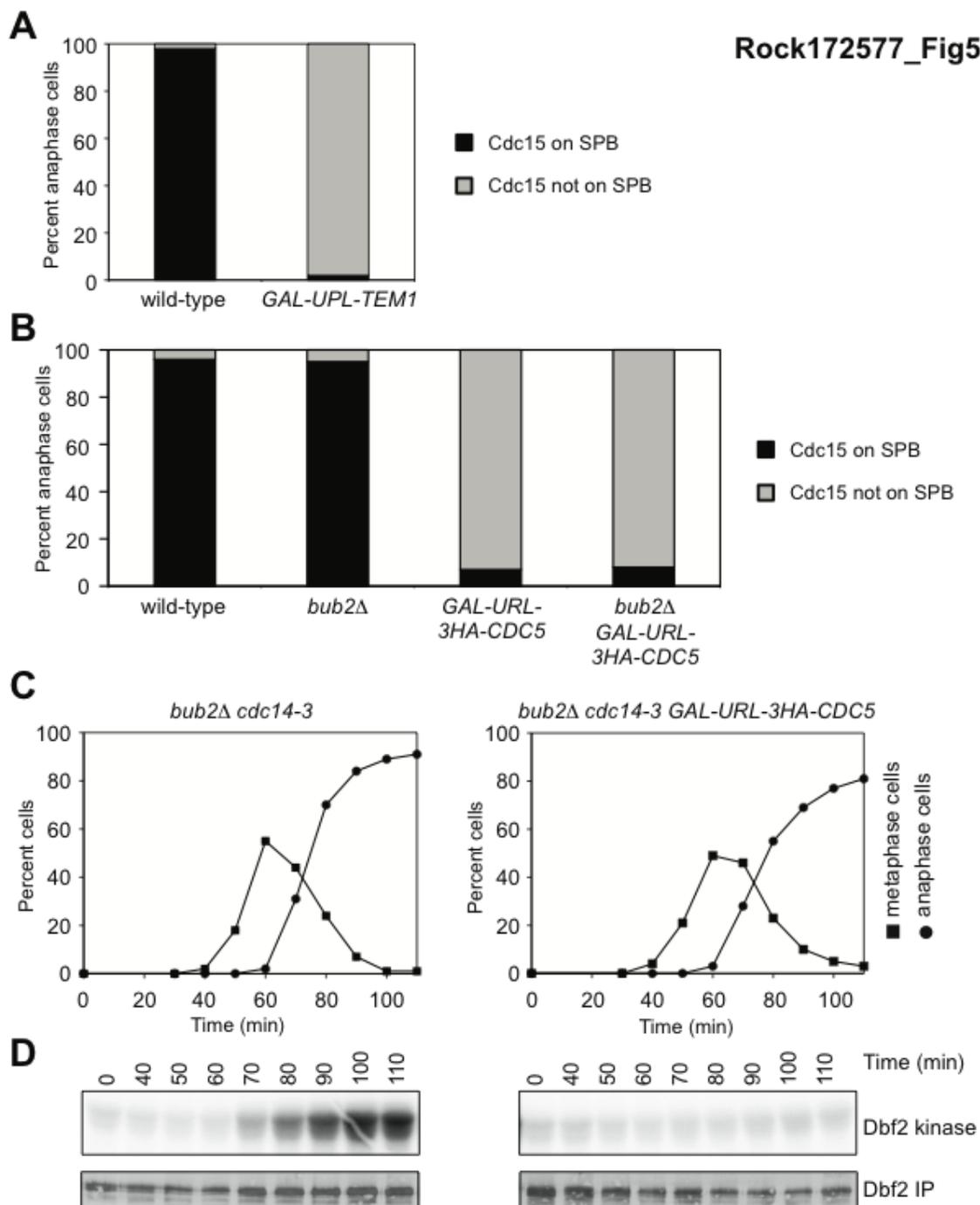
A

wild-type

tem1Δ CDC15-UP
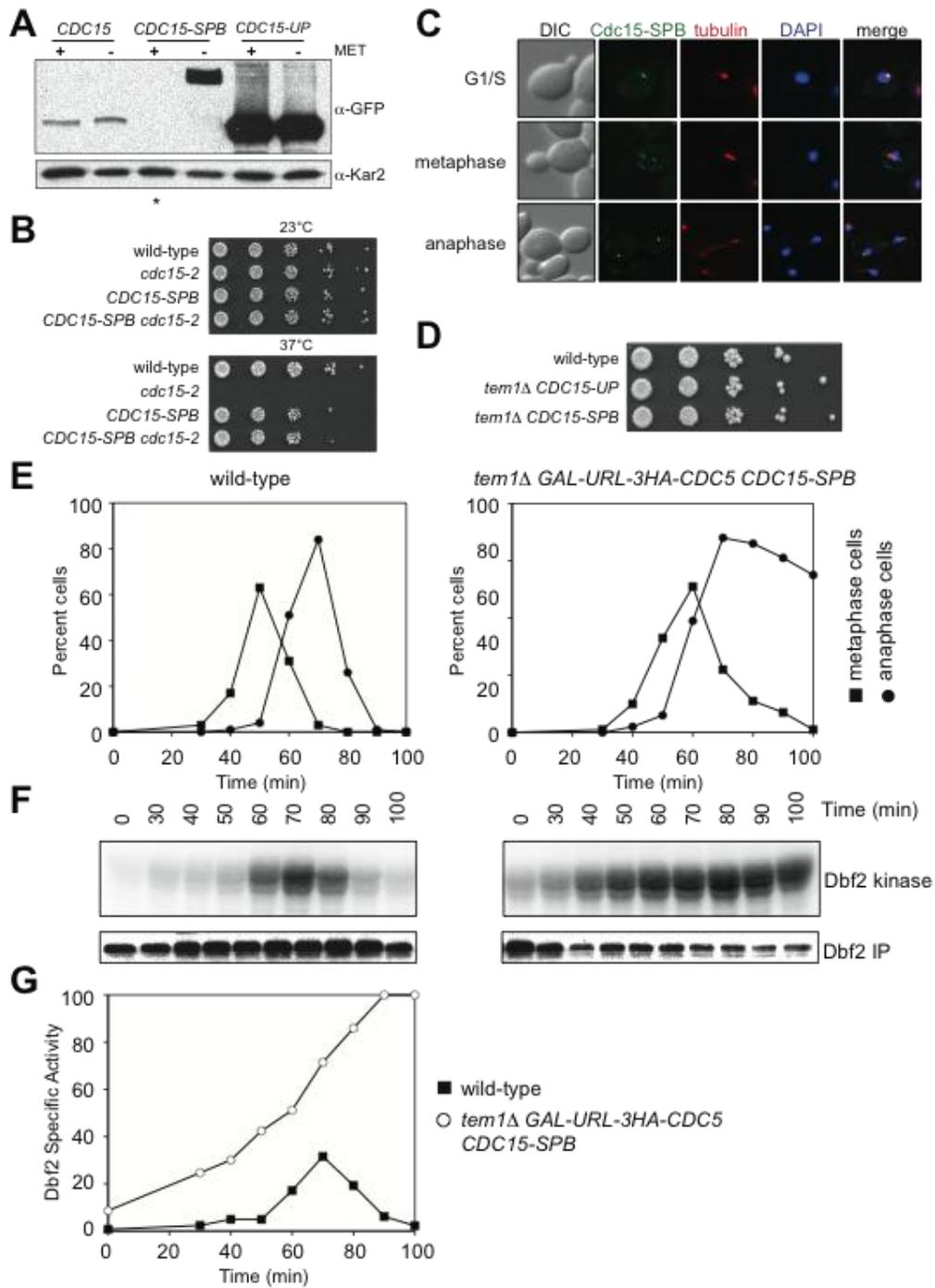
tem1Δ CDC15-UP spo12Δ bns1Δ

tem1Δ CDC15-UP slk19Δ

B

tem1Δ CDC15-UP

tem1Δ CDC15-UP cdc14-3

■ metaphase cells
● anaphase cells

C

Time (min)

