Spatial and Temporal Coordination of Genome Segregation with Activation of the Mitotic Exit Network

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Abstract

In budding yeast, an essential Hippo-like signal transduction cascade known as the Mitotic Exit Network (MEN) governs the final cell cycle transition, the mitosis to G1 transition. To ensure the accurate execution of mitosis, MEN activity is coordinated with other cellular events. The work presented in this thesis elucidates the coordination of genome segregation with MEN activation. We first identified a novel essential role for Polo kinase Cdc5 in MEN activation. Cdc5 functions in parallel to the GTPase Tem1 to recruit the MEN kinase Cdc15 to SPBs, which is both necessary and sufficient for MEN signaling. This work thus defined Cdc15 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. Finally, we characterize the role of the scaffold Nud1 in the assembly of active MEN signaling complexes at spindle pole bodies (SPBs). We found that the assembly of such complexes requires the phosphorylation of the MEN scaffold Nud1. Phosphorylation of Nud1 in mitosis is essential for the SPB recruitment of terminal MEN kinase Dbf2 and its coactivator Mob1. We further show that the Hippo-like kinase Cdc15 phosphorylates Nud1. Finally, we present evidence that Mob1 is a novel class of phosphopeptide binding domains. Thus, Cdc15-dependent phosphorylation of the scaffold Nud1 creates a docking site for the Mob1 coactivator, thereby resulting in the recruitment of Dbf2 to SPBs and firing of the MEN.

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Chapter I: Introduction

Summary

The transmission of genetic information from one generation to the next requires the faithful duplication and segregation of the genome. Accomplishing this task necessitates the coordination of many mitotic processes both in time and space. The topic of this thesis concerns the spatiotemporal coordination of genome segregation with the final cell cycle transition, the mitosis to G1 transition, in the budding yeast *S. cerevisiae*. This transition is governed by a conserved Hippo-like signaling pathway known as the Mitotic Exit Network (MEN). This introduction will discuss the mechanisms of mitotic exit in budding yeast, followed by a discussion of the two signaling pathways (the FEAR network and the MEN) that regulate this process. Following this, roles for the mitotic exit machinery, the FEAR network, and the MEN will be discussed in the context of both meiosis in budding yeast and mitosis in higher eukaryotes. Finally, this introduction will conclude with a summary of the findings presented in this thesis.

The transmission of genetic information from one generation to the next requires the faithful duplication and segregation of the genome. Accomplishing this task requires the coordination of many mitotic processes both in time and space. Successful completion of mitosis requires the temporal ordering of chromosome duplication, spindle assembly, chromosome segregation, spindle disassembly, and cytokinesis. Moreover, as many mitoses (all mitoses in budding yeast) occur within the context of a polarized cell division, the mitotic spindle must be properly oriented with respect to intrinsic or extrinsic polarity cues. The fidelity of mitotic processes is ensured both by the remarkable precision by which the cell cycle machinery carries out its functions as well as by checkpoints that monitor the progress of key cell cycle events. If a problem with the cell cycle arises, checkpoints halt the cell cycle to allow the cell time to correct the defect. Errors in the coordination of these mitotic processes can lead to both genetic and epigenetic changes and are strongly associated with cancer (reviewed in (Kops et al. 2005; Gonzalez 2007; Gordon et al. 2012)).

An overview of mitosis

The cell cycle machinery regulating chromosome segregation is conserved in all eukaryotes. At the core of eukaryotic mitosis are protein kinases known as cyclin dependent kinases (CDKs). Activation of CDKs, which are composed of a catalytic kinase subunit and a regulatory cyclin subunit, drive the entry and progression through mitosis (reviewed in (Morgan 1997)).

Preparations for chromosome segregation begin in S phase. Prior to DNA replication, protein complexes known as cohesin complexes are deposited on the chromosomes. As cells pass through S phase and duplicate their genome, these cohesin complexes are in some way transformed into the cohesive structures that link duplicated sister chromatids and facilitate their bi-orientation on the mitotic spindle (reviewed in (Nasmyth and Haering 2009)). Also during this phase of the cell cycle, the spindle pole body (SPB, the yeast equivalent of the centrosome) duplicates and separates to form a short spindle (reviewed in (Lim et al. 2009)).

Upon completion of chromosome duplication and assembly of a bipolar spindle, duplicated sister chromatids bi-orient on the mitotic spindle. The fidelity of this process is ensured by the spindle assembly checkpoint, a checkpoint signaling pathway that inhibits the activity of an E3 ubiquitin ligase known as the Anaphase Promoting Complex/Cyclosome (APC/C) coupled to its specificity factor Cdc20. Upon successful bi-orientation of all chromosomes, checkpoint signaling is relieved and the APC/C^{Cdc20} targets the anaphase inhibitor Securin for destruction (Figure 1). Destruction of Securin liberates the protease Separase which then cleaves the cohesin subunit Scc1/Mcd1, thereby initiating the onset of chromosome segregation (reviewed in (Peters 2006)).

Upon completion of chromosome segregation, cells exit from mitosis. This final cell cycle transition is defined by the disassembly of the mitotic spindle, nuclear envelope reformation, chromosome decondensation, cytokinesis, and the removal

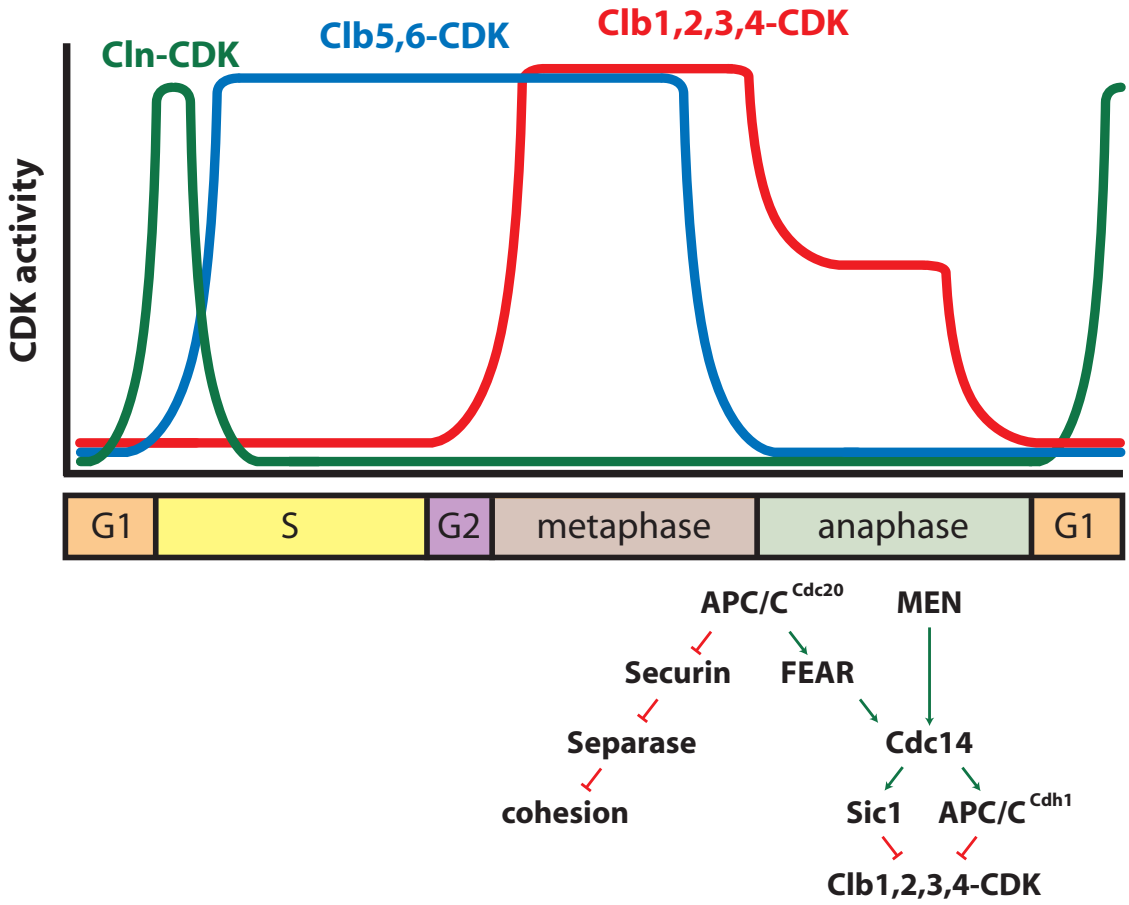

Figure 1. Mitosis in budding yeast

Activation of Clb1,2,3,4-CDK drives the cell into mitosis. At the metaphase to anaphase transition, activation of the APC/C^{Cdc20} targets the anaphase inhibitor Securin for destruction. Destruction of Securin liberates the protease Separase which then cleaves the cohesin subunit Scc1/Mcd1, thereby initiating the onset of chromosome segregation. Activation of Separase by the APC/C^{Cdc20} also activates the FEAR network and initiates the release of Cdc14 from the nucleolus. Activation of the MEN in late anaphase drives sustained Cdc14 release, which then dephosphorylates and activates both the Clb-CDK inhibitor Sic1 and the APC/C specificity factor Cdh1, thereby resulting in the inactivation of Clb-CDK and exit from mitosis.

of mitotic determinants. The removal of mitotic determinants and the resetting of the cell to a G1 like state creates conditions permissive for cytokinesis as well as the loading of pre-replicative complexes onto the origins of DNA (reviewed in (Stegmeier and Amon 2004)). In all eukaryotes studied to date, exit from mitosis requires the inactivation of mitotic CDKs. In the absence of mitotic CDK downregulation, cells arrest in late anaphase. Mitotic CDK inactivation is achieved shortly after the metaphase to anaphase transition in higher eukaryotes and is accomplished primarily through ubiquitin-mediated proteolysis of the cyclin B (Clb) CDK regulatory subunits.

Mechanisms of mitotic CDK inactivation in *S. cerevisiae*

Unlike in higher eukaryotes, in budding yeast a significant pool of mitotic CDK activity remains after the metaphase to anaphase transition and persists until anaphase chromosome segregation is complete (reviewed in (Stegmeier and Amon 2004)). Clb-CDK inactivation in late anaphase is controlled by the essential phosphatase Cdc14. Cells lacking Cdc14 activity arrest in late anaphase with high mitotic CDK activity. Cdc14 promotes Clb-CDK downregulation by two primary mechanisms: (1) Cdc14 dephosphorylates the APC/C specificity factor Cdh1 thereby stimulating the APC/C-dependent destruction of Clb cyclins (Jaspersen et al. 1998; Visintin et al. 1998; Zachariae et al. 1998). (2) Cdc14 dephosphorylates the Clb-CDK inhibitor Sic1 and the transcription factor Swi5, resulting in the stabilization of Sic1 and Swi5-dependent activation of *SIC1* transcription (Jaspersen et al. 1998; Visintin

et al. 1998). Furthermore, Cdc14 counteracts CDK activity by dephosphorylating a host of CDK substrates, allowing for the rapid resetting of cells to the G1 state.

Regulation of Cdc14 during mitosis in *S. cerevisiae*

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; Sullivan and Uhlmann 2003). FEAR network promoted Cdc14 release from the nucleolus is transient: in the absence of a functional Mitotic Exit Network (MEN; see next), Cdc14 becomes re-sequestered in the nucleolus during late anaphase and cells fail to exit from mitosis. While not essential, FEAR network-mediated release of Cdc14 from the nucleolus is crucial for the accurate execution of anaphase (reviewed in (Rock and Amon 2009)). Cdc14 release from the nucleolus during late anaphase is promoted by the MEN, which drives the sustained release of Cdc14 in both the nucleus and the cytoplasm and results in exit from mitosis (reviewed in (Stegmeier and Amon 2004)).

The FEAR Network

Work over the last ten years has identified a number of proteins that function together to regulate the release of Cdc14 from the nucleolus during early anaphase. These proteins, collectively known as the FEAR network, include Separase, the Separase binding protein Slk19, Polo kinase Cdc5, a protein of unknown function Spo12, the replication fork block protein Fob1, PP2A phosphatase bound to its regulatory subunit Cdc55, Zds1 and Zds2, histone modifications such as H2B ubiquitylation and H3 methylation, and CDKs associated with the cyclins Clb1 and Clb2 (Pereira et al. 2002; Stegmeier et al. 2002; Yoshida et al. 2002; Sullivan and Uhlmann 2003; Visintin et al. 2003; Azzam et al. 2004; Stegmeier et al. 2004; Queralt et al. 2006; Queralt and Uhlmann 2008; Hwang and Madhani 2009). We do not fully understand how these proteins function together to bring about the release of Cdc14 from the nucleolus but a mechanism is beginning to emerge.

The association between Cdc14 and its nucleolar-localized inhibitor Cfi1/Net1 appears to be regulated by phosphorylation (Shou et al. 1999; Visintin et al. 1999; Shou et al. 2002; Yoshida and Toh-e 2002; Visintin et al. 2003). Prior to anaphase, Cdc14 and Cfi1/Net1 are in an unphosphorylated or hypophosphorylated state (Figure 2). During this stage of the cell cycle, Fob1 stabilizes the Cdc14 - Cfi1/Net1 interaction. At anaphase onset, APC/C activation results in the degradation of the Separase-inhibitor Securin, thereby resulting in the activation of the Separase – Slk19 complex. By a poorly understood mechanism that involves the proteins Zds1 and Zds2, the Separase – Slk19 complex is thought to downregulate $PP2A^{Cdc55}$

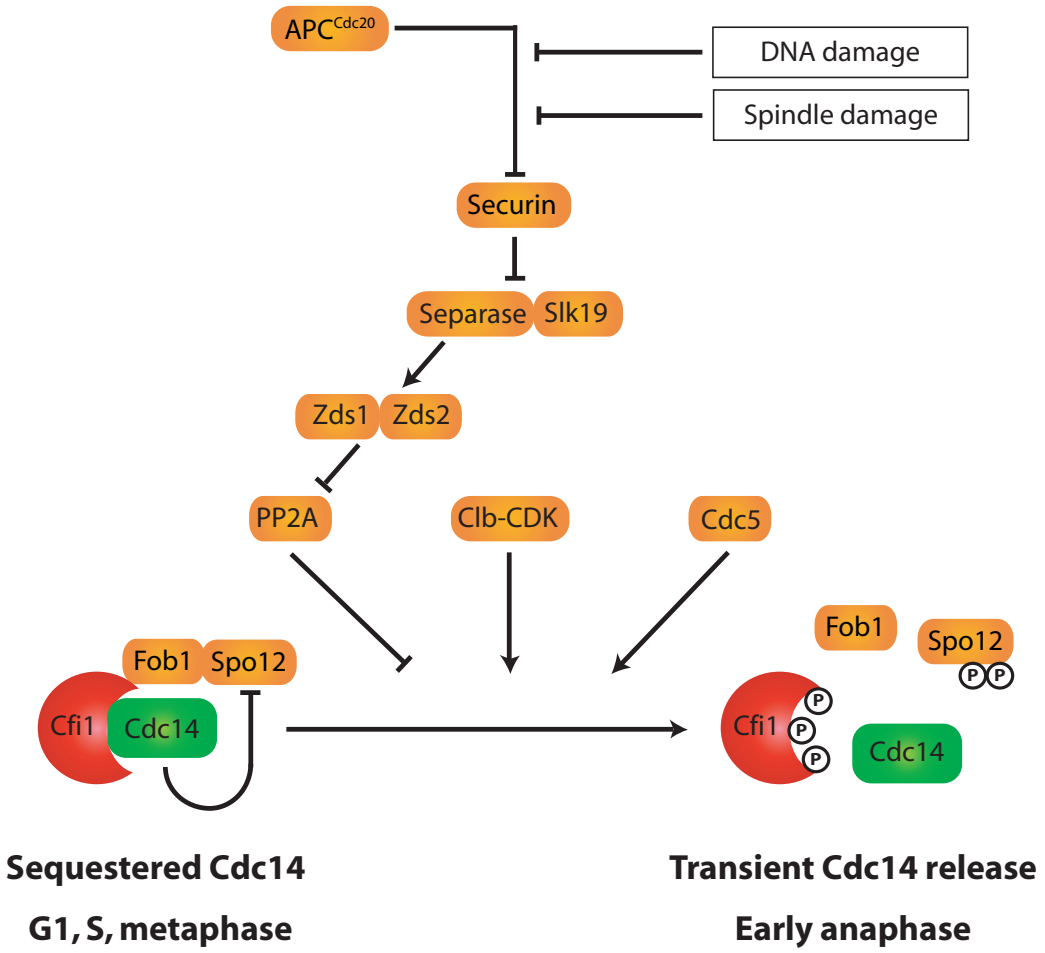

Figure 2. The FEAR Network

Cdc14 release from the nucleolus during early anaphase is mediated by the FEAR network and results in a pulse of Cdc14 activity that coordinates multiple anaphase events. See text for details.

phosphatase activity. This downregulation of $PP2A^{Cdc55}$ phosphatase activity allows Clb1-CDK and Clb2-CDK to phosphorylate Cfi1/Net1. Clb1-CDK and Clb2-CDK are also responsible for the phosphorylation of Spo12. It appears, however, that it is Cdc14, rather than PP2A, that restrains Spo12 phosphorylation in cell cycle stages prior to anaphase (Tomson et al. 2009).

Cdc5 is essential for the release of Cdc14 from the nucleolus (Pereira et al. 2002; Stegmeier et al. 2002; Visintin et al. 2003). The mechanism by which this Polo kinase functions in the FEAR network is not yet understood and is clouded by the fact that Cdc5 acts positively in both the FEAR network and the MEN. Epistasis analysis places Cdc5 downstream of and/or in parallel to Separase - Slk19. Cdc5 promotes the phosphorylation of Cdc14 and Cfi1/Net1 *in vivo*, which is at least in part due to Cdc5 dependent degradation of the CDK inhibitory kinase Swe1 (Liang et al. 2009). Furthermore, Cdc5 can dissociate the Cdc14 – Cfi1/Net1 complex *in vitro* (Shou et al. 2002; Yoshida and Toh-e 2002). Ultimately, phosphorylation of Cfi1/Net1 is thought to decrease its affinity for Cdc14 while phosphorylation of Spo12 is thought to promote, in part, the dissociation of Fob1 from the Cdc14 - Cfi1/Net1 complex.