Dbf2 kinase

Dbf2 IP

D

■ *tem1Δ CDC15-UP*
○ *tem1Δ CDC15-UP cdc14-3*

A *tem1Δ CDC15-UP*

DIC | Cdc15 | tubulin | DAPI | merge

G1/S

metaphase

anaphase

Percent cells

■ Cdc15 on SPB ■ Cdc15 not on SPB

B

DIC | Cdc15 | tubulin | DAPI | merge

tem1Δ CDC15-UP

tem1Δ CDC15-UP cdc5-as1

Percent anaphase cells

■ Cdc15 on SPB ■ Cdc15 not on SPB

C

DIC | Cdc15 | Spc42 | DAPI | merge

tem1Δ CDC15-UP

tem1Δ CDC15-UP CDC5-UP

Percent metaphase cells

■ Cdc15 on SPB ■ Cdc15 not on SPB

A

Rock172577_Fig5

■ Cdc15 on SPB

▨ Cdc15 not on SPB

B

■ Cdc15 on SPB

▨ Cdc15 not on SPB

C

■ metaphase cells

● anaphase cells

D

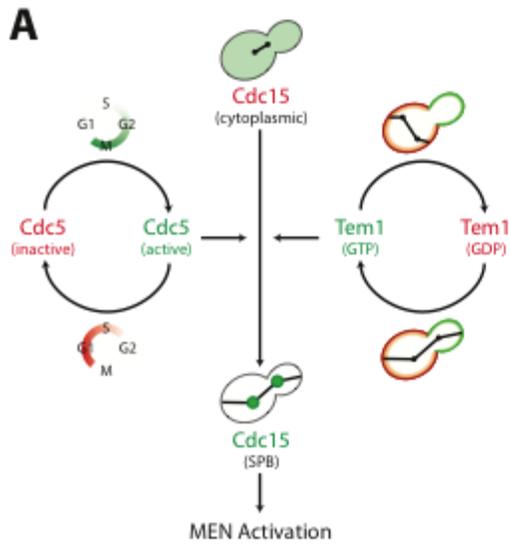**Supplemental Materials:**

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-CNM67 (*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-CNM67* (*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-Independnet 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.

Table S1. Table of Yeast Strains

A2270	*MAT a, dyn1::URA3, bub2:: HIS3, CDC14-HA, ade2-1, ura3, trp1-1, his3-11, 14, can1-100, GAL, psi+*
A2444	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, CDC14-3HA, dyn1::URA3*
A2587	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+*
A2597	*MATalpha, cdc15-2, leu2-3, ura3, trp1-1, omns*
A2747	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, CDC14-3HA, DBF2-3MYC*
A20791	*MATa, bub2::HIS3, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, CDC15-eGFP::KanMX6*
A20935	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, CDC15-eGFP::KanMX6*
A21924	*MATa, ade2-1, leu2-3, ura3-1, trp1-1, his3-11,15, can1-100, GAL, leu2::CDC15-HA3-LEU2, cdc15::TRP-GAL-GFP-CDC15(1-750)-HIS, DBF2-3MYC, CDC14-3HA*
A22670	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1, PGPD-CDC15::NatMX6, DBF2-3MYC, CDC14-3HA*
A23387	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1, PGPD-CDC15::NatMX6, slk19::kanMX6, CDC14-3HA, DBF2-3MYC*
A23392	*MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1, PGPD-CDC15::NatMX6, spo12::HIS3, bns1::KanMX6, CDC14-3HA, DBF2-3MYC*
A23657	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1 (copy # unknown), PGPD-CDC15::NatMX6, dyn1::URA3, CDC14-3HA*
A23716	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1, PGPD-CDC15::NatMX6, esp1-1, DBF2-3MYC, CDC14-3HA*
A23712	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1 (copy # unknown), PGPD-CDC15::NatMX6, DBF2-3MYC*
A23782	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1, PGPD-CDC15::NatMX6, DBF2-3MYC*
A24305	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1, PGPD-CDC15::NatMX6, cdc5-7, DBF2-3MYC, CDC14-3HA*
A24508	*MATa, ade2-1, leu2-3, ura3-1, trp1-1, his3-11,15, can1-100, GAL, leu2::CDC15-HA3-LEU2, cdc15::TRP-GAL-GFP-CDC15(1-750)-HIS, cdc5-as1 (cdc5L158G), DBF2-3myc, CDC14-3HA*
A24695	*MATa, ade2-1, leu2-3, ura3-1, trp1-1, his3-11,15, can1-100, GAL, leu2::CDC15-HA3-LEU2, cdc15::GAL-GFP-CDC15::TRP, cdc5-as1 (cdc5L158G), DBF2-3MYC, CDC14-3HA*
A24698	*MATa, ade2-1, leu2-3, ura3-1, trp1-1, his3-11,15, can1-100, GAL, leu2::CDC15-HA3-LEU2, cdc15::GAL-GFP-CDC15::TRP, DBF2-3MYC, CDC14-3HA*