The FEAR network harbors two key regulators of chromosome segregation, Separase and Cdc5. This allows for the coordination of FEAR network activation and chromosome segregation. Activation of Separase at the metaphase – anaphase transition both initiates chromosome segregation and FEAR network activation, thereby ensuring that Cdc14 activation does not occur prior to genome

partitioning. Importantly, the essential nature of Separase in FEAR network activation also ensures that FEAR network activation is responsive to insults such as mitotic spindle damage and DNA damage, both of which stabilize Securin.

The Mitotic Exit Network

Sustained Cdc14 release in late anaphase is controlled by the MEN. The core MEN components consist of the Ras-like GTPase Tem1, the Tem1 GTPase activating protein (GAP) Bub2-Bfa1, the Hippo-like kinase Cdc15, the Warts-like kinase Dbf2, and the Dbf2-coactivator Mob1 (Figure 3).

The MEN is a conserved Hippo-like signal transduction cascade and is essential for exit from mitosis (reviewed in (Stegmeier and Amon 2004)). As with Cdc14, loss of function mutations in the core MEN genes results in a late anaphase arrest with high CDK activity. MEN activation is thought to occur at spindle pole bodies (SPBs), the yeast equivalent of the centrosome (Rock and Amon 2011; Valerio-Santiago and Monje-Casas 2011). Central to MEN activation is the Hippo-like kinase Cdc15. Cdc15 is activated coordinately by both the small Ras-like GTPase Tem1 as well as by Polo kinase Cdc5.

As cells enter metaphase, the GTPase Tem1 is loaded onto SPBs (Bardin et al. 2000; Molk et al. 2004). During this phase of the cell cycle, Tem1 is thought to be kept in an inactive GDP-bound form by action of the two-component GTPase activating protein (GAP) complex Bub2-Bfa1 (Fesquet et al. 1999; Visintin and Amon 2001;

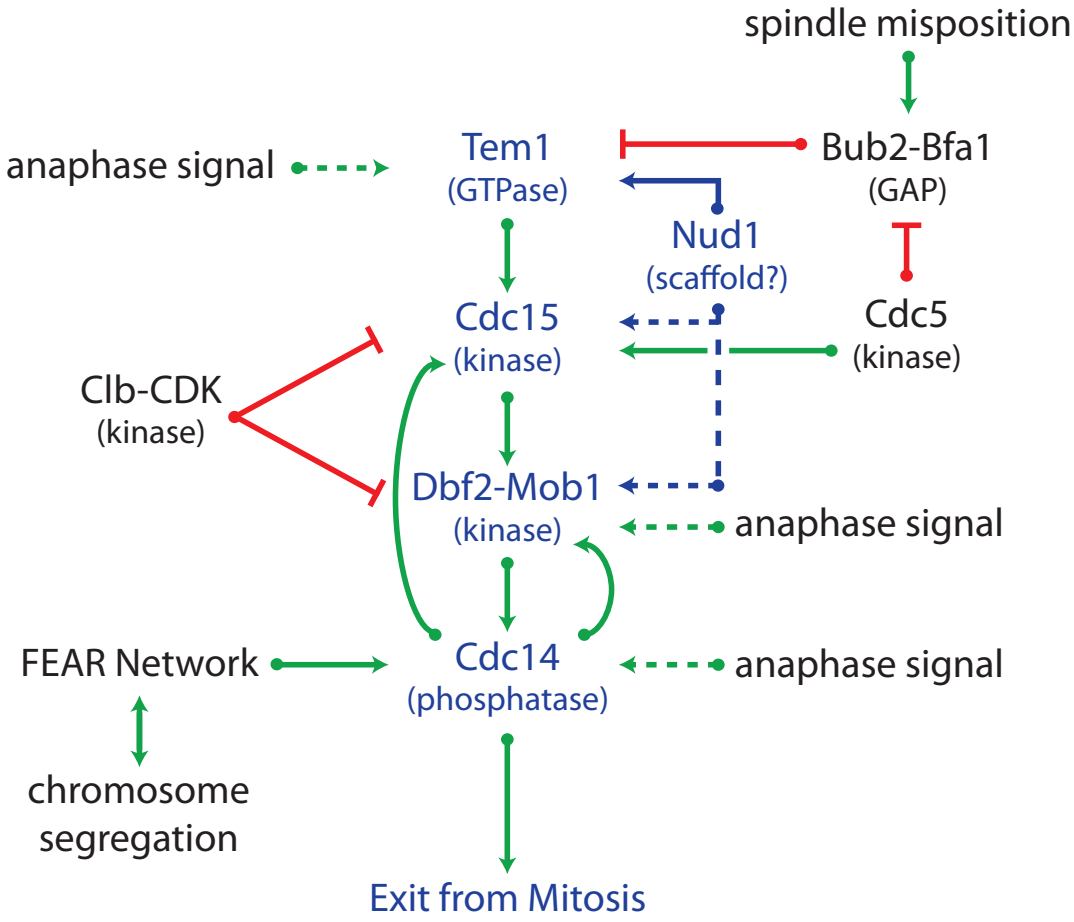

Figure 3. The Mitotic Exit Network (MEN)

Cdc14 release from the nucleolus during late anaphase is mediated by the MEN. MEN activity is tightly coordinated with genome segregation and is controlled by multiple signals. 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.

Geymonat et al. 2002). As cells pass the metaphase to anaphase transition, Bfa1 is phosphorylated by Cdc5, which lowers Bub2-Bfa1 GAP activity towards Tem1 (Hu et al. 2001; Geymonat et al. 2003). While it is clear that the Bub2-Bfa1 GAP complex restricts Tem1 activity in metaphase, the precise mechanism(s) by which Tem1 is activated in anaphase is unknown. Tem1-GTP is then thought to bind to and recruit the kinase Cdc15 to SPBs (Asakawa et al. 2001). In addition to playing a non-essential role in Bub2-Bfa1 GAP inactivation, Cdc5 plays an essential role in activating the MEN in parallel to Tem1. As cells pass the metaphase to anaphase transition, Cdc5 phosphorylates an as yet unidentified target to promote Cdc15 SPB localization (Rock and Amon 2011). Highlighting the importance of Cdc15 SPB association, the sole essential MEN activating function of both Tem1 and Cdc5 is recruiting Cdc15 to SPBs (Rock and Amon 2011).

The SPB-resident scaffold Nud1 is essential for the SPB localization of Tem1, Cdc15, and Dbf2-Mob1 and thus plays a critical role in the activation of the MEN (Gruneberg et al. 2000; Visintin and Amon 2001). Once localized to SPBs, Cdc15 phosphorylates the scaffold Nud1. Phosphorylated Nud1 in turn is recognized by the phosphopeptide binding pocket of the Mob1 coactivator, which results in the recruitment of Dbf2-Mob1 to SPBs (J. M. R. unpublished observations). Once localized to poles, both Dbf2 and Mob1 are phosphorylated by Cdc15, resulting in the activation of Dbf2 kinase activity (Mah et al. 2001). Cdc15-dependent 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 then act on its substrates (Mohl et al. 2009). It is important to note, however, that while Dbf2-Mob1 kinase activity is necessary for Cdc14 release in anaphase, it is not sufficient for release in earlier phases of the cell cycle. Thus, a regulatory mechanism downstream of and/or in parallel to Dbf2-Mob1 ensures that MEN function does not occur until anaphase (Rock and Amon 2011). Ultimately, activation of the MEN in late anaphase is essential for the sustained release of Cdc14 from the nucleolus, which then promotes exit from mitosis.

Upon completion of exit from mitosis, the MEN is deactivated and Cdc14 returns to the nucleolus. Failure to return Cdc14 to the nucleolus results in a severe defect in Clb-CDK activation in the following cell cycle (Visintin et al. 1998; Shou et al. 1999; Visintin et al. 1999). Two primary mechanisms contribute to the inactivation of the MEN: the APC/C bound to its specificity factor Cdh1 targets the Polo kinase Cdc5 for ubiquitin-mediated proteolysis (Visintin et al. 2008); and Cdc5-dependent phosphorylation of the Tem1 GAP Bub2-Bfa1 is reversed, potentially by the phosphatase Cdc14, thereby stimulating GAP activity and inactivating Tem1 (Hu et al. 2001; Pereira et al. 2002; Geymonat et al. 2003).

Regulation of MEN activity

The mitotic processes encompassing exit from mitosis, i.e. spindle disassembly, nuclear envelope reformation, chromosome decondensation, and cytokinesis are irreversible. Therefore, the decision to exit from mitosis must be tightly coordinated with other cell cycle events. The mechanisms by which MEN activity and exit from

mitosis are temporally and spatially coordinated with genome segregation are beginning to be understood.

Coordination of MEN activity with chromosome segregation through the FEAR network

The FEAR network harbors two key regulators of chromosome segregation, Separase and Cdc5. This allows for the coordination of FEAR network activation and chromosome segregation. As elaborated on page 22, FEAR-network promoted Cdc14 release participates in a positive feedback loop to stimulate MEN activity. The early anaphase release of Cdc14 dephosphorylates Cdc15, thereby promoting its ability to associate with SPBs and activate Dbf2-Mob1 (Jaspersen and Morgan 2000; Stegmeier et al. 2002). Cdc14 also dephosphorylates Mob1, thereby enhancing Dbf2-Mob1 kinase activity (Konig et al. 2010). As all of the core MEN components are phospho-proteins, it is likely that Cdc14 has multiple targets that collectively function in this positive feedback loop. Thus, the requirement for FEAR network firing for the switch-like activation of the MEN in late anaphase temporally links full MEN activation with the onset of chromosome segregation.

Restricting exit from mitosis in response to unattached kinetochores & DNA damage

In the event of defects in the attachment of chromosomes to the mitotic spindle or DNA damage, exit from mitosis must be delayed to allow the cell time to repair these errors. Two checkpoint signaling pathways, known as the spindle assembly

checkpoint (SAC) and the DNA damage checkpoint, monitor defects in the attachment of chromosomes to the mitotic spindle and the presence of DNA damage, respectively (reviewed in (Clarke and Bachant 2008; Putnam et al. 2009)). Both checkpoints prevent the metaphase to anaphase transition by inhibiting the activity of the APC/C bound to its specificity factor Cdc20, thereby stabilizing the anaphase inhibitor Securin. In addition, by poorly understood mechanisms, both checkpoints activate the Tem1 GAP Bub2-Bfa1. Thus, the activation of the SAC and DNA damage checkpoints prevents both the activation of the FEAR and the MEN, thereby ensuring that exit from mitosis does not occur until all chromosomes are damage free and are correctly attached to the mitotic spindle.

Coordination of MEN activity with spindle position

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; Pereira and Schiebel 2005; Maekawa et al. 2007). Kin4 is a protein kinase that functions in opposition to Cdc5, phosphorylating Bfa1 and thus rendering the GAP insensitive to Cdc5-dependent inhibitory phosphorylation. Lte1 is a protein of poorly defined biochemical activity but functions, at least in large part, to prevent activity of Kin4 in the bud (Bertazzi

et al. 2011; Falk et al. 2011). 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.

Roles of Cdc14 activated by the FEAR network

Although not essential for progression through mitosis, Cdc14 released by the FEAR network regulates multiple aspects of chromosome segregation (Figure 4). Interestingly, during early anaphase Cdc14 dephosphorylates its targets when overall Clb-CDK activity is high, raising the possibility that local high concentrations of active Cdc14 or locally repressed Clb-CDK activity is critical to regulate and coordinate anaphase events. FEAR network promoted Cdc14 activation ensures that the segregation of all chromosomes is initiated simultaneously, it is required for the efficient segregation of the rDNA, positioning of the anaphase nucleus, proper regulation of mitotic spindle dynamics, localization of spindle proteins, and activation of the MEN. In a sense, the FEAR network can be viewed as the "fine tuner of anaphase events," bringing accuracy to the system.

Sharpening the metaphase – anaphase transition

The onset of anaphase is marked by the rapid loss of sister chromatid cohesion, resulting in the synchronous segregation of sister chromatids. This rapid loss in sister

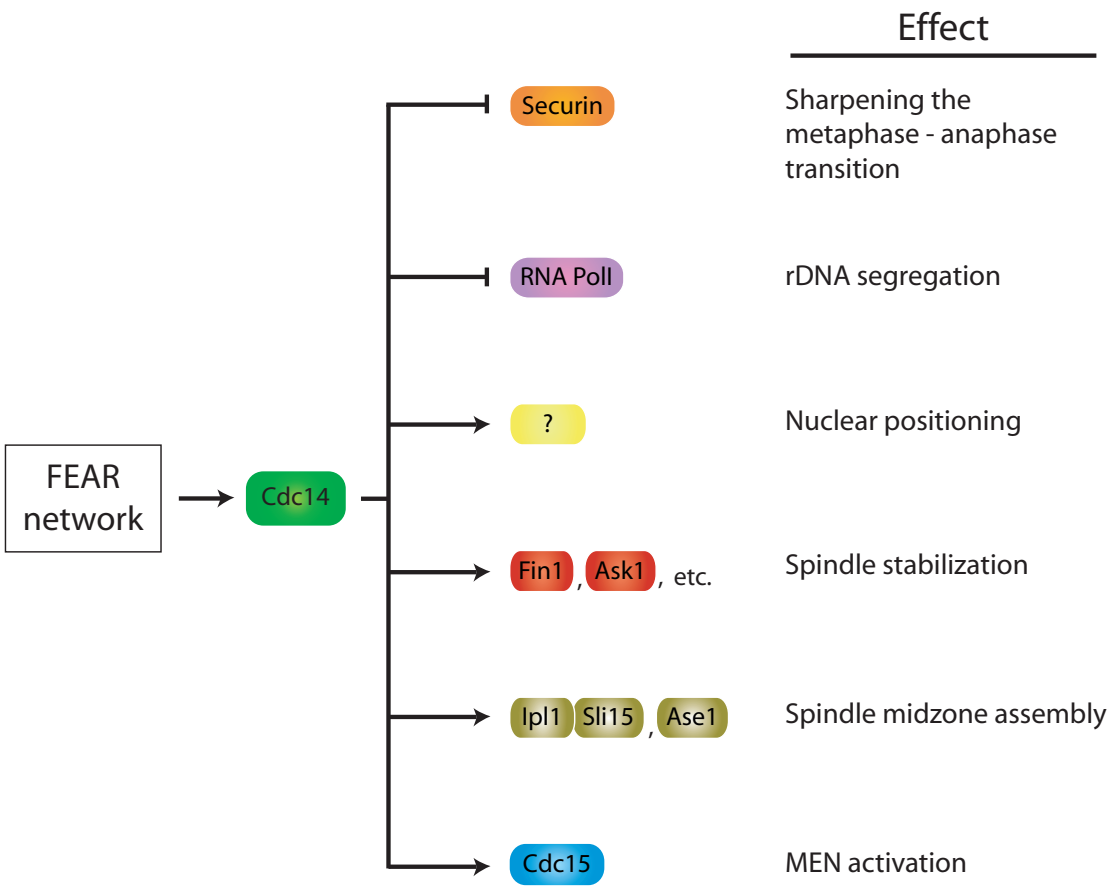

Figure 4. Functions of FEAR

Cdc14 released by the FEAR network regulates multiple aspects of chromosome segregation (see text for details).

chromatid cohesion is driven, in large part, by the activation of Separase, which then cleaves a subunit of the cohesin complex. Separase activation at the metaphase-anaphase transition occurs as a result of APC/C dependent destruction of Securin. The mechanisms by which the switch-like activation of Separase is made are beginning to be understood. Securin is phosphorylated by Clb-CDKs and two of these phosphorylation events decrease the efficiency of Securin ubiquitination by the APC/C, at least *in vitro*. Cdc14 dephosphorylates these CDK marks on Securin, thus suggesting the presence of a positive feedback loop (Holt et al. 2008). The initial degradation of Securin activates the Separase – Slk19 complex, which then promotes the early anaphase release of Cdc14. Cdc14 in turn dephosphorylates Securin, resulting in enhanced APC/C dependent Securin degradation. When this positive feedback loop is disrupted, anaphase chromosome segregation is less abrupt and occurs with a significantly increased error rate.

Segregation of the rDNA

Activation of Separase at the metaphase-anaphase transition results in cohesin cleavage and initiates chromosome segregation. While cleavage of the cohesin complexes is sufficient for segregation of most parts of the genome, the rDNA repeats, which encode the rRNA and that nucleate the nucleolus, are late to segregate during anaphase and are linked through additional cohesin independent mechanisms. The physical nature of these cohesin independent chromosome linkages remains a mystery. What is known, however, is that the

resolution of these linkages is dependent on Cdc14 activated by the FEAR network. Inactivation of either results in the defective segregation of the rDNA but not other parts of the genome (Buonomo et al. 2003; D'Amours et al. 2004). Activation of Cdc14 in early anaphase results in the silencing of transcription of rDNA by RNA PolI and the exclusion of RNA PolI subunits from the nucleolus (Clemente-Blanco et al. 2009). This anaphase-specific silencing of rDNA transcription is thought to allow for the recruitment of condensins, cohesin-like complexes that mediate the condensation of chromosomes, to the rDNA and facilitate both the compaction and segregation of this genomic region.

Nuclear positioning

In budding yeast, the nucleus is positioned at the bud neck during metaphase. At the onset of anaphase, microtubule motors associated with the cytoplasmic microtubules emanating from the daughter bound spindle pole body (SPB; the yeast equivalent of the centrosome) pull the nucleus into the bud. Concomitantly, microtubule motor proteins associated with the mitotic spindle push the two SPBs apart. The end result of anaphase-specific microtubule associated forces is the equal distribution of the genetic material between the mother and daughter cells.