A25175	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1, PGPD-CDC15::NatMX6, PMET25-Cdc5dN70::URA3, CDC14-3HA, DBF2-3MYC*
A25222	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, GAL-CDC15::TRP1, PGPD-CDC15::NatMX6, MET-CDC20::URA3, DBF2-3MYC, CDC14-3HA*
A25515	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, (GAL-CDC15-eGFP::KanMX6)::TRP1, NatMX6::PGPD-CDC15-eGFP::His3MX6, DBF2-3MYC, CDC14-3HA*
A25594	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, cdc15::TRP-GAL-GFP-CDC15(1-750)-HIS, ura3:pRS306-mCherry-TUB1::URA3, cdc5-as1 (cdc5L158G)*
A25596	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, cdc15::TRP-GAL-GFP-CDC15(1-750)-HIS, ura3::pRS306-mCherry-TUB1::URA3*
A25630	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, (GAL-CDC15-eGFP::KanMX6)::TRP1, NatMX6::PGPD-CDC15-eGFP::His3MX6, ura3::pRS306-mCherry-TUB1::URA3*
A25633	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, (GAL-CDC15-eGFP::KanMX6)::TRP1, NatMX6::PGPD-CDC15-eGFP::His3MX6, ura3::pRS306-mCherry-TUB1::URA3, cdc5-as1*
A25661	*MATa, ade2-1, leu2-3, ura3-1, trp1-1, his3-11,15, can1-100, GAL, tem1::KanMX6, cdc15::GAL-GFP-CDC15::TRP, ura3::pRS306-mCherry-TUB1::URA3, cdc5-as1 (cdc5L158G), DBF2-3MYC, CDC14-3HA*
A25662	*MATa, ade2-1, leu2-3, ura3-1, trp1-1, his3-11,15, can1-100, GAL, tem1::KanMX6, cdc15::GAL-GFP-CDC15::TRP, ura3::pRS306-mCherry-TUB1::URA3, DBF2-3MYC, CDC14-3HA*
A25744	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, (GAL-CDC15-eGFP::KanMX6)::TRP1, NatMX6::PGPD-CDC15-eGFP::His3MX6, SPC42-mCherry:NatMx6*
A25983	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, (GAL-CDC15-eGFP::KanMX6)::TRP1, NatMX6::PGPD-CDC15-eGFP::His3MX6, SPC42-mCherry:NatMx6, PMET25-Cdc5dN70::URA3*
A26379	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, kin4::TRP1, lte1::KanMX6, CDC14-3HA, DBF2-3MYC*
A26396	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, PMET3-CDC15-eGFP-CNM67::LEU2, CDC14-3HA, DBF2-3MYC*
A26413	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, cdc15-2, PMET3-CDC15-eGFP-CNM67::LEU2*
A26417	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, PMET3-CDC15-eGFP-CNM67::LEU2, DBF2-3MYC*
A26418	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, PMET3-CDC15-eGFP-CNM67::LEU2, CDC14-3HA, DBF2-3MYC*
A26419	*MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, PMET3-CDC15-eGFP-CNM67::LEU2*
A26480	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, CDC15-eGFP::KanMX6, bub2::HIS3, ura3::pRS306-mCherry-TUB1::URA3*

A26481	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, CDC15-eGFP::KanMX6, ura3::pRS306-mCherry-TUB1::URA3*
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*
A26558	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, CDC15-eGFP::KanMX6, bub2::HIS3, cdc5::GAL-URL-3HA-CDC5::KanMX6, ura3::pRS306-mCherry-TUB1::URA3*
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*
A27051	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, tem1::KanMX6, PMET3-CDC15-eGFP-CNM67::LEU2, cdc5::GAL-URL-3HA-CDC5::kanMX, CDC14-3HA, DBF2-3MYC*
A27055	*MATa, ade2-1, can 1-100, his3-11,12, leu2-3,112, trp1-1, ura3-1, tem1::GAL-UPL-TEM1:TRP1, CDC15-eGFP::KanMX6, ura3::pRS306-mCherry-TUB1::URA3*

Table S2. Table of Plasmids

pA226   YIplac204-*GAL-CDC15*
pA1469   pRS306-*mCherry-TUB1*
pA1813   YIplac211-*PMET25-CDC5ΔN70*
pA1880   YIplac204-*PMET3-CDC15-eGFP-CNM67*

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'-aataAAGCTTCGGATGCAAGGGTTCGAATC-3') and (5'-aataCTGCAGGGATGGGGGTAATAGAATTG-3') from A2587 genomic DNA (PCR product 1); the N-terminally truncated (70 amino acids) *CDC5* ORF was amplified with primers (5'-aataCTGCAGAAAATGCCACCTTCATTAATCAAAACAAG-3') and (5'-CATGGCAATTTTGAATAGATATAG-3') from A2587 genomic DNA genomic DNA (PCR product 2). PCR product 1 was digested with HindIII and PstI; PCR product 2 was digested with PstI and XbaI; plasmid YIplac211was digested with HindIII and XbaI (Gietz and Sugino 1988). Fragments were three way ligated to yield: YIplac211-*MET25-CDC5ΔN70.*

pA1880: *CDC15-eGFP-CNM67* was cloned under the control of the *MET3* promoter using the following strategy. *PMET3* was amplified with primers (5'-TTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATGAAACTGAGTAAGAT GCTCAGAATAC-3') and (5'-GAGTCAAGTTGACTCTATCGGTATCGGCCATACTGTTCATCCTAGGGTTAATTATAC TTTATTCTTG-3') with a *PMET3* containing plasmid as template; *CDC15-eGFP* was amplified with primers (5'-ATGAACAGTATGGCCGATACC-3') and (5'-GCCACCACCAGAGCCACCTCCACCAGAACCTCCACCACCTAGTTTGTACAATTCAT CAATACCATG-3') with A20791 genomic DNA as PCR template; and *CNM67* was amplified with primers (5'-CTAGGTGGTGGAGGTTCTGGTGGAGGTGGCTCTGGTGGTGGCATGACTGATTTCG ATTTAATG-3') and (5'-

TAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGGAACCCCTAAAAGCTCA
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-CNM67* allele.
Plasmids were recovered from resulting Trp+ colonies and sequence confirmed to
contain mutation-free *PMET3-CDC15-eGFP-CNM67. PMET3-CDC15-eGFP-CNM67*
was then subcloned into the SphI & 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
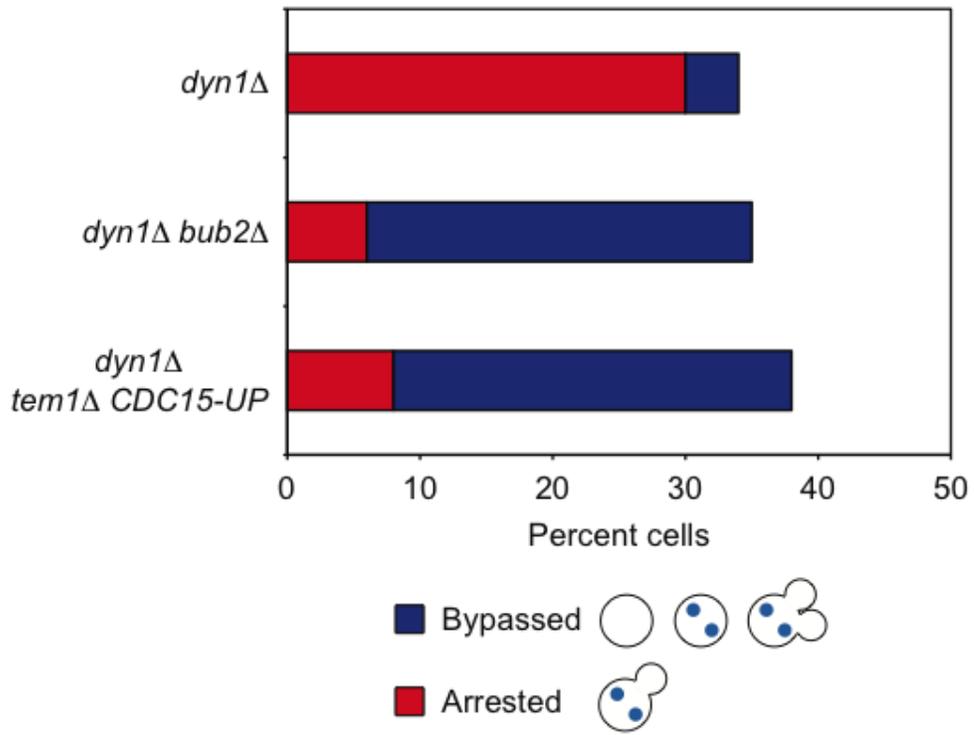connected by an elongated spindle.