In cells lacking Separase function the cohesin complexes are not cleaved and sister chromatids fail to segregate. Interestingly, in the majority of these Separase deficient cells the undivided nucleus is pulled into the bud, the future daughter cell. The reason for this inheritance pattern is that cells that lack Separase function,

while proficient in generating a daughter directed cytoplasmic microtubule pulling force, fail to generate or maintain a mother directed cytoplasmic microtubule pulling force. This asymmetric force pattern, coupled with the inability to cleave cohesin complexes and thus properly elongate the mitotic spindle, results in the undivided nucleus being pulled into the daughter cell. While the mechanism by which this mother directed pulling force is generated remains unknown, its activation is dependent on FEAR network components and Cdc14 (Ross and Cohen-Fix 2004). Thus, during a normal cell cycle, Cdc14 released by the FEAR network directs the establishment or maintenance of a mother directed pulling force that, together with the daughter directed pulling force and elongating spindle, faithfully segregates the genetic material.

Spindle dynamics, stability, and midzone assembly

Microtubules, the basic components of the microtubule cytoskeleton, are highly dynamic structures. Microtubule dynamics increases as cells enter mitosis. This increase is brought about by mitotic CDK activity and is thought to promote both the formation of the mitotic spindle and the attachment of chromosomes to the assembling spindle. At the onset of anaphase, microtubule dynamics decreases dramatically and results in the stabilization of the anaphase spindle and chromosome segregation. This is the result, at least in part, of the activation of Cdc14 by the FEAR network (Higuchi and Uhlmann 2005; Woodbury and Morgan 2007). Cdc14 dephosphorylates a number of microtubule binding proteins, e.g. Ask1

and Fin1, which then allows these proteins to interact with and stabilize the elongating spindle.

In addition to regulating microtubule dynamics, FEAR network mediated Cdc14 activation also controls the assembly of the spindle midzone. The spindle midzone is the site of overlap between the interpolar microtubules. Numerous proteins are recruited to this site to stabilize this fragile region of the elongating anaphase spindle. The spindle midzone also plays an important role in cytokinesis, particularly in higher eukaryotes. FEAR network driven Cdc14 activation results in the dephosphorylation of a host of proteins, including the microtubule bundling protein Ase1 (Khmelinskii et al. 2009). Ase1 dephosphorylation then results in the proper localization of this protein to the spindle midzone. Cdc14 also dephosphorylates Sli15, which leads to the targeting of the Ipl1 - Sli15 - Bir1 complex, the conserved Aurora B – INCENP - Survivin chromosomal passenger complex in higher eukaryotes, to the spindle midzone (Pereira and Schiebel 2003). Together, Ase1 and Sli15 - Ipl1 - Bir1 recruit additional factors that stabilize the anaphase spindle, critical among them being the Separase – Slk19 complex. In the absence of proper targeting of these components to the spindle midzone, the elongating anaphase spindle frequently breaks.

Activation of the MEN

FEAR network mutants display a 10 - 20 minute delay in exit from mitosis (an approximately 10 - 20% increase in the length of the *S. cerevisiae* cell cycle). The

reason for this delay is that the lack of FEAR network dependent Cdc14 release delays the activation of a positive feedback loop that stimulates Cdc14 release. The MEN kinase Cdc15 is phosphorylated during cell cycle stages prior to anaphase and these phosphorylation events have been shown to inhibit the ability of Cdc15 to associate with the SPB and thus activate the MEN. The early anaphase release of Cdc14 dephosphorylates Cdc15, thereby stimulating MEN activity and further promoting Cdc14 release and mitotic exit (Jaspersen and Morgan 2000; Stegmeier et al. 2002). As all of the core MEN components are phospho-proteins, it is likely that Cdc14 has multiple targets that collectively function in this positive feedback loop. Consistent with this hypothesis, Mob1 was recently shown to be a substrate for both CDK and Cdc14, with Cdc14-dependent dephosphorylation contributing to the activation of DBf2-Mob1 kinase activity (Konig et al. 2010).

Roles of Cdc14 activated by the MEN

Cdc14 activated by the MEN is primarily responsible for promoting Clb-CDK downregulation and exit from mitosis, as this cell cycle transition does not occur in the absence of Cdc14 function. Cdc14 promotes Clb-CDK downregulation by two primary mechanisms: (1) Cdc14 dephosphorylates the APC/C specificity factor Cdh1 thereby stimulating the APC/C-dependent destruction of Clb cyclins (Jaspersen et al. 1998; Visintin et al. 1998; Zachariae et al. 1998). (2) Cdc14 dephosphorylates the Clb-CDK inhibitor Sic1 and the transcription factor Swi5, resulting in the stabilization of Sic1 and Swi5-dependent activation of *SIC1* transcription (Jaspersen et al. 1998; Visintin et al. 1998). Furthermore, Cdc14 counteracts CDK activity by

dephosphorylating a host of CDK substrates, allowing for the rapid resetting of cells to the G1 state. Several observations, however, indicate that Cdc14 and the MEN play additional roles in mitosis (see below).

Additional roles of Cdc14 & the MEN in mitosis

In addition to their essential role in exit from mitosis, Cdc14 and the MEN also regulate cytokinesis (reviewed in (Meitinger et al. 2012)). When the role for the MEN in exit from mitosis is bypassed, either by the overexpession of the CDK inhibitor Sic1 or the weakening of the Cdc14-Cfi1/Net1 interaction, severe cytokinesis defects result (Shou et al. 1999; Luca et al. 2001). Consistent with a role for Cdc14 and the MEN in cytokinesis, Cdc14, Cdc15, Dbf2-Mob1, and Cdc5 all localize to the bud neck during late anaphase. Interestingly, Cdc14 release is essential for the localization of Dbf2-Mob1 to the bud neck (Frenz et al. 2000; Yoshida and Toh-e 2001). While the molecular details of the role of Cdc14 and the MEN in cytokinesis are largely unknown, a mechanism is beginning to emerge. A trimeric complex between the SH3-domain containing protein Cyk3, the conserved F-BAR protein Hof1, and the C2-domain protein Inn1 is thought to coordinate actomyosin ring (AMR) contraction and septum extension during cytokinesis. Inn1 was found to be a substrate of both CDK and Cdc14, with Cdc14-dependent dephosphorylation thought to contribute to the formation of the Inn1-Cyk3 complex (Palani et al. 2012). In addition, Dbf2-Mob1 was found to phosphorylate Hof1 which releases Hof1 from the septin ring. Once released from the septin ring, Hof1 is recruited to

the AMR, where it then promotes AMR contraction and membrane ingression (Meitinger et al. 2011).

In addition to a role for the MEN in cytokinesis, a recent report suggests that the MEN functions in metaphase to regulate spindle positioning (Hotz et al. 2012). Spindle positioning in budding yeast is mediated by two partially redundant pathways: the Dyn1 and Kar9 pathways. Kar9 (APC in mammals) interacts indirectly with both actin cables and microtubules by binding to the type V myosin, Myo2, and the microtubule binding protein Bim1 (EB1 in mammals). Through these interactions, Kar9 directs the movement of astral microtubule plus ends along actin cables toward the bud. Arrival at the bud tip results in the anchoring of the astral microtubules in the bud and, by a poorly understood mechanism, alignment of the mitotic spindle. Central to the success of this process is the asymmetric recruitment of Kar9 to a single SPB. Recent work has shown that, in the absence of MEN signaling in metaphase, Kar9 asymmetry is lost, with Kar9 inappropriately localizing to both SPBs. It was proposed that the MEN kinase Dbf2-Mob1 phosphorylates Kar9, which results in the stable association of Kar9 with a single SPB (Hotz et al. 2012). In the absence of MEN signaling, the loss of Kar9 asymmetry results in a defect in spindle positioning.

The implication of the MEN in the positioning of the metaphase spindle raises the important question of how exit from mitosis is restrained if MEN activity arises in metaphase. Standard markers of MEN activity include Cdc15 and Mob1 localization

to SPBs and Dbf2-Mob1 kinase activity. By all three of these markers, the MEN is not significantly activated until anaphase. Thus, it is possible that low levels of Dbf2-Mob1 kinase activity in metaphase are sufficient for spindle positioning but full Dbf2-Mob1 activation in late anaphase is necessary for exit from mitosis. Furthermore, it is important to note that while Dbf2-Mob1 kinase activity is necessary for Cdc14 release in anaphase, it is not sufficient for release in earlier phases of the cell cycle. An undefined regulatory mechanism downstream of and/or in parallel to Dbf2-Mob1 ensures that MEN function does not occur until anaphase (Rock and Amon 2011).

Functions of Cdc14, the FEAR network, and MEN in budding yeast meiosis

Meiosis is a specialized gamete-producing cell division in which a single round of DNA replication is followed by two sequential rounds of chromosome segregation, termed meiosis I and meiosis II. During meiosis I, homologous chromosomes are segregated from each other and during meiosis II sister chromatids segregate. The transition from meiosis I to meiosis II represents an unusual form of cell cycle exit in that there is not an intervening DNA synthesis phase.

The FEAR network and Cdc14 play the same role in meiosis as they do in mitosis, that is they accomplish Clb-CDK down-regulation and exit from the chromosome segregation phase. However, while the FEAR network is important but not essential for Clb-CDK downregulation during mitosis, it is absolutely essential to bring about the proper transition from meiosis I to meiosis II (Buonomo et al. 2003;

Marston et al. 2003). In the absence of FEAR network or Cdc14 function, meiotic cells fail to downregulate Clb-CDK activity at the end of meiosis I which leads to a severe delay in meiosis I spindle disassembly. In addition, Cdc14 and FEAR network mutants exhibit a "mixed" chromosome segregation pattern, which is characterized by some chromosomes segregating in a meiosis I-like pattern and others segregating in a meiosis II-like pattern. This unusual chromosome segregation pattern appears to result from meiotic events being uncoupled. Despite cells arresting in anaphase I, meiosis II chromosome segregation events continue to occur, leading to some chromosomes undergoing both meiotic divisions on the same anaphase I spindle. These findings suggest that Cdc14 and the FEAR network ensure that the two meiotic divisions occur on two sequentially built spindles.

It is interesting to note that unlike in mitosis, the MEN is dispensable for exit from meiosis I (Kamieniecki et al. 2005; Gordon et al. 2006; Pablo-Hernando et al. 2007). The meiosis I to meiosis II transition is unique in that Clb-CDK activity needs to be lowered to a sufficient extent such that the meiosis I spindle disassembles but kept sufficiently high to prevent the formation of pre-replicative complexes (preRCs) and any subsequent DNA replication. MEN-mediated Cdc14 activation leads to complete inactivation of Clb-CDKs and resetting of the cell to the G1 state. Cdc14 released by the FEAR network lowers Clb-CDK activity, perhaps in a localized manner and/or for certain critical substrates, which may just accomplish this balancing act. Employing the FEAR network for Cdc14 activation may lower CDK activity sufficiently for

meiosis I spindle disassembly to occur but not low enough to allow assembly of preRCs onto origins of replication.

Functions of Cdc14, the FEAR network, and MEN in higher eukaryotes

All eukaryotes appear to have homologues of Cdc14 and many of the FEAR and MEN network components. While it appears that the mechanisms by which many of these proteins function is conserved across species, their output has been rewired to accomplish divergent cellular tasks.

Cdc14 homologues

Cdc14 belongs to a family of highly conserved dual-specificity phosphatases that are found across eukaryotes (reviewed in (Mocciaro and Schiebel 2010)). While it appears that some Cdc14 homologues play a role in the regulation of CDK activity, the primary functions of most Cdc14 proteins have diverged. For example Clp1p, the *S. pombe* Cdc14 homolog, primarily regulates mitotic entry and coordinates cytokinesis with the initiation of the next cell cycle (Cueille et al. 2001; Trautmann et al. 2001). *C. elegans* and *X. laevis* Cdc14 have also been suggested to regulate cytokinesis, although other findings in *C. elegans* have disputed this conclusion (Gruneberg et al. 2002; Krasinska et al. 2007). Neither of the two avian orthologs of Cdc14 are essential, and the lack of either ortholog does not cause any obvious defects in cell-cycle progression, mitotic entry, chromosome segregation, mitotic exit or cytokinesis (Mocciaro et al. 2010). The molecular functions of human Cdc14 homologues has been particularly contentious, with proposed roles in centriole

duplication, cytokinesis, DNA damage repair, and mitotic exit (Kaiser et al. 2002; Mailand et al. 2002; Bassermann et al. 2008; Berdougo et al. 2008; Wu et al. 2008). The most recent evidence suggests that neither human Cdc14A nor Cdc14B are essential but their function is required for the efficient repair of DNA following damage (Mocciaro et al. 2010).

The FEAR network in higher eukaryotes

It is a long-standing question as to whether or not a FEAR-like network functions in other organisms. All eukaryotes appear to have homologues of many of the budding yeast FEAR network components (Separase, polo-like kinase Cdc5, Clb-CDK, PP2A). In *S. pombe*, it was demonstrated that fission yeast FEAR network homologues do not regulate the localization of the Cdc14 homologue Clp1p/Flp1p (Chen et al. 2006). The signal transduction pathways that regulate Cdc14 homologue activation in metazoans are not yet known and there has yet to be any assessment for FEAR network contribution to Cdc14 activation in any other organism. It is possible that fission yeast and mammals do not require a FEAR network that brings about localized antagonism of mitotic CDK activity as, unlike in budding yeast, the bulk of Clb cyclin degradation and hence mitotic CDK downregulation occurs globally and abruptly at the metaphase – anaphase transition.

MEN-like signaling pathways in higher eukaryotes

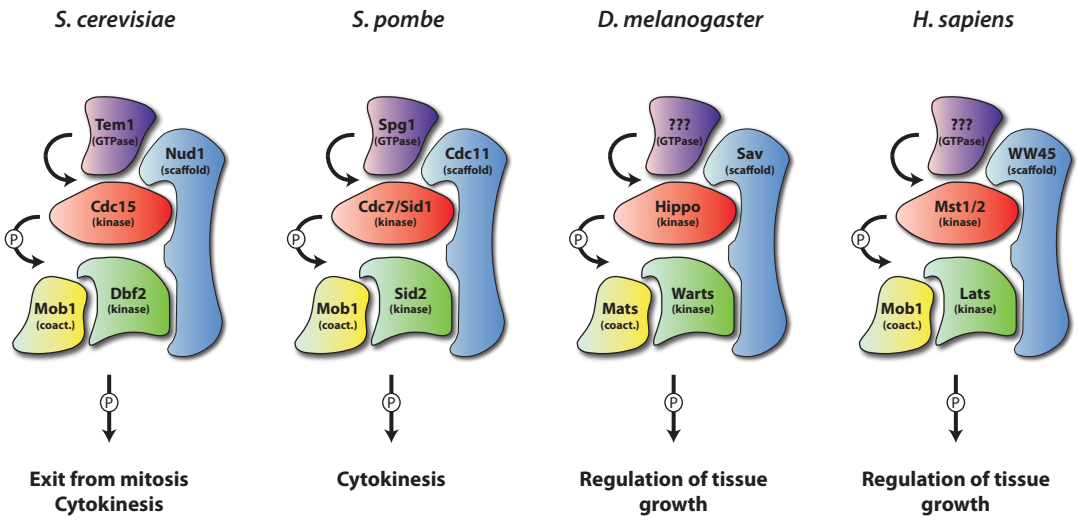

Figure 5. MEN-like signaling in other eukaryotes

The MEN is conserved across eukaryotes. In *S. pombe*, this signaling pathway is known as the SIN and plays an essential role in cytokinesis. In *D. melanogaster*, the Hippo pathway is an essential tumor suppressor signaling cascade that coordinates tissue growth with cell proliferation and apoptosis. The Hippo pathway appears to be playing a similar role in humans. See text for details.

MEN-like signaling cascades are conserved across eukaryotes (Figure 5). The orthologous pathway in fission yeast is known as the Septation Initiation Network (SIN) and regulates cytokinesis. Like the MEN, at the core of SIN signaling is the Cdc15-like kinase Cdc7p. Cdc7p is activated by both the Tem1 GTPase homolog Spg1p as well as by the Cdc5 kinase homolog Plo1p (Sohrmann et al. 1998; Mulvihill et al. 1999). Whether, as in the MEN, Cdc7p functions as a coincidence detector of both Spg1p and Plo1p is unknown. Cdc7p recruitment to SPBs in anaphase results in the activation of the Dbf2-Mob1-like kinase complex Sid2-Mob1. Active Sid2-Mob1 then translocates to the medial ring and triggers acotomyosin ring constriction and septum formation following chromosome segregation. As part of a checkpoint mechanism, Sid2-Mob1 also phosphorylates the Cdc14-like phosphatase Clp1p which results in the retention of Clp1p in the cytoplasm and ensures the successful completion of cytokinesis prior to commitment to the following mitosis (Chen et al. 2008).

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.). 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 (Luca et al. 2001; Wilmeth et al.). It will be very interesting to explore whether or not the regulatory principles of this conserved signaling cascade defined in budding and fission yeasts are true in higher eukaryotes.

Findings of the work presented in this dissertation

This thesis presents three main findings. First, we describe the unexpected finding that the GTPase Tem1 is not the sole central switch in MEN regulation. We show that Polo kinase Cdc5 plays an essential role in parallel to Tem1 to recruit the Hippo-like kinase Cdc15 to SPBs. In this context, Cdc15 functions as a coincidence detector, integrating temporal (Cdc5 and Tem1 activity) and spatial (Tem1 activity) signals to ensure that exit from mitosis occurs only after proper genome partitioning. Second, we describe our findings on the role Cdc15 SPB localization plays in the activation of the MEN. We identify the SPB-resident scaffold Nud1 as an essential Cdc15 target. Phosphorylation of Nud1 by Cdc15 results in the recruitment of Dbf2-Mob1 to SPBs, at which point Cdc15 phosphorylates and activates the Dbf2-Mob1 complex. Lastly, we describe results that implicate Mob1-coactivator proteins as a novel family of phosphopeptide binding domains.

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

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Rock, J.M. and Amon, A. 2011. Cdc15 integrates Tem1 GTPase-mediated spatial signals with Polo kinase-mediated temporal cues to activate mitotic exit. *Genes Dev* **25**(18): 1943-1954.

Summary

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 15B 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

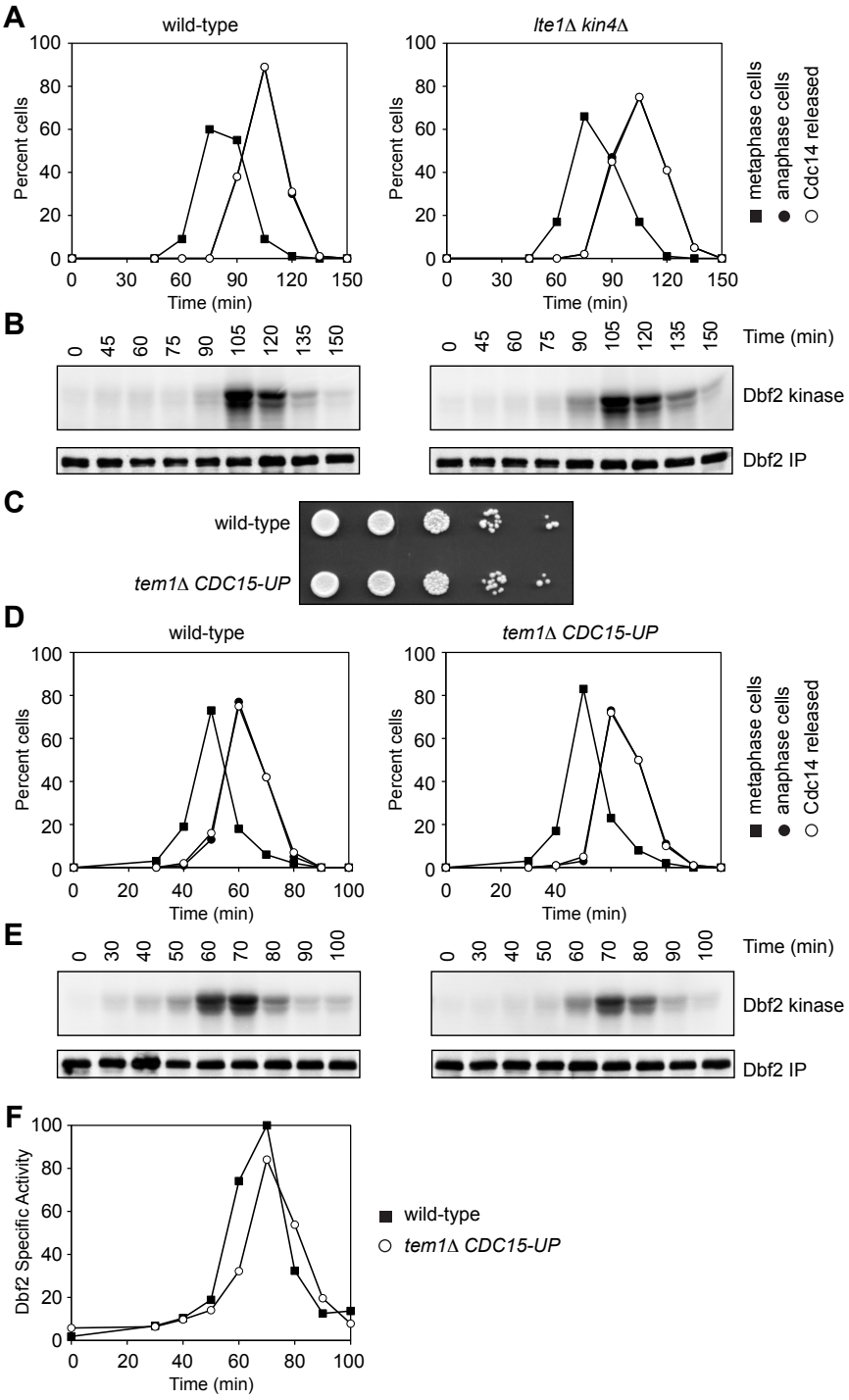

Figure 1: Anaphase-specific Activation of the MEN in the Absence of *TEM1*

(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.

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); Figure 2). As in *bub2Δ* cells, *tem1Δ CDC15-UP* cells did not arrest in anaphase in response to spindle mis-position (Figure 2). 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 15B). 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

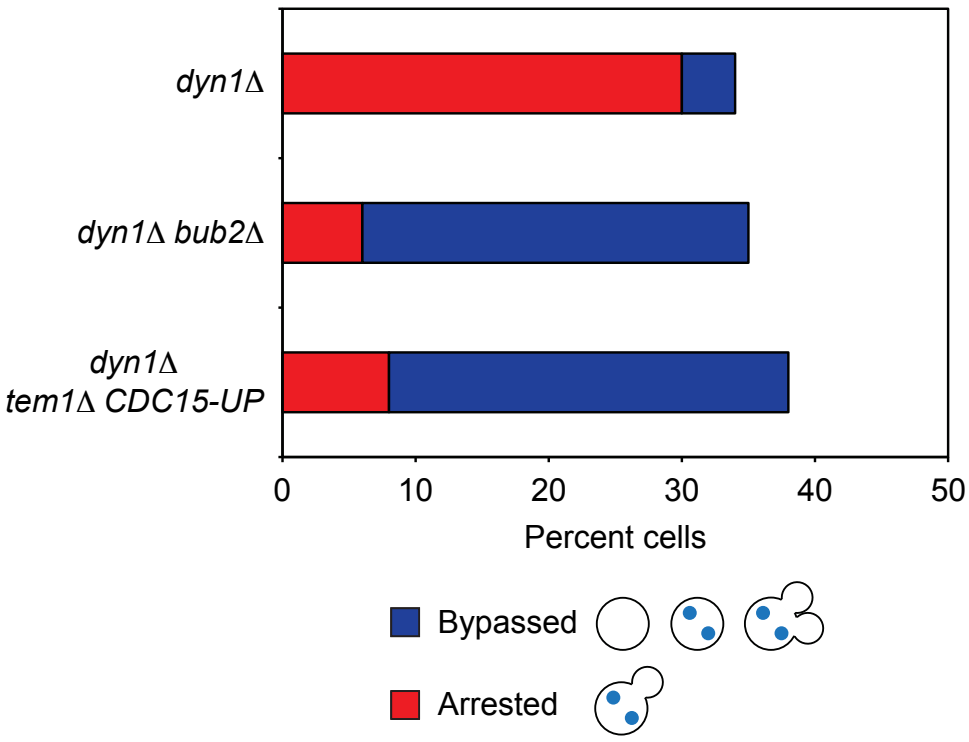

Figure 2: *tem1Δ CDC15-UP* Cells are Spindle Position Checkpoint Defective.
dyn1Δ (A2444)*, bub2Δ dyn1Δ* (A2270)*, and tem1Δ CDC15-UP dyn1Δ* (A23657) cells were grown to mid-exponential phase at 30°C and then incubated for 24 h at 14°C. Cells were fixed and the number of nuclei in cells was determined. 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".

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 3A). 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 3B-D, Figure 4). 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 (Figure 4), 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

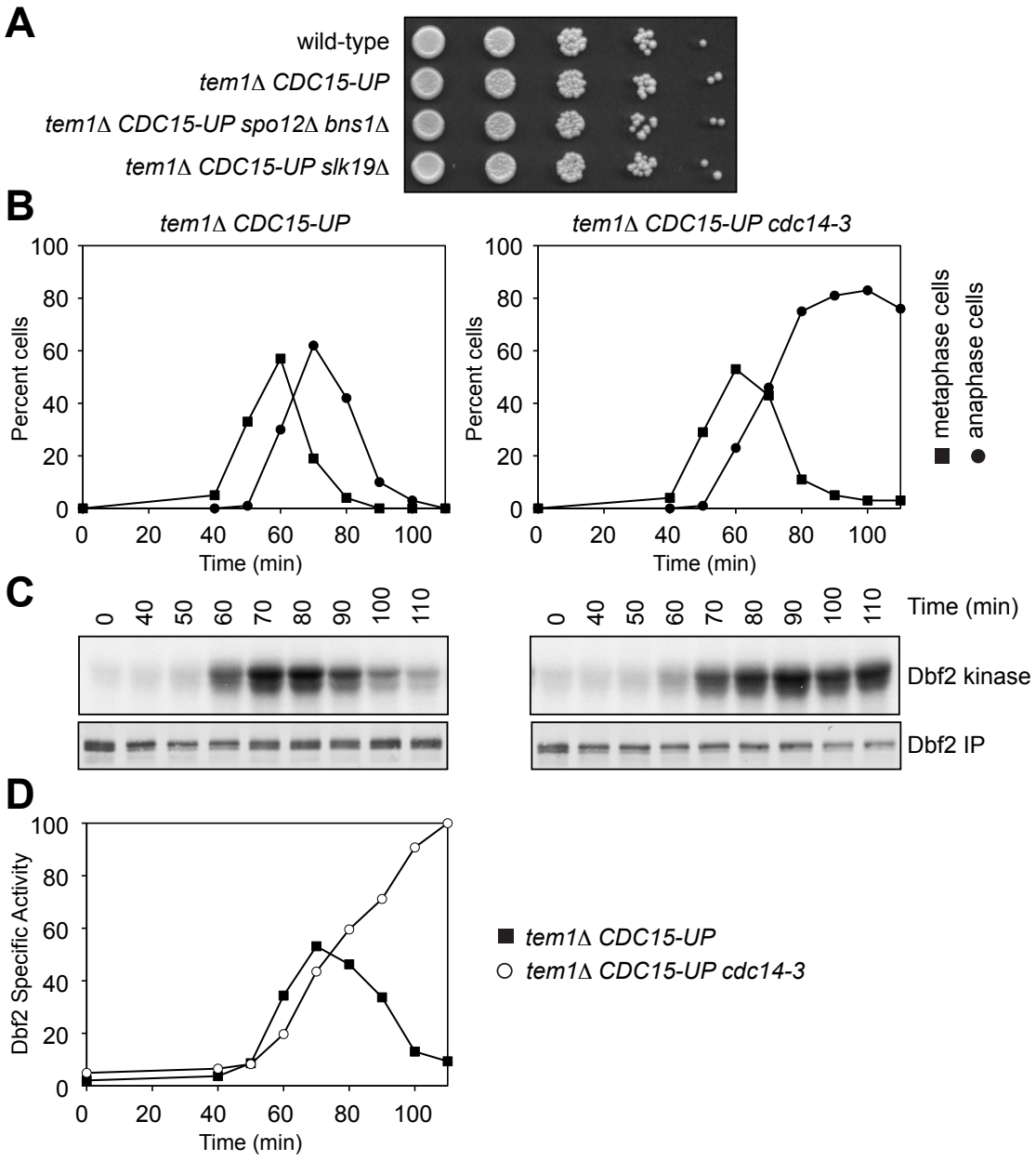

Figure 3: The FEAR Network is Not Required for Dbf2 activity in *tem1Δ CDC15-UP* Cells

(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.

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 (Figure 5). 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 (Figure 5) 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

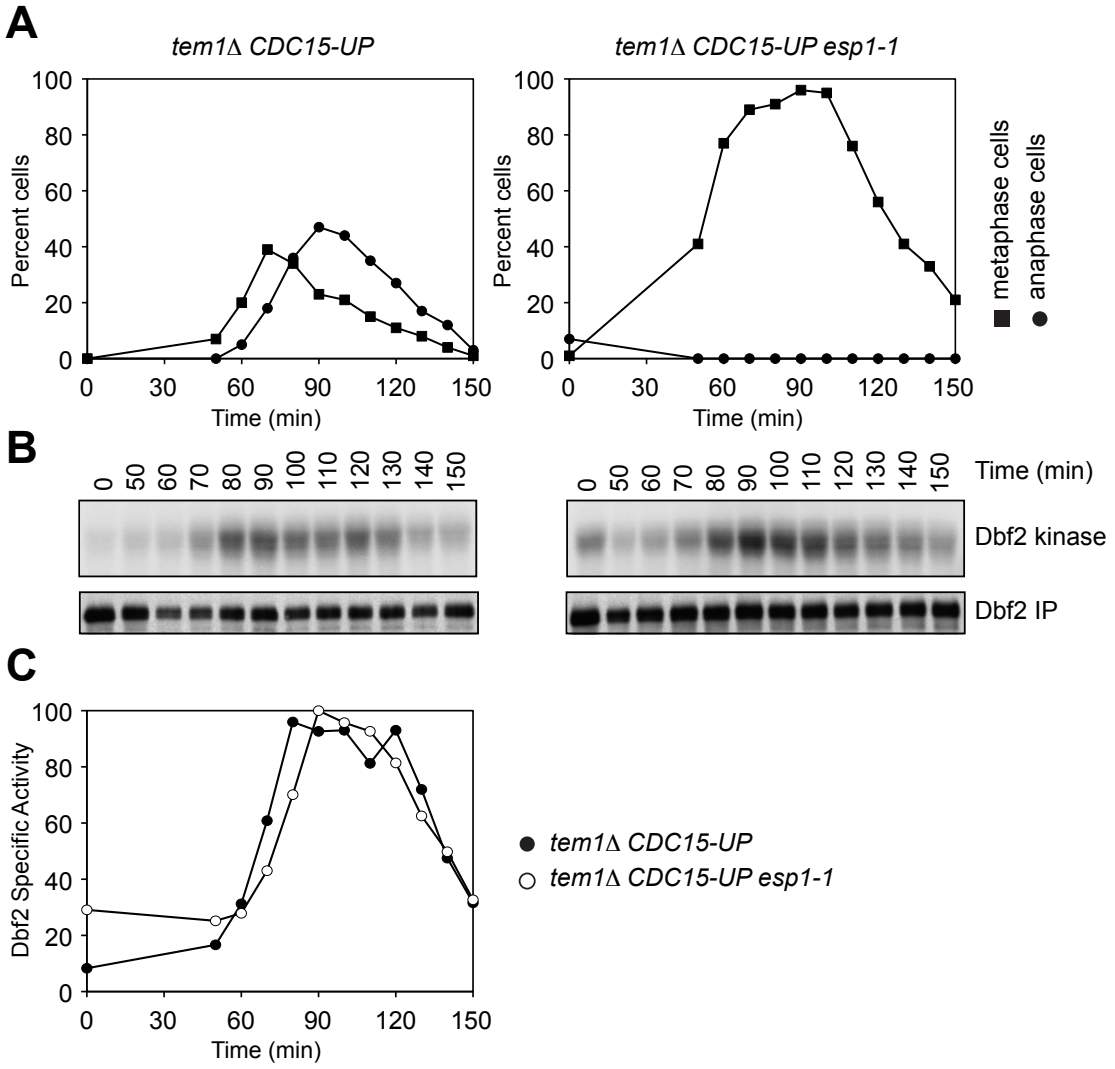

Figure 4: Separase is Not Required for Dbf2 activity in *tem1Δ CDC15-UP* Cells

(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.

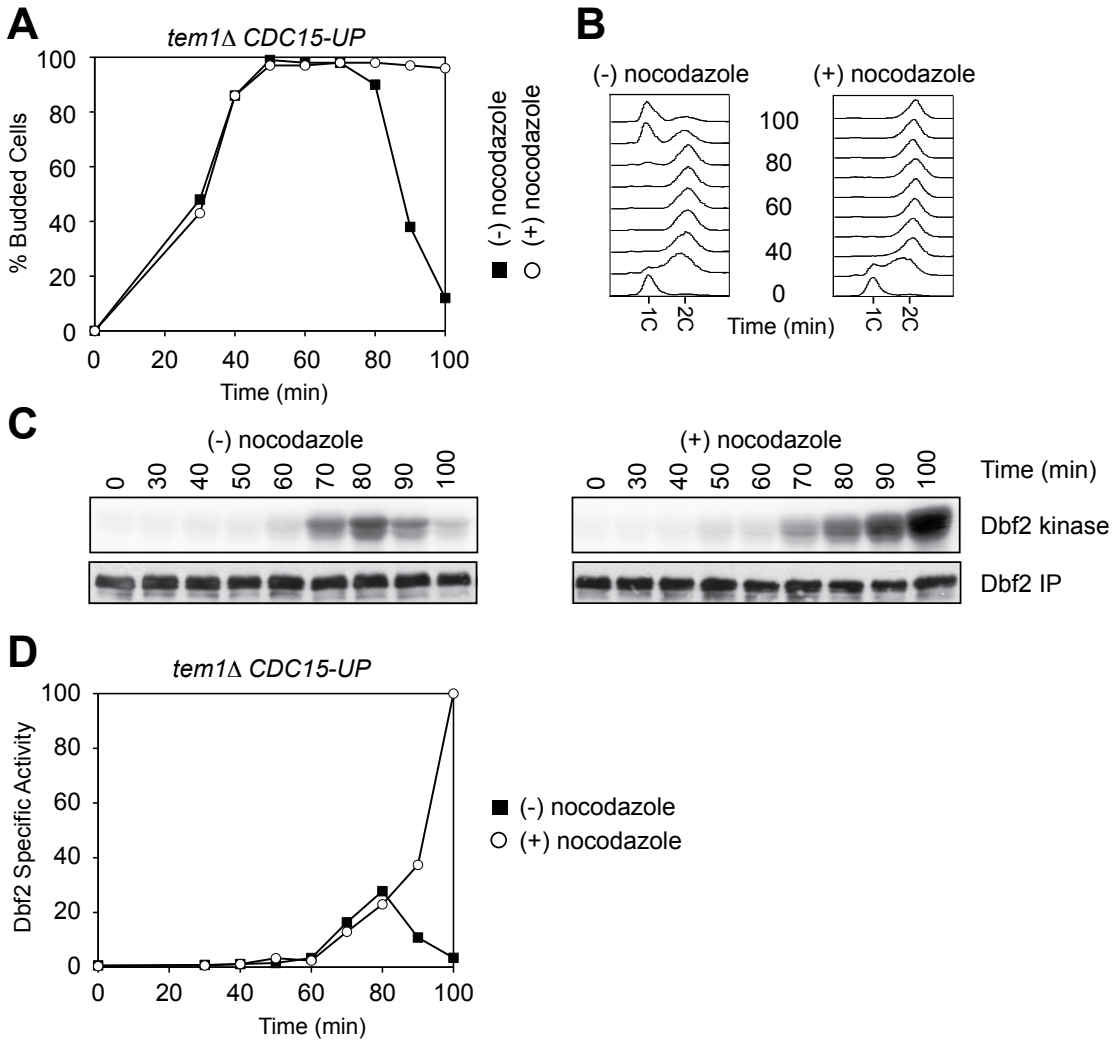

Figure 5: Anaphase Entry is not Required for MEN Activity in the Absence of *TEM1*

(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.