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
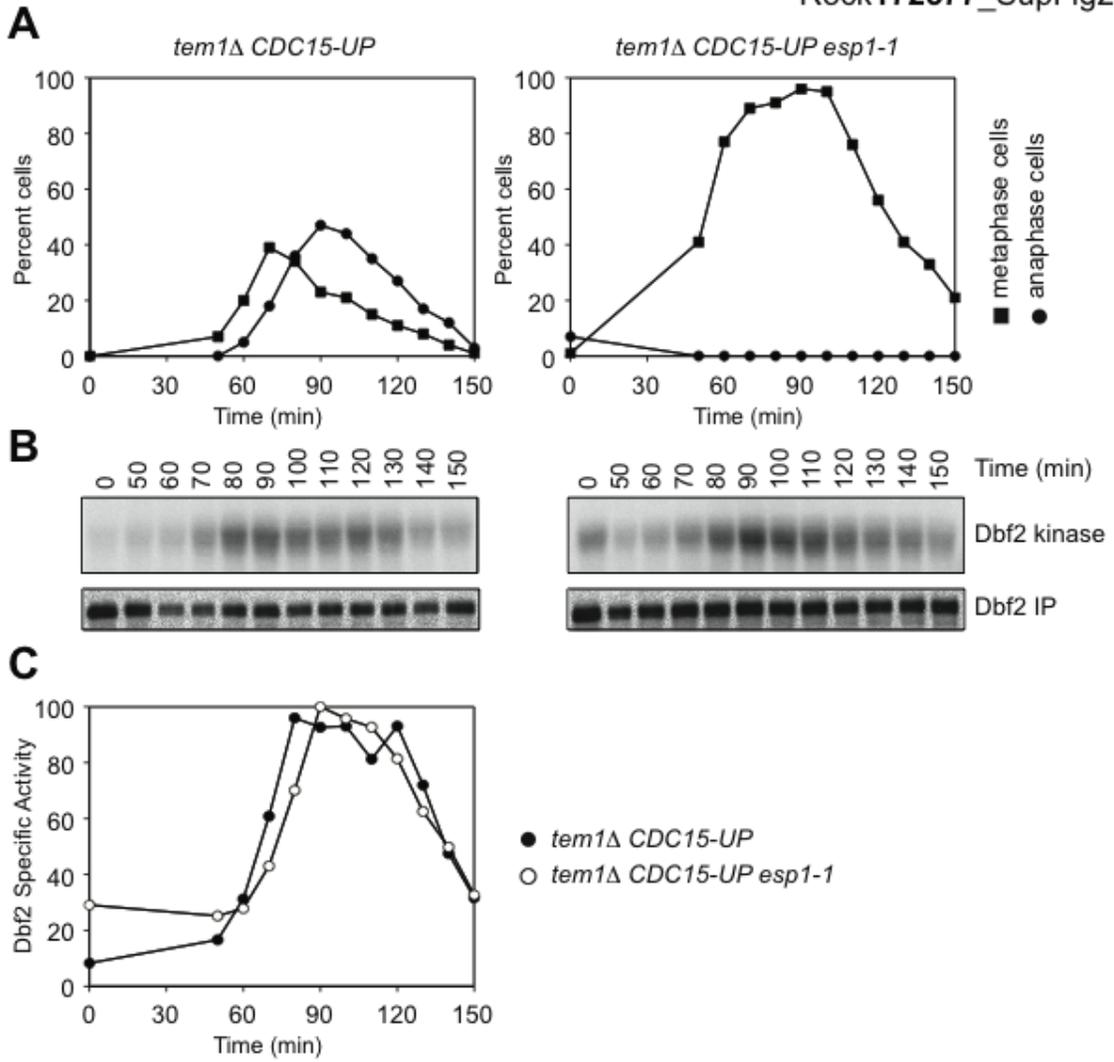nuclei in the mother cell body were counted as arrested.