(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 6A, 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 6C, 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 7A). 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

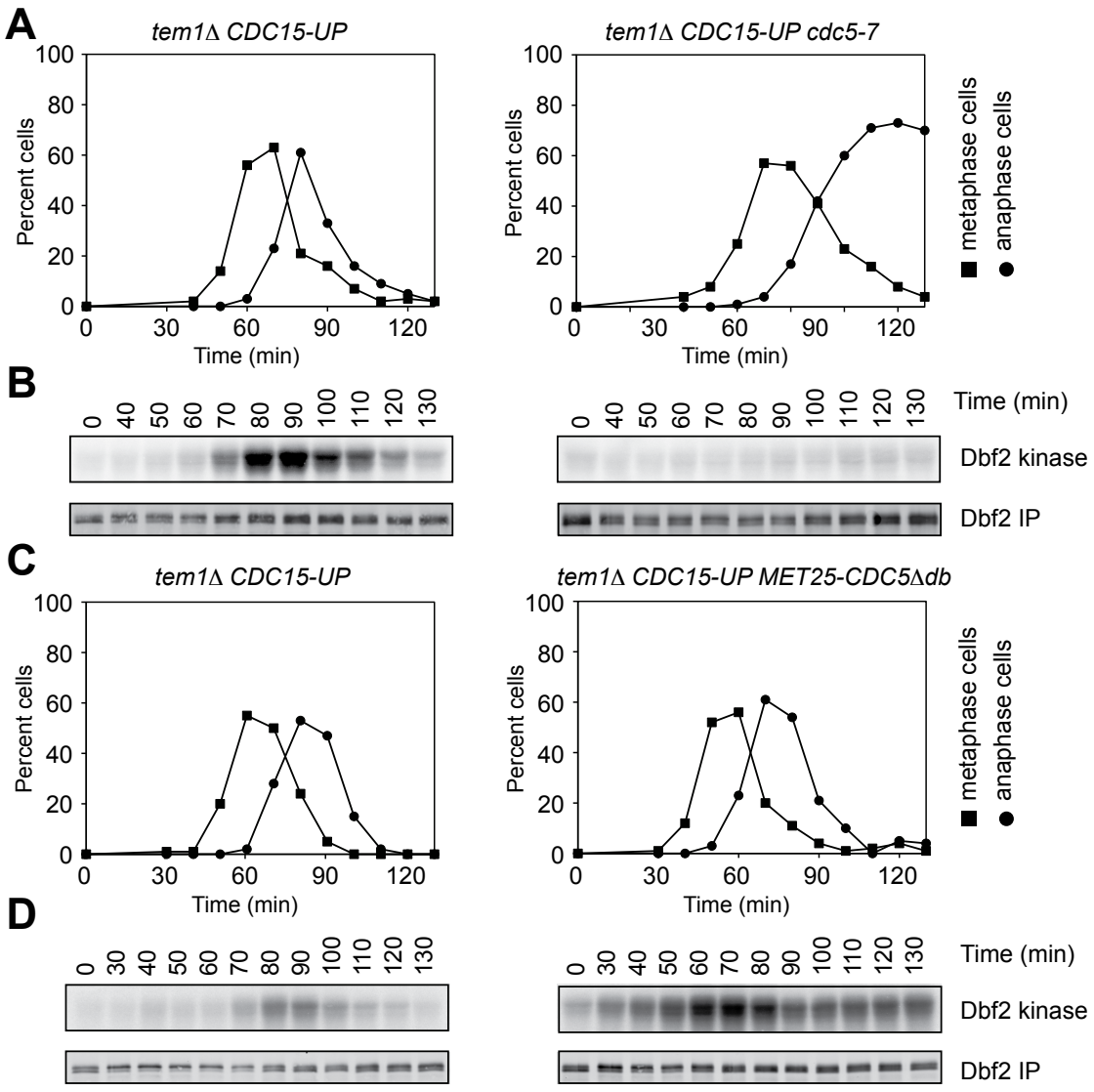

Figure 6: Polo-like kinase Cdc5 Controls MEN Activity in the Absence of *TEM1*

(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.

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 7B). As *CDC5* is sufficient to activate the MEN in the absence of Tem1 (Figure 6), 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 7C). 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 8A). *CDC5* was also essential for Cdc15 association with SPBs. Cdc15 did not localize

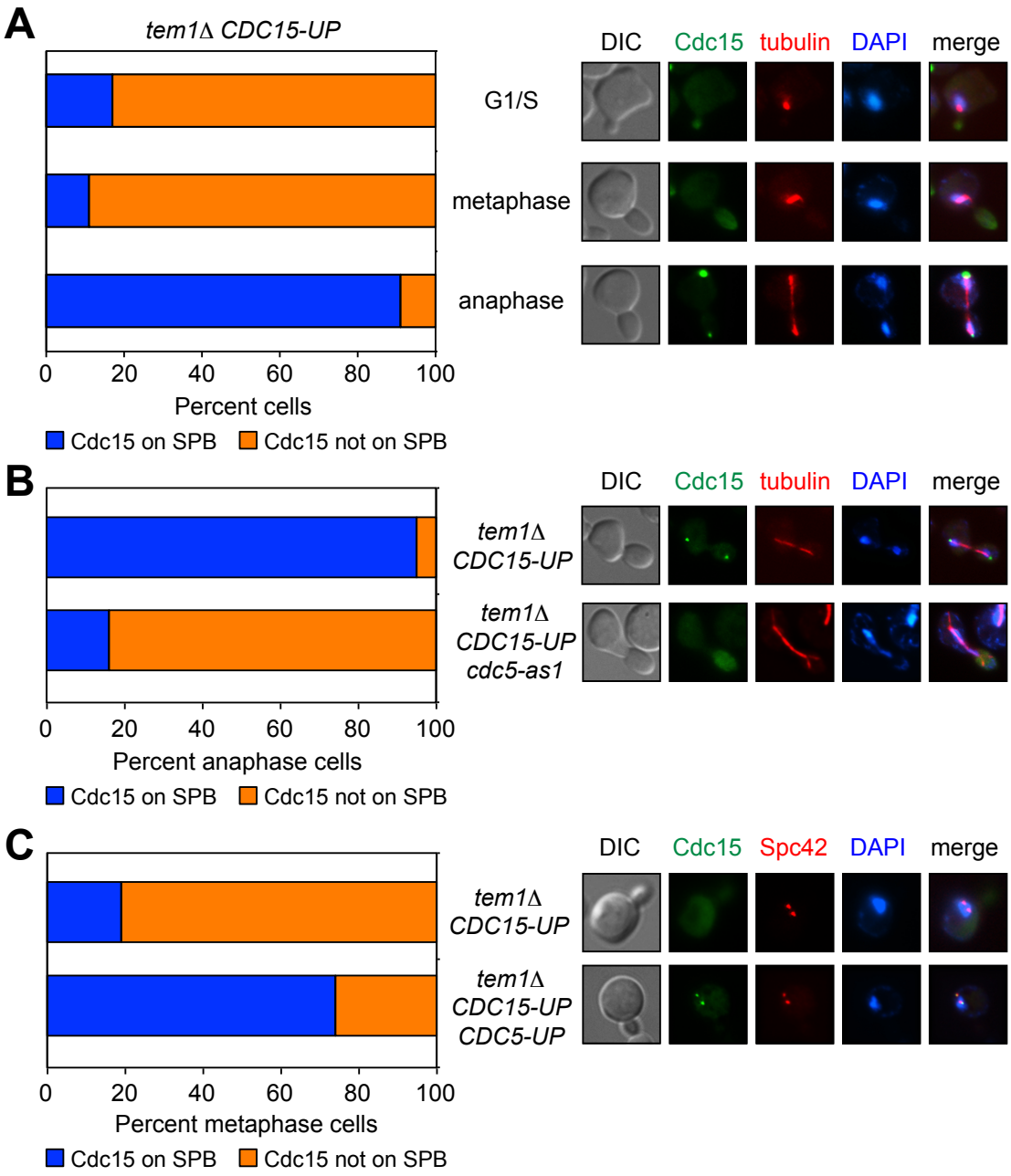

Figure 7: Cdc5 Promotes Localization of Cdc15 to SPBs

(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.

to SPBs in anaphase cells depleted of Cdc5 (Figure 8B). Similar results were obtained in *bub2Δ* cells depleted of Cdc5 (Figure 8B). Importantly, depletion of Cdc5 did not affect Tem1 localization to the SPB (Figure 9). 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 8C, D). Consistent with the Cdc15 localization observations, Dbf2-Mob1 was not activated in the *cdc14-3 bub2Δ* strain depleted of Cdc5 (Figure 8C, 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.

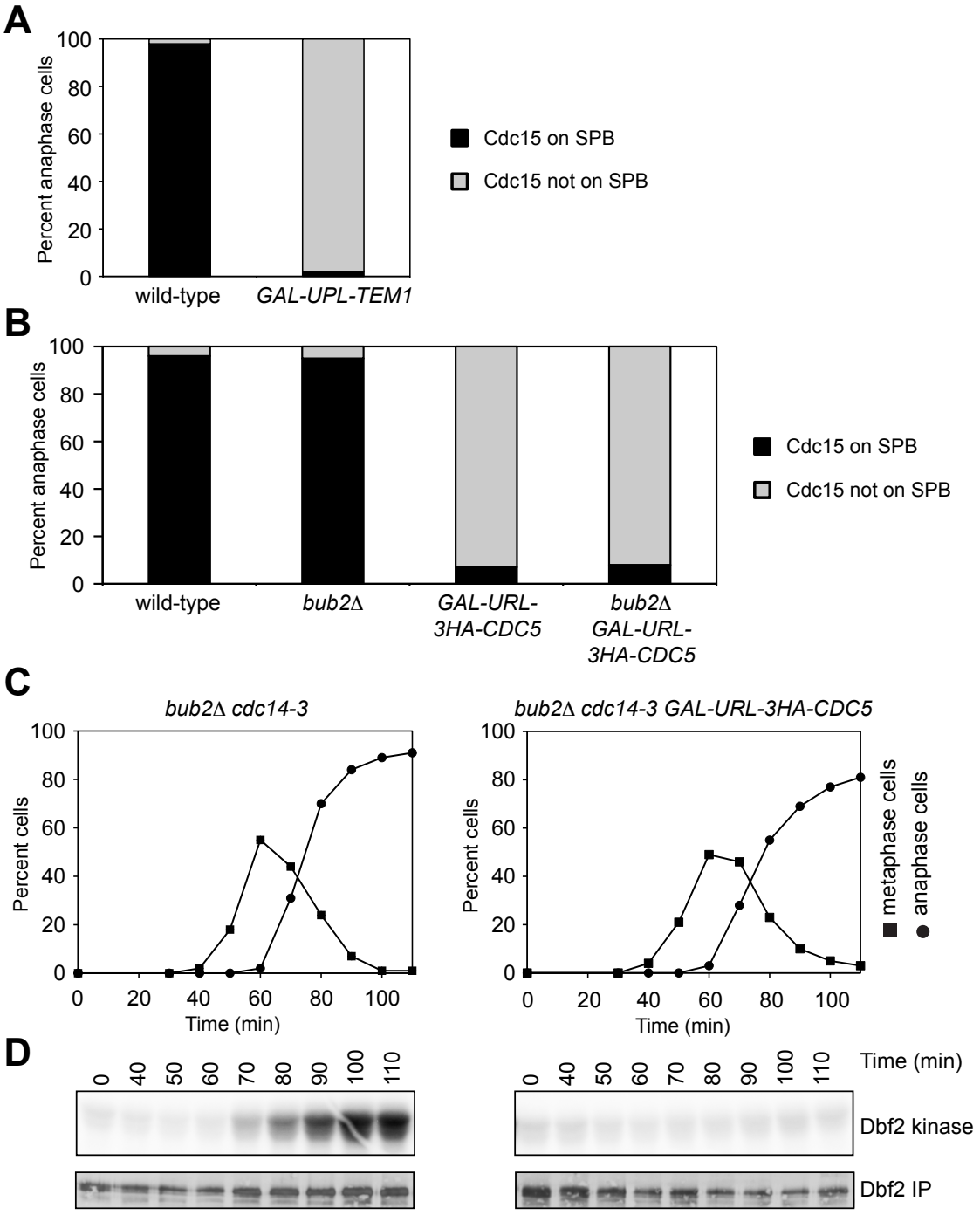

Figure 8: Cdc15 Functions as a Coincidence Detector of Tem1 and Cdc5 Activity

(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.

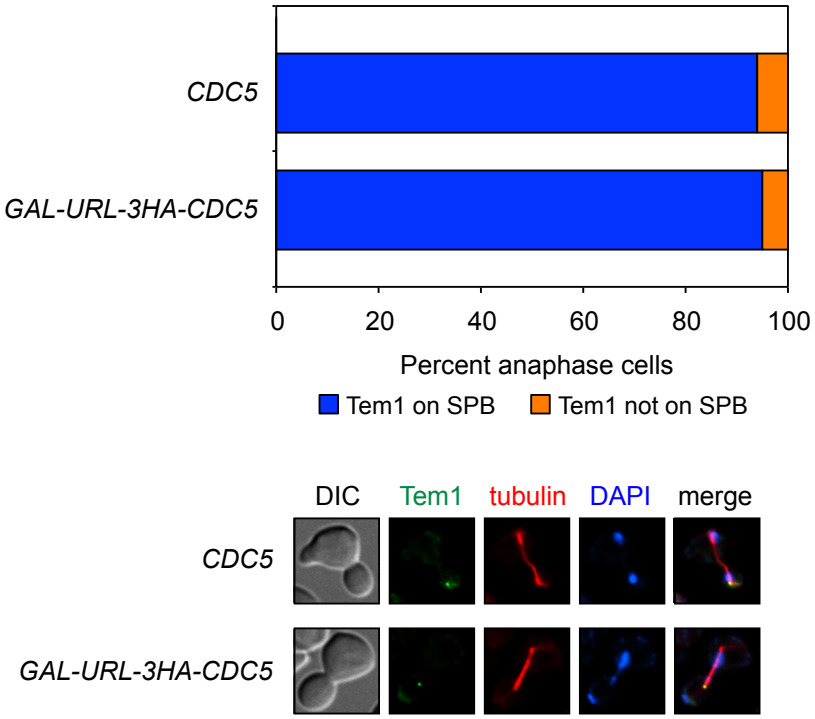

Figure 9: Cdc5 is Not Required for Tem1 SPB Localization

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.

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 10C) but was not detectable by Western blot analysis (Figure 10A, 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 10B). 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 10C, Figure 11). 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 10D), 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 10E, F, G with Figures 8D; (Visintin and Amon 2001)). Moreover, Dbf2 kinase activity was both premature and hyperactive in this strain (Figure 10F, G). Similar results were obtained in wild-type cells expressing the Cdc15-SPB fusion (Figure 12).

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

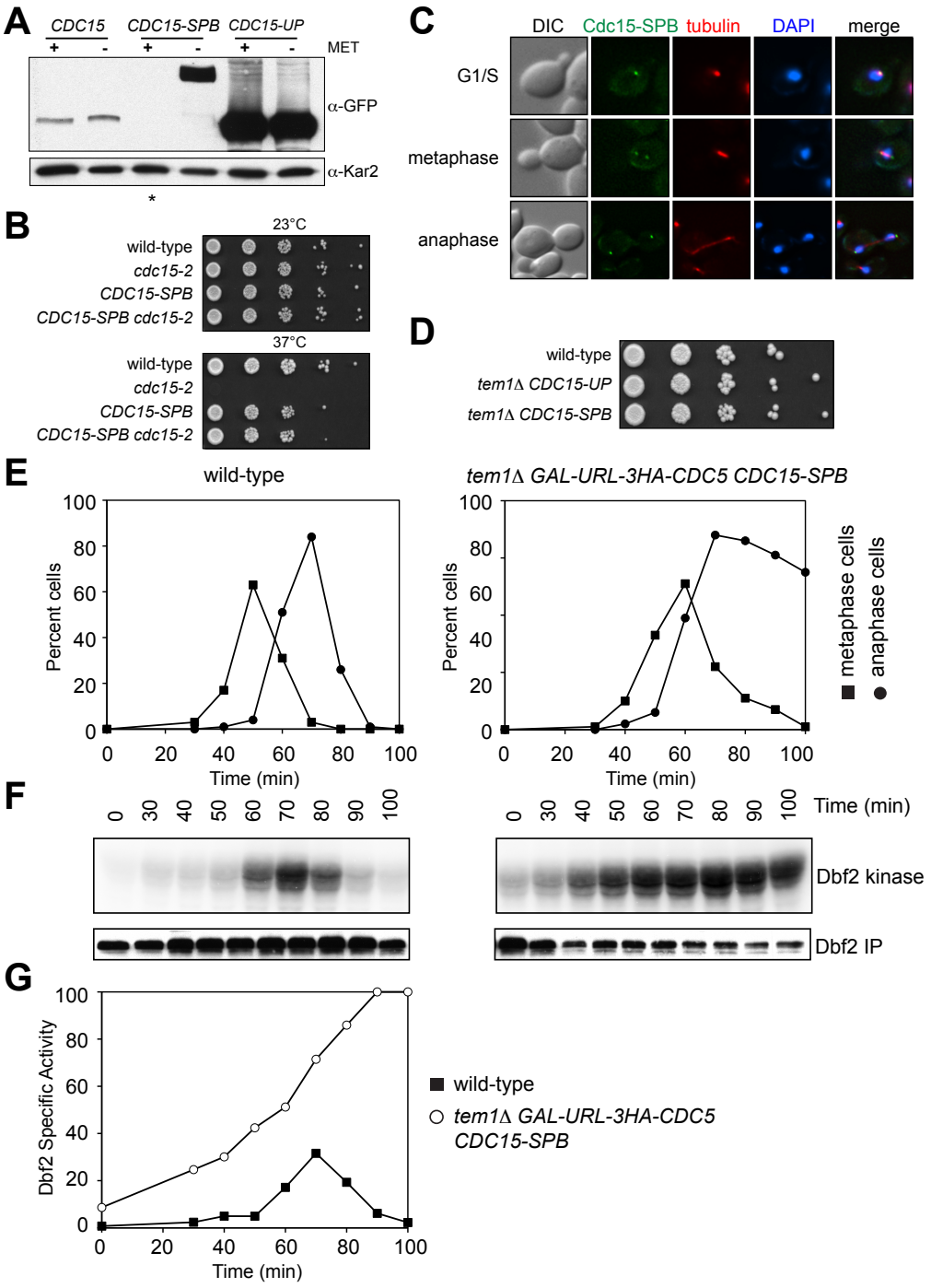

Figure 10: Targeting Cdc15 to SPBs Bypasses the Need for *TEM1* and *CDC5* in MEN Activation

(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.

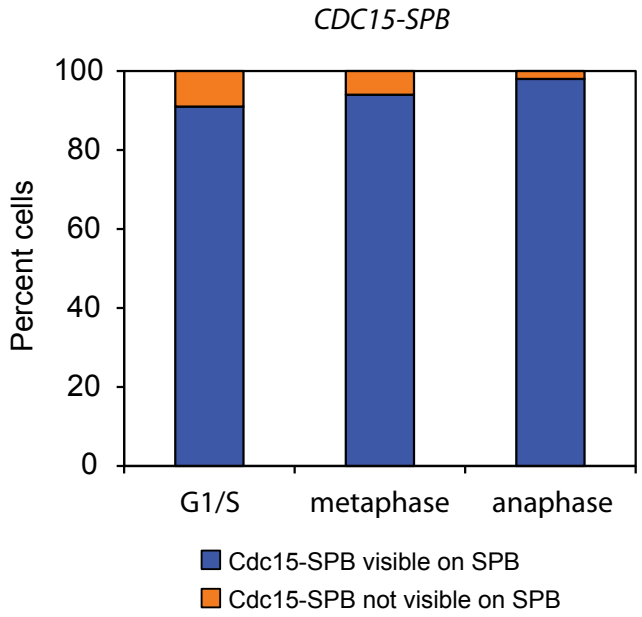

Figure 11: Cdc15-SPB localizes to the SPB constitutively throughout the cell cycle
pMET3-CDC15-eGFP-CNM67 (*CDC15-SPB*; A26486) cells containing a mCherry-Tub1 fusion protein were grown to log phase in YEPRG medium supplemented with 8 mM methionine and imaged after a brief paraformaldehyde fixation. 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).

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 (Figure 13A). 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 13B-G).

Interestingly, Dbf2 kinase activity still fluctuates during the cell cycle in cells in which Cdc15 localizes to SPBs constitutively (Figure 10, 12-14). 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 (Figure 12, Figure 14). 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

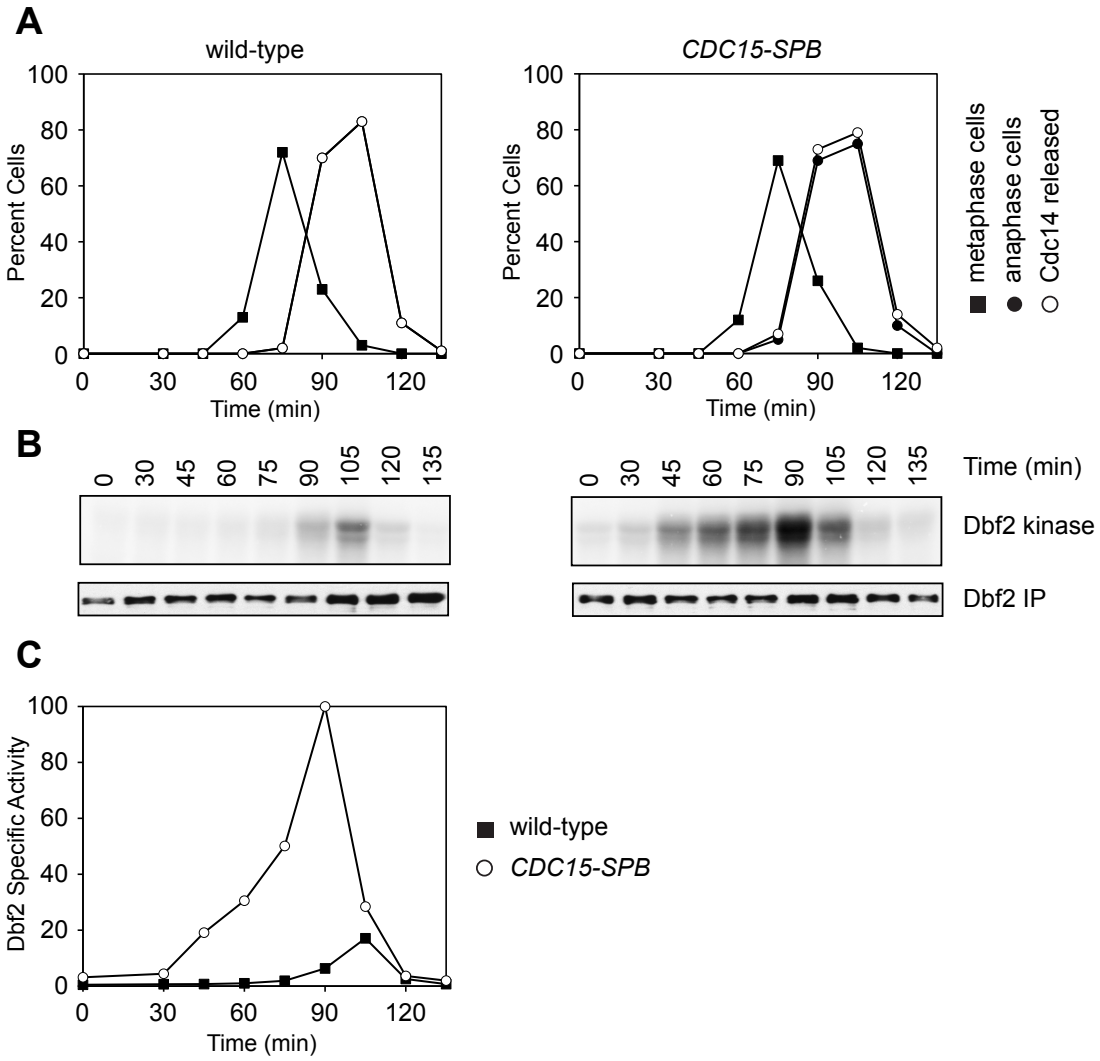

Figure 12: Dbf2-Mob1 Kinase Activity is Not Sufficient for Cdc14 Release Prior to Anaphase

(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.

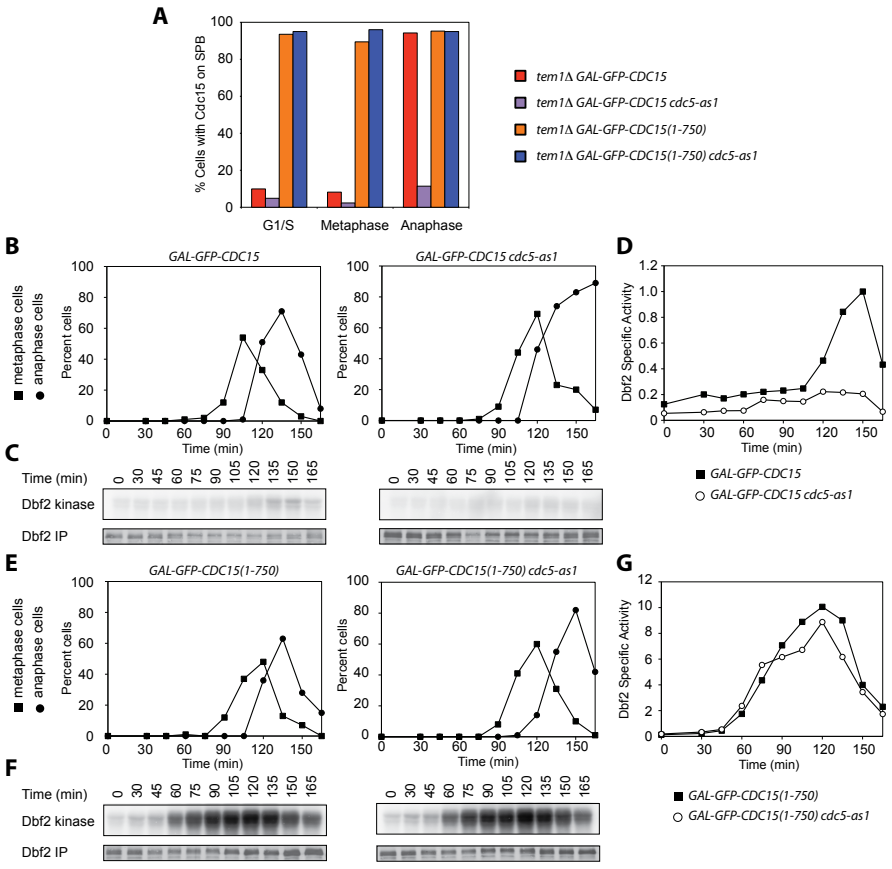

Figure 13: Removal of the C-terminal 274 Amino Acids of Cdc15 Results in Constitutive Cdc15 SPB Targeting and Tem1 and Cdc5-Independnet Activation of the MEN

(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.

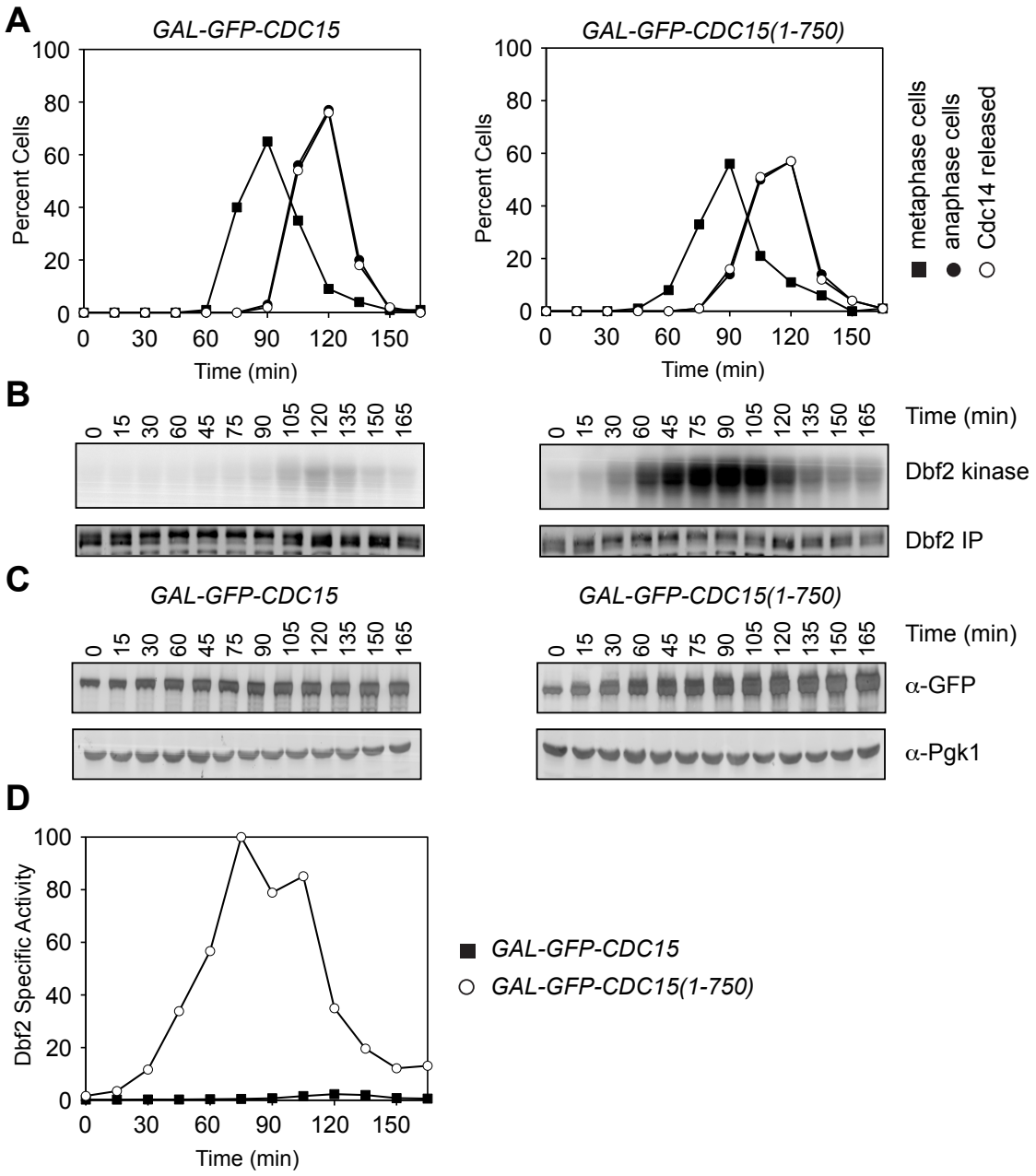

Figure 14: Overexpression of Cdc15(1-750) hyperactivates Dbf2-Mob1 but does not Result in the Premature Release of Cdc14 from the Nucleolus

(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.

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 15A), 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 (Figure 2). 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-dependent Dbf2-Mob1 activation. Although we do

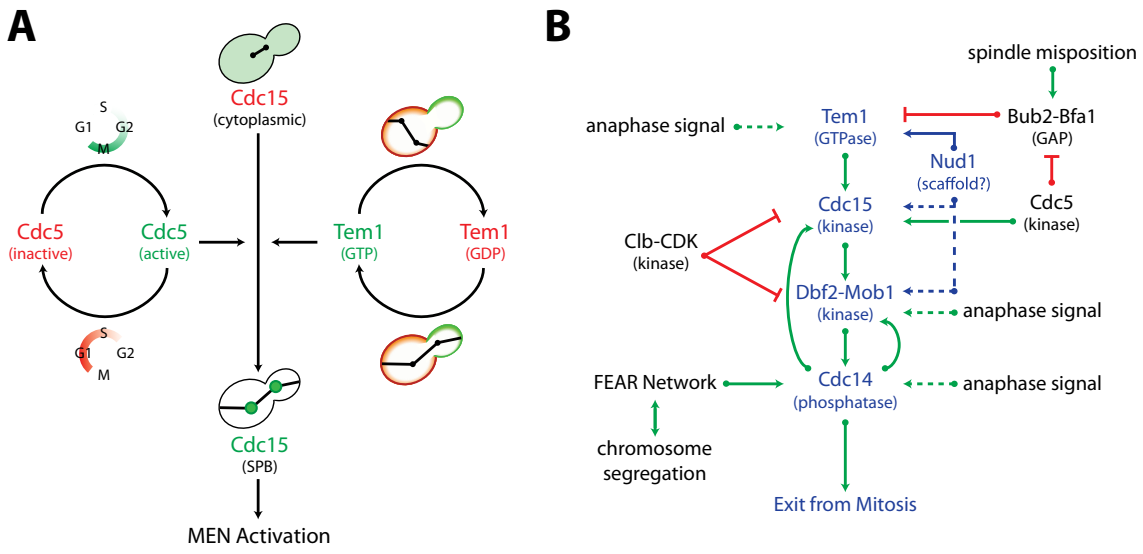

Figure 15: A Model for the Coordination of Exit from Mitosis with Spatial and Temporal Cues

(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.

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 15). 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 (Figures 10, 12-14). 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 (Figure 12-14). 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 15B). 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.

Experimental Procedures

Yeast Strains and Growth Conditions

All strains are derivatives of W303 (A2587) and are listed in Table 1. Growth conditions are described in the figure legends.

Plasmid Construction

All plasmids used in this study are listed in Table 2.

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′-

TTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATGAAACTGAGTAAGATGCTCA GAATAC-3') and (5'-GAGTCAAGTTGACTCTATCGGTATCGGCCATACTGTTCATCCTAGGGTTAATTATACTTTATT CTTG-3') with a *PMET3* containing plasmid as template; *CDC15-eGFP* was amplified with primers (5'-ATGAACAGTATGGCCGATACC-3') and (5'-GCCACCACCAGAGCCACCTCCACCAGAACCTCCACCACCTAGTTTGTACAATTCATCAATAC CATG-3') with A20791 genomic DNA as PCR template; and *CNM67* was amplified with primers (5'-CTAGGTGGTGGAGGTTCTGGTGGAGGTGGCTCTGGTGGTGGCATGACTGATTTCGATTTAA TG-3') and (5'-TAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGGAACCCCTAAAAGCTCATAGTA GCAG-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.

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.

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.

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).

Table 1. 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 2. Table of Plasmids

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

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Chapter III: Exit from mitosis is governed by phosphorylation-dependent nucleation of MEN signaling complexes

Jamie M. Keck, Michele H. Jones, Catherine C.L. Wong, John R. Yates III, and Mark Winey performed the mass spectrometric analysis of Nud1. Daniel Lim and Michael B. Yaffe performed Mob1 phosphopeptide binding assays.

Summary

Scaffold-assisted kinase cascades guide cellular decision-making. In budding yeast, an essential signal transduction cascade known as the Mitotic Exit Network (MEN) governs the mitosis to G1 transition. The MEN is conserved across eukaryotes and in metazoans is known as the Hippo tumor-suppressor pathway. We define here the order of MEN activation *in vivo*. MEN-signaling requires an unusual two-step process. The kinase Cdc15 first phosphorylates the scaffold Nud1 to create phospho-docking sites for the Mob1 coactivator. Nud1 phosphorylation in turn recruits the effector kinase Dbf2 to spindle pole bodies, where Cdc15 then activates Dbf2-Mob1. We further show that the Mob1 coactivator is a novel class of phosphoserine/threonine binding domains and that this phosphopeptide binding property allows for the phosphorylation-dependent recruitment of Dbf2-Mob1 to Nud1. Our findings uncover a novel mechanism of Hippo-like signaling pathway activation and we speculate that aspects of this regulation are likely to be conserved in higher eukaryotes.