Supplemental References

Bachmair, A., Finley, D., and Varshavsky, A. 1986. In vivo half-life of a protein is a function of its amino-terminal residue. *Science* **234**(4773): 179-186.

Charles, J.F., Jaspersen, S.L., Tinker-Kulberg, R.L., Hwang, L., Szidon, A., and Morgan, D.O. 1998. The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in S. cerevisiae. *Curr Biol* **8**(9): 497-507.

Gietz, R.D. and Sugino, A. 1988. New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**(2): 527-534.

Janke, C., Magiera, M.M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., and Knop, M. 2004. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**(11): 947-962.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* **14**(10): 953-961.

Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. 1998. The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae. *EMBO J* **17**(5): 1336-1349.
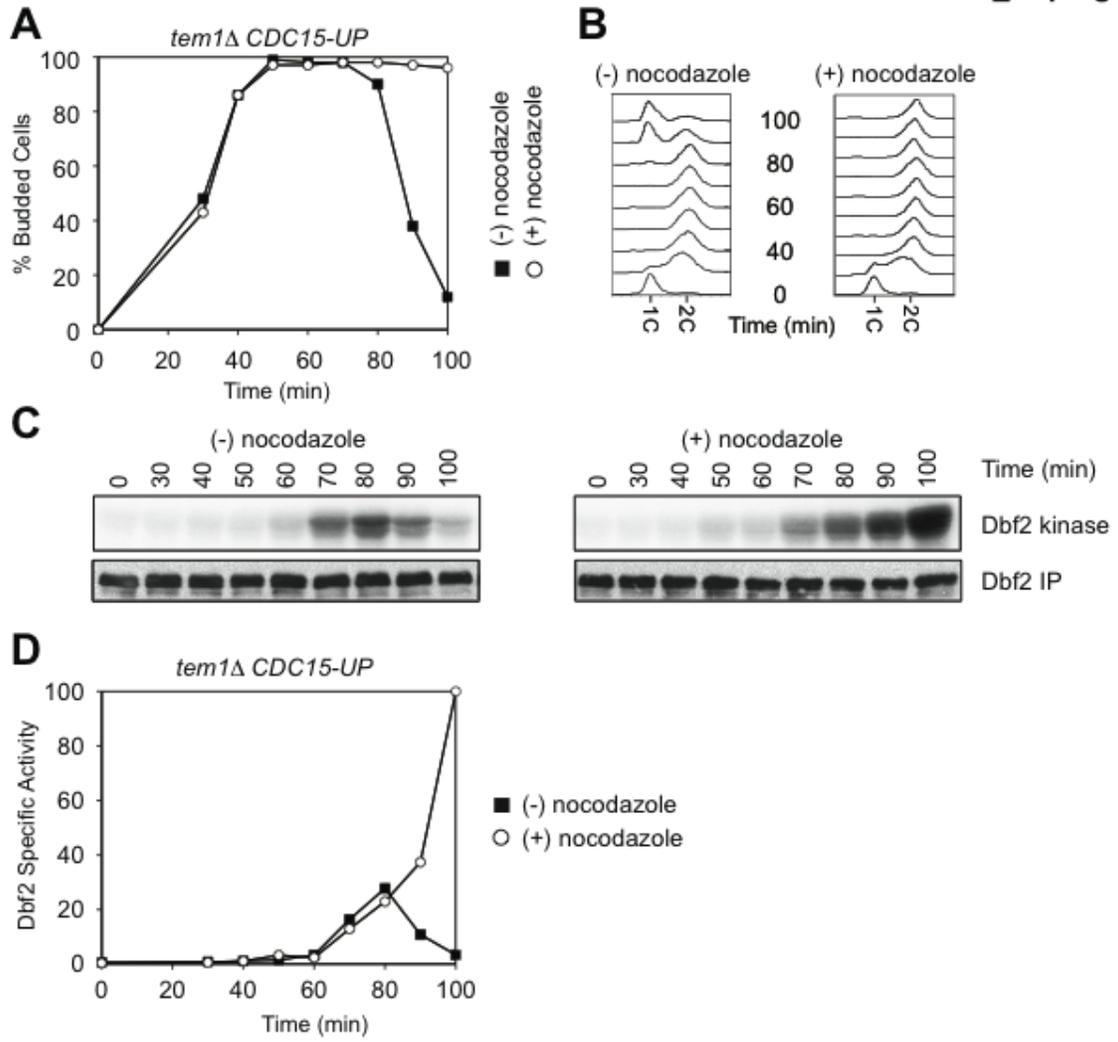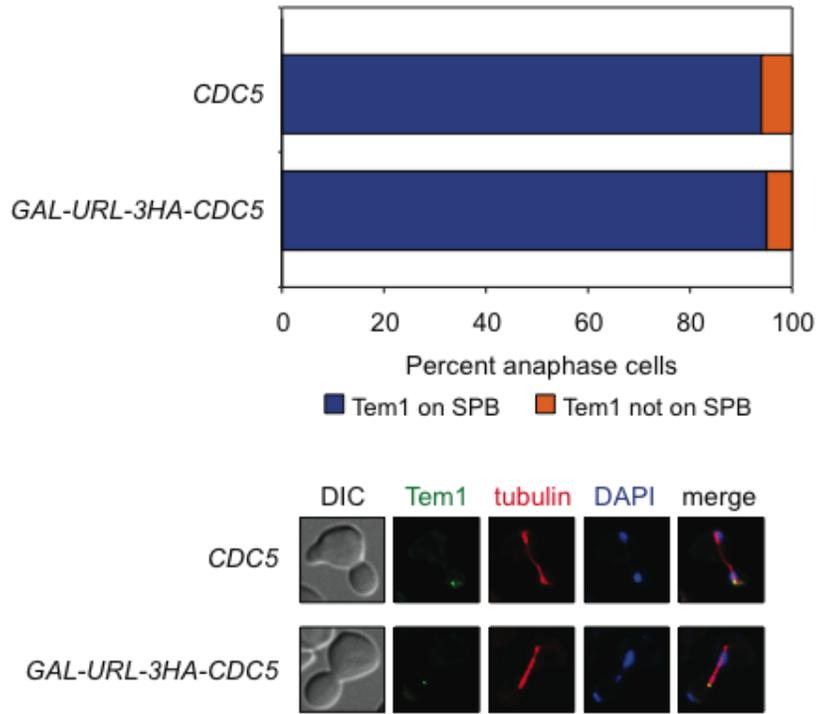
A