Introduction

Activation of signaling cascades involves the ordered assembly of macromolecular complexes. Generation of these signaling complexes must be tightly controlled in both space and time. The use of protein scaffolds and phosphorylation-dependent protein-protein interactions is central to this spatiotemporal control. In the budding yeast *S. cerevisiae*, an essential signal transduction cascade known as the Mitotic Exit Network (MEN) governs the final cell cycle transition, the mitosis to G1 transition (Stegmeier and Amon, 2004). The MEN is conserved across eukaryotes and in metazoa is known as the Hippo tumor-suppressor pathway (Harvey and Tapon, 2007; Hergovich and Hemmings, 2012). Whereas the MEN directs exit from mitosis, the Hippo pathway has been repurposed to regulate organ size and regeneration (Zhao et al., 2011). While the functions of these two pathways have diverged, the core signaling module has been conserved from yeast to humans.

In yeast, the MEN directs exit from mitosis by promoting the downregulation of cyclin-dependent kinases (CDKs) (Jaspersen et al., 1998). The MEN activates the CDK-counteracting phosphatase Cdc14 by liberating it from its nucleolar-localized inhibitor. Once active, Cdc14 downregulates CDK activity by multiple mechanisms (Stegmeier and Amon, 2004). CDK inactivation results in exit from mitosis, which is defined by the disassembly of the mitotic spindle, decondensation of chromosomes, cytokinesis, and the resetting of the cell to a G1-like state. Given the central role of the MEN in initiating these events, it is not surprising that MEN activity is precisely coordinated with other cell cycle events such as chromosome segregation and nuclear position.

It is likely that all essential MEN signaling proteins have been discovered, but much remains to be learned about the mechanisms by which the activity of this pathway is regulated. During

mitosis, assembly of MEN signaling complexes occurs at spindle pole bodies (SPBs), the yeast equivalent of the centrosome (Rock and Amon, 2011; Valerio-Santiago and Monje-Casas, 2011). The Hippo-like kinase Cdc15 functions as the signal integrator of the MEN. Cdc15 is activated by both the Ras-like GTPase Tem1 as well as by the Polo kinase Cdc5 (Rock and Amon, 2011). The coincident action of both Tem1 and Cdc5 is required for Cdc15 recruitment to SPBs. Tem1 localizes to SPBs and is thought to bind to and recruit Cdc15 to this organelle (Asakawa et al., 2001; Bardin et al., 2000; Molk et al., 2004). Concurrently, Cdc5 phosphorylates an as yet unidentified target to promote Cdc15 SPB localization. The importance of Cdc15 localization to SPBs is highlighted by the finding that the sole essential MEN activating function of both Tem1 and Cdc5 is recruiting Cdc15 to SPBs (Rock and Amon, 2011).
Once localized to SPBs, Cdc15 phosphorylates both the LATS-like kinase Dbf2 and its activating subunit Mob1 (Mah et al., 2001). Activation of Dbf2-Mob1 then leads to the sustained release of Cdc14 from the nucleolus (Stegmeier and Amon, 2004). The mechanism whereby Cdc14 is released is not yet understood in detail but is at least in part mediated by Dbf2-Mob1 phosphorylation of Cdc14 (Mohl et al., 2009).

Multiple signals control MEN activity to ensure that exit from mitosis is coordinated with chromosome segregation and other mitotic events. MEN activity is regulated by the position of the spindle within the dividing cell. When the anaphase spindle is not correctly aligned along the mother – daughter cell axis, MEN activity is inhibited (Yeh et al., 1995; Bardin et al., 2000; Pereira et al., 2000). Spindle realignment and subsequent entry of the Tem1-bearing SPB into the bud then activates the MEN. MEN activity is also temporally linked to the phase of the cell cycle (Rock and Amon, 2011). This multitude of signals regulating the MEN makes it clear that restricting MEN activity to the right time and place is essential for successful cell division.

Indeed, inappropriate activation of the MEN uncouples genome segregation and exit from mitosis and results in cell death (Visintin et al., 1998; Wang et al., 2000).

To understand the mechanisms by which MEN activity is coordinated with other cellular processes, we must understand how MEN signaling complexes are assembled at SPBs. The SPB-resident scaffold Nud1 plays a critical role in this process. Nud1 is essential for Tem1, Cdc15, and Dbf2-Mob1 localization to SPBs (Gruneberg et al., 2000; Visintin and Amon, 2001). Nud1 also recruits the γ–tubulin receptor Spc72 to the SPB, thereby serving to organize astral (cytoplasmic) microtubules and coordinate the positioning of the mitotic spindle with MEN activation (Gruneberg et al., 2000). How Nud1 promotes the proper spatiotemporal assembly of the MEN is unknown. We show here that rather than functioning as a passive platform onto which the MEN assembles, Nud1 is an dynamic participant in MEN signaling module assembly, activation, and signal transmission. Nud1 is phosphorylated by Cdc15. Phosphorylated Nud1 in turn recruits Dbf2-Mob1 to SPBs, where it is then activated by Cdc15. We further show that the Mob1 activating subunit is a novel class of phosphoserine/threonine binding domains and that this phosphopeptide binding property allows for the phosphorylation-dependent recruitment of Dbf2-Mob1 to Nud1. This two-step mode of activation of Dbf2-Mob1 *in vivo*- recruitment to the scaffold through the generation of phospho-docking sites followed by phosphorylation and activation of the downstream kinase- has important implications for our understanding of MEN-like signaling pathways.

Results

Mitosis-specific phosphorylation is essential for Nud1 function.

To determine the role of Nud1 in MEN signaling we tested the hypothesis that mitosis-specific phosphorylation is important for Nud1 function. This line of investigation was motivated by

the observation that Nud1 phosphorylation increases during mitosis (Figure 1A-C, (Keck et al., 2011; Maekawa et al., 2007; Park et al., 2008) and the recent comprehensive phosphorylation map of the budding yeast SPB (Keck et al., 2011). Mass-spectrometry identified 50 phosphorylation sites in Nud1, 38 of which are present during mitosis (Figure 1A, Table 1, (Keck et al., 2011)). As with many other multisite phosphorylated proteins, phosphorylation sites in Nud1 are not distributed evenly across the protein but rather are found in clusters (Figure 1A, (Schweiger and Linial, 2010)). 38 of the 50 identified phosphorylation sites occur within three dense clusters: two clusters in the N-terminus and one in the central region of Nud1. These sites did not occur within any known domains or motifs as identified by Pfam (the only identified motifs in Nud1 are a series of leucine-rich repeats in the C-terminal half of Nud1).

To determine the importance of Nud1 phosphorylation in MEN signaling, we generated a *NUD1* allele in which all 42 serines and threonines that were phosphorylated in mitotic cells were mutated to alanine (henceforth *nud1-42A)*. This allele contains mutations in the 38 high confidence mitotic phosphorylation sites as well as mutations in 4 lower confidence sites (Table 1). The nud1-42A protein was stable and localized to SPBs but phosphorylation during mitosis was reduced (Figure 1B – D). This is best seen in cells progressing through the cell cycle in a synchronous manner upon release from a pheromone-induced G1 arrest (Figure 1B,C). The residual phosphorylation of the nud1-42A protein could be due to tyrosine phosphorylation (Nud1 is phosphorylated on at least 1 tyrosine residue (Keck et al., 2011)), phosphorylation of sites not covered by the mass spectrometry analysis, and/or alternative site usage by Nud1 kinases. The fact that nud1-42A is present at wild-type levels, is a substrate for mitotic kinases, and localizes to SPBs indicates that the overall folding and structure of the protein are not affected by the alanine substitutions.

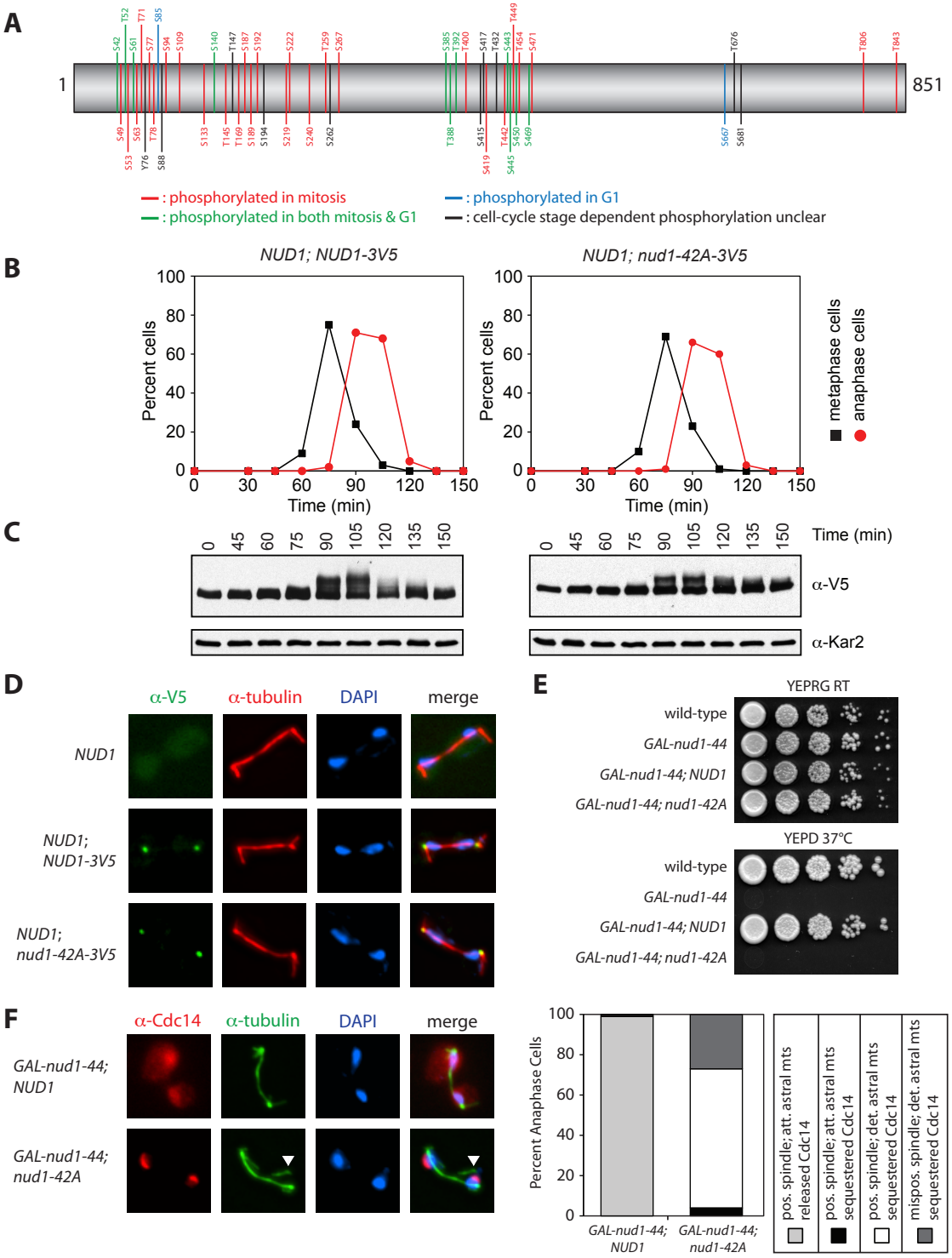

Figure 1: Phosphorylation of the scaffold Nud1 is essential

(A) Schematic of Nud1 phosphorylation sites identified by mass spectrometry.

(B, C) *NUD1; NUD1-3V5* (A27463) and *NUD1; nud1-42A-3V5* (A29128) cells 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 (black closed squares, B) and anaphase spindles (red closed circles, B) was determined at the indicated times. Nud1-3V5 and nud1-42A-3V5 protein levels were monitored by western blot analysis (C). Kar2 was used as a loading control.

(D) Exponentially growing *NUD1; NUD1-3V5* (A27463), *NUD1; nud1-42A-3V5* (A29128), or the no tag control (A2587) cells were prepared for immunofluorescence with anti-V5 antibodies (α-V5) and anti-tubulin antibodies (α-tubulin) to detect Nud1 and α-tubulin, respectively. 4'6-diamidino-2-phenylindole (DAPI) was used to stain DNA.

(E) Wild-type (A2587), *GAL-nud1-44* (A29248), *GAL-nud1-44; NUD1-3V5* (A29685), and *GAL-nud1-44; nud1-42A-3V5* (A29500) cells were spotted on YEP plates containing either galactose and raffinose (YEPRG) or glucose (YEPD) and incubated at room temperature (YEPRG) or 37°C (YEPD). Approximately 3 X 10^4 cells were deposited in the first spot and each subsequent spot is a 10-fold serial dilution. The pictures shown depict 1.5 days of growth (YEPD) or 3 days of growth (YEPRG).

(F) *GAL-nud1-44; NUD1-3V5* (A29878) and *GAL-nud1-44; nud1-42A-3V5* (A29881) cells containing a 3HA-Cdc14 fusion protein were incubated for 2 hours in YEP medium containing glucose at 37°C and then prepared for immunofluorescence with anti-HA antibodies (α-HA) and anti-tubulin antibodies (α-tubulin) to detect Cdc14 and α-tubulin, respectively. Cells were scored as having either correctly positioned (anaphase spindle aligned along the mother-daughter cell axis) or mispositioned anaphase spindles, the presence or absence of detached astral microtubules, and released or sequestered Cdc14. Representative images of anaphase cells are shown. The white arrowhead marks a detached astral microtubule.

Despite wild-type expression levels and localization patterns, the *nud1-42A* allele was not able to complement the temperature sensitive lethality of cells expressing the conditional *nud1-44* allele. At the restrictive temperature, *nud1-44* cells arrest in late anaphase because the MEN is inactive (Adams and Kilmartin, 1999; Visintin and Amon, 2001). Introducing the *nud1-42A* allele into the *nud1-44* strain not only failed to restore growth at 37°C, it caused lethality at the permissive temperature (data not shown). Thus, to examine the effects of the *nud1-42A* allele on MEN activity we placed the temperature sensitive *nud1-44* allele under the galactose-inducible/glucose-repressible *GAL1-10* promoter to generate a *GAL-nud1-44 nud1-42A* strain. While the wild-type *NUD1* allele allowed *GAL-nud1-44* cells to grow on glucose-containing medium (YEPD) at 37°C, the *nud1-42A* allele failed to support growth under these conditions (Figure 1E). Examining the terminal phenotype revealed that *GAL-nud1-44 nud1-42A* cells arrested in late anaphase with Cdc14 sequestered in the nucleolus (Figure 1F). Such an arrest phenocopies MEN loss-of-function mutants (Jaspersen et al., 1998; Shou et al., 1999; Visintin et al., 1999). In addition, a significant fraction of *GAL-nud1-44 nud1-42A* cells showed mispositioned anaphase spindles and nearly all anaphase cells harbored detached astral microtubules (Figure 1F). We conclude that *nud1-42A* is a loss-of-function allele and that mitosis-specific phosphorylation is essential Nud1 to promote both MEN activation and astral microtubule organization. We chose to first pursue the role of Nud1 phosphorylation in MEN activation followed by an analysis of the role of Nud1 phosphorylation in spindle positioning.

Nud1 phosphorylation is essential for Dbf2-Mob1 SPB localization and kinase activity.

To determine how the mitosis-specific phosphorylation of Nud1 affects MEN signaling, we monitored the activity of the most downstream MEN kinase, Dbf2-Mob1. *GAL-nud1-44 NUD1* and *GAL-nud1-44 nud1-42A* cells were induced to undergo a synchronous cell cycle in glucose-containing medium at 37°C. Dbf2 kinase activity and Cdc14 release from the nucleolus

occurred during anaphase in *GAL-nud1-44 NUD1* cells, but *GAL-nud1-44 nud1-42A* cells arrested in anaphase with sequestered Cdc14 and inactive Dbf2-Mob1 (Figure 2A,B). The early anaphase release of Cdc14 in *GAL-nud1-44 nud1-42A* cells is a result of FEAR network activation (Pereira et al., 2002; Stegmeier et al., 2002). We conclude that mitosis-specific phosphorylation of the scaffolding protein Nud1 is essential for the activation of the most downstream MEN kinase, Dbf2-Mob1.

The localization of the core MEN components Tem1, Cdc15, Dbf2, and Mob1 to SPBs is essential for Dbf2-Mob1 activation (Rock and Amon, 2011; Valerio-Santiago and Monje-Casas, 2011). To test whether the failure of *GAL-nud1-44 nud1-42A* cells to activate Dbf2-Mob1 was due to a defect in recruiting core MEN components to SPBs, we examined Tem1, Cdc15, Dbf2, and Mob1 localization. To perform these localization studies, we needed to generate a strain in which *nud1-42A* was the sole source of *NUD1* in the cell. The reason such a strain was desired was that we had previously observed intragenic complementation between the *nud1-42A* and *cdc18-1* alleles (*cdc18-1* is a temperature sensitive allele of *NUD1*, data not shown). Furthermore, Nud1 is known to oligomerize via its C-terminus (Elliott et al., 1999). A strain in which *nud1-42A* was the sole source of *NUD1* in the cell would obviate concerns that any observed MEN component localization to SPBs was the result of intragenic complementation, be it partial or complete.