tem1Δ CDC15-UP

tem1Δ CDC15-UP esp1-1
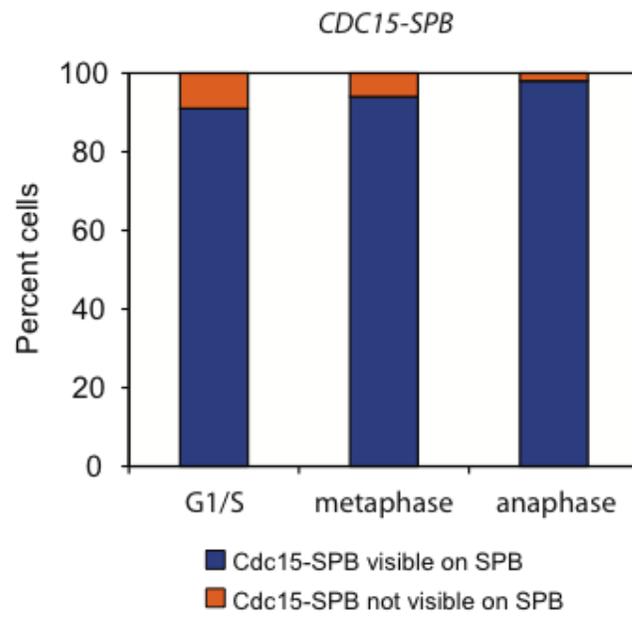
■ metaphase cells
● anaphase cells

B

Dbf2 kinase

Dbf2 IP

C

● *tem1Δ CDC15-UP*
○ *tem1Δ CDC15-UP esp1-1*

A tem1Δ CDC15-UP

B (-) nocodazole / (+) nocodazole

C (-) nocodazole / (+) nocodazole

Dbf2 kinase

Dbf2 IP

D tem1Δ CDC15-UP

- (-) nocodazole
- (+) nocodazole

Rock172577_SupFig4

Percent anaphase cells

■ Tem1 on SPB ■ Tem1 not on SPB

CDC15-SPB

Rock**172577**_SupFig6

A

B

C