We were able to construct a strain with *nud1-42A* as the sole source of *NUD1* by introducing a constitutively active Dbf2 allele (*DBF2-HyA*) to activate the MEN and by restoring astral microtubule anchorage by expressing an *SPC72-CNM67* fusion (Geymonat et al., 2009; Gruneberg et al., 2000). It should be noted that the suppression of the lethality of a *nud1-42A* allele by the combination of a constitutively active *DBF2* allele and a *SPC72-CNM67* allele

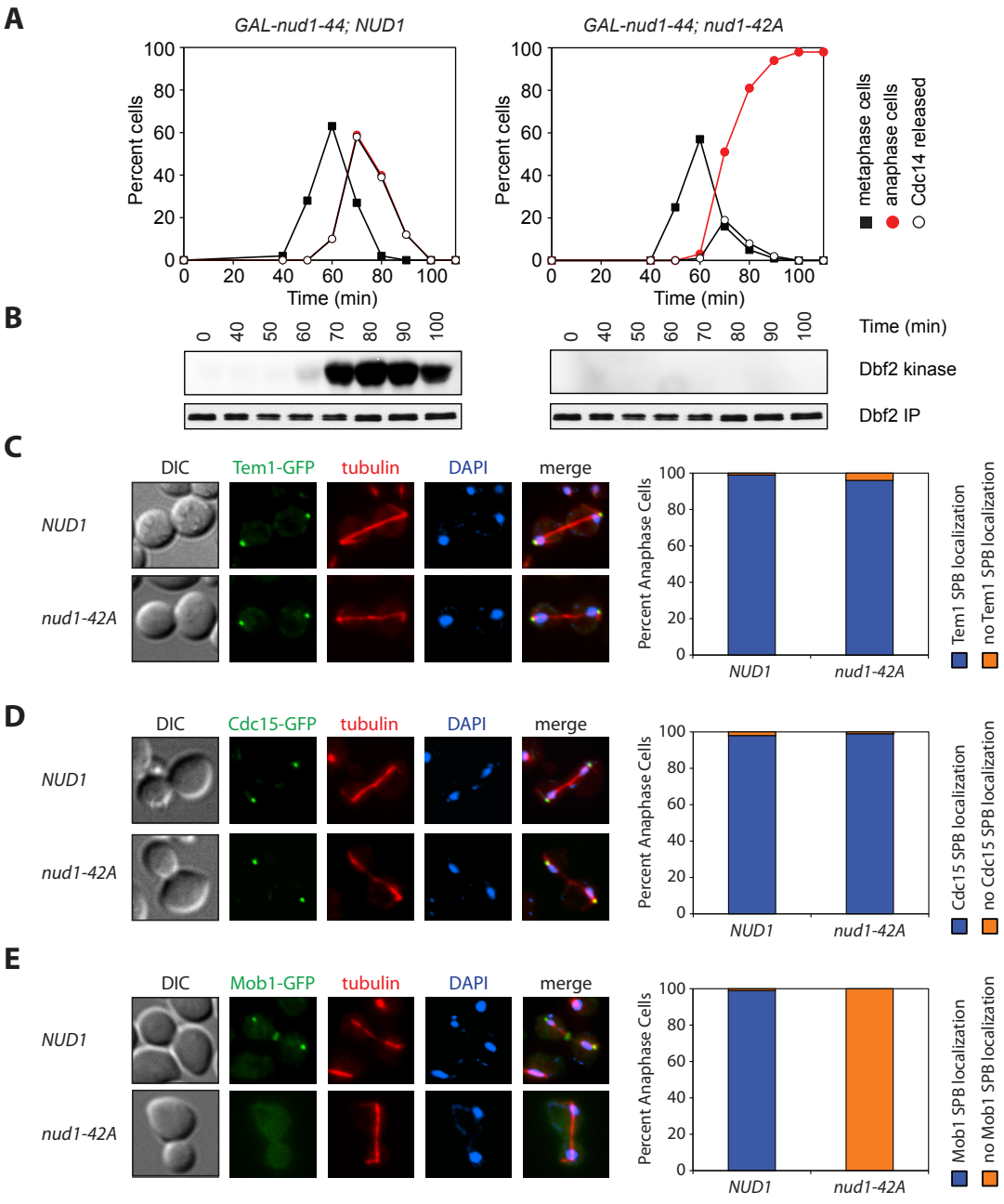

Figure 2: Nud1 phosphorylation is essential for Dbf2-Mob1 recruitment to SPBs and MEN activation

(A, B) *GAL-nud1-44; NUD1-3V5* (A29878) and *GAL-nud1-44; nud1-42A-3V5* (A29881) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium at room temperature. 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 YEPD 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 (black closed squares, A), anaphase spindles (red 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) *nud1Δ; NUD1-3V5 Dbf2-HyA SPC72-CNM67 TEM1-GFP* (A29899) and *nud1Δ; nud1-42A-3V5 Dbf2-HyA SPC72-CNM67 TEM1-GFP* (A29679) cells containing an mCherry-Tub1 fusion protein were grown to log phase SC-Leu medium and imaged after a brief paraformaldehyde fixation. Cell cycle stage was determined based on spindle morphology and correlated with Tem1 localization at SPBs (n ≥ 100 cells). Representative images of anaphase cells are shown.

(D) *nud1Δ; NUD1-3V5 Dbf2-HyA SPC72-CNM67 CDC15-eGFP* (A28650) and *nud1Δ; nud1-42A-3V5 Dbf2-HyA SPC72-CNM67 CDC15-eGFP* (A29506) cells containing an mCherry-Tub1 fusion protein were grown and scored as in Figure 2C. Representative images of anaphase cells are shown.

(E) *nud1Δ; NUD1-3V5 Dbf2-HyA SPC72-CNM67 MOB1-eGFP* (A29450) and *nud1Δ; nud1-42A-3V5 Dbf2-HyA SPC72-CNM67 MOB1-eGFP* (A29722) cells containing an mCherry-Tub1 fusion protein were grown and scored as in Figure 2C. Representative images of anaphase cells are shown.

indicates that the two major defects associated with the *nud1-42A* allele are MEN activation and astral microtubule organization. The coexpression of *DBF2-HyA* and *SPC72-CNM67* in otherwise wild-type cells did not significantly perturb MEN component localization (data not shown). In this genetic background, *nud1-42A*, like wild-type Nud1, localized to SPBs constitutively throughout the cell cycle (Figure 3A).

We did not observe any significant differences in Bfa1 (the Tem1 GAP complex protein) nor Tem1 localization between *NUD1* and *nud1-42A* cells (Figure 3B; Figure 2C). Both proteins localize to SPBs in metaphase and become enriched on the SPB that is pulled into the daughter during anaphase. In late anaphase, the two proteins localize to both SPBs (Bardin et al., 2000; Molk et al., 2004). Cdc15 localization also was not affected by the *nud1-42A* allele (Figure 2D). Cdc15 localizes to both SPBs during anaphase in both wild-type and *nud1-42A* cells (Konig et al., 2010; Visintin and Amon, 2001). In contrast, Mob1 and Dbf2 localization was affected. In wild-type cells, Mob1 and Dbf2 localize to both SPBs in anaphase (Luca et al., 2001; Pereira et al., 2002; Visintin and Amon, 2001) but both proteins were absent from SPBs in *nud1-42A* cells (Figure 2E, Figure 3C). Thus, the mitosis-specific phosphorylation of the scaffolding protein Nud1 is specifically required for recruiting Dbf2-Mob1 but not the other MEN components to SPBs.

Phosphorylation of Nud1 S53, S63, and T78 is essential for Dbf2-Mob1 localization to SPBs and exit from mitosis.

Phosphorylation of Nud1 in mitosis is essential for both Dbf2-Mob1 activation and proper astral microtubule organization. We next sought to generate separation-of-function alleles by narrowing down the phosphorylation sites required for Dbf2-Mob1 SPB recruitment. To do this, we took advantage of the fact that overexpression of the *nud1-42A* allele causes lethality.

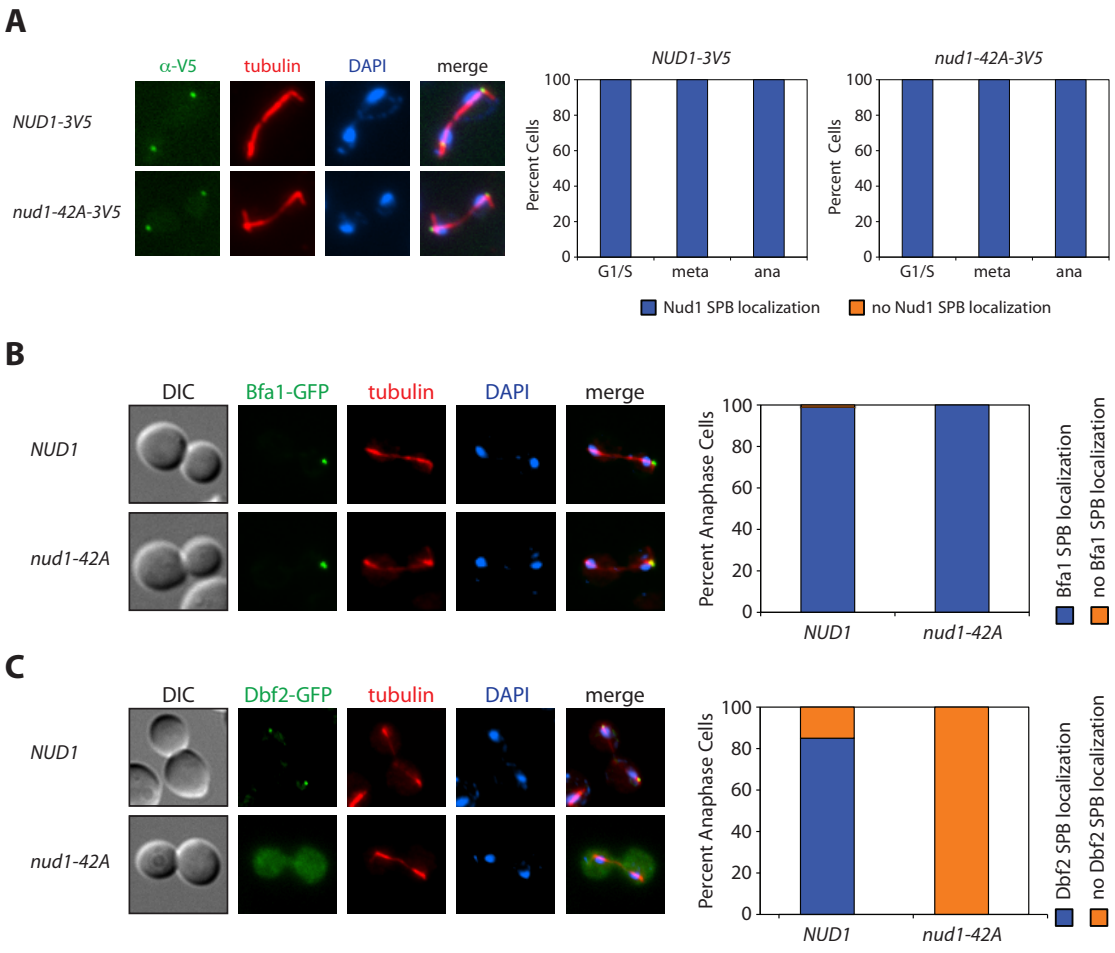

Figure 3: MEN component localization in the *nud1-42A* mutant.

(A) Exponentially growing *nud1Δ; NUD1-3V5* (A29412) and *nud1Δ; nud1-42A-3V5* (A29508) cells were prepared for immunofluorescence with anti-V5 antibodies (α-V5) and anti-tubulin antibodies (α-tubulin) to examine Nud1 and tubulin localization, respectively. 4'6-diamidino-2-phenylindole (DAPI) was used to stain DNA. Cell cycle stage was determined based on spindle morphology and correlated with Nud1 localization at SPBs (n ≥ 100 cells). Representative images of anaphase cells are shown.

(B) *nud1Δ; NUD1-3V5 Dbf2-HyA SPC72-CNM67 BFA1-eGFP* (A29733) and *nud1Δ; nud1-42A-3V5 Dbf2-HyA SPC72-CNM67 BFA1-eGFP* (A29730) cells containing an mCherry-Tub1 fusion protein were grown to log phase SC-Leu medium and imaged after a brief paraformaldehyde fixation. Cells were scored as in Figure 3A. Representative images of anaphase cells are shown.

(C) *nud1Δ; NUD1-3V5 Dbf2-HyA SPC72-CNM67 DBF2-eGFP* (A29711) and *nud1Δ; nud1-42A-3V5 Dbf2-HyA SPC72-CNM67 DBF2-eGFP* (A29682) cells containing an mCherry-Tub1 fusion protein

were grown to log phase SC-Leu medium and imaged and scored as in Figure 3A. Representative images of anaphase cells are shown.

While overexpression of wild-type *NUD1* did not grossly perturb cell growth, *nud1-42A* caused lethality when overexpressed from the *GAL1-10* promoter, even in cells that also harbor a wild-type *NUD1* allele (Figure 4A). *GAL-nud1-42A* cells arrested in anaphase with Cdc14 sequestered in the nucleolus (data not shown), indicating that the overexpressed nud1-42A protein interferes with MEN function.

We first generated a *nud1* allele in which only those serine and threonine residues that are specifically phosphorylated in mitosis (i.e. excluding sites that are phosphorylated in both mitosis and G1) were mutated to alanine (Table 1). This allele, *nud1-27A*, could not complement the growth defect of *GAL-nud1-44* cells grown in YEPD at 37°C and caused lethality when overexpressed from the *GAL* promoter (Figure 4A, data not shown). We subsequently created hybrid *NUD1* alleles harboring a subset of these phosphorylation site mutants and identified critical residues by virtue of whether or not they caused lethality when overexpressed (see Experimental Procedures). This analysis revealed that Nud1 T78 was especially critical (Figure 4A,B). Cells overexpressing the *nud1-T78A* allele exhibited a severe mitotic exit delay as judged by an increase in the percentage of cells with anaphase spindles (Figure 4C). Cells expressing *nud1-T78A* as the sole source of *NUD1* were also defective in exit from mitosis and Mob1 SPB recruitment (data not shown). Mutating S53 or S63 to alanine substantially exacerbated the mitotic exit defect caused by overexpression of the *nud1-T78A* allele: a *NUD1* allele carrying alanine substitutions of residues S53, S63, and T78 (hereafter referred to as *nud1-3A*) caused an anaphase arrest (Figure 5A, Figure 4C) and could not complement the lethality of a *GAL-nud1-44* allele grown at 37°C on YEPD medium (Figure 5B). The anaphase delay of a *NUD1* allele that included the S53A and S63A mutations (but was wild-type for T78) was minor (Figure 4C). Thus, Nud1 T78 phosphorylation is critical for exit from mitosis, whereas phosphorylation of S53 and S63 play important but more minor roles.

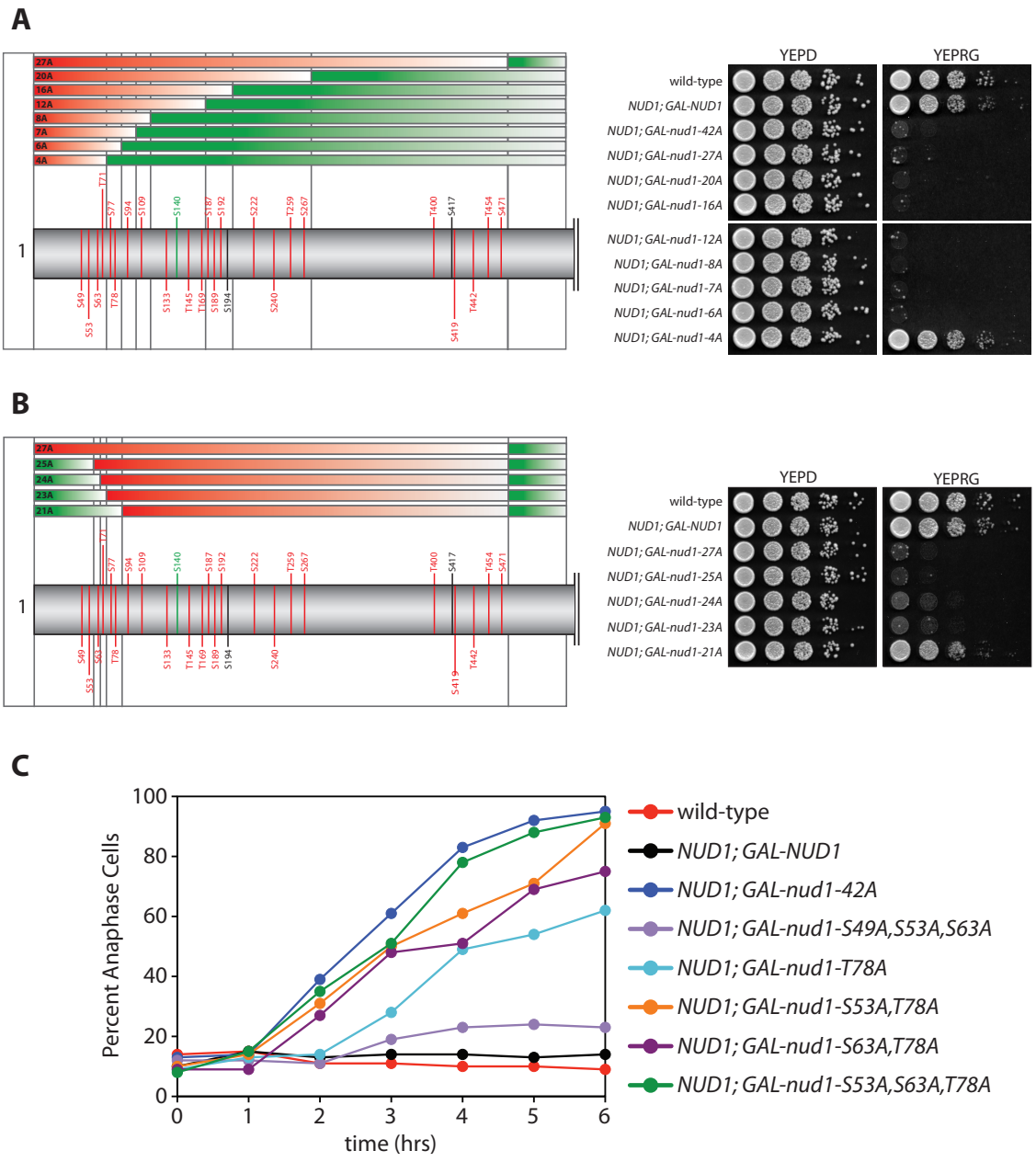

Figure 4: Identification of Nud1 phosphosites necessary for Dbf2-Mob1 localization at SPBs.

(A) Wild-type (A2587), *NUD1; GAL-NUD1-3V5* (A27931), *NUD1; GAL-nud1-42A-3V5* (A29239), *NUD1; GAL-nud1-27A-3V5* (A27933), *NUD1; GAL-nud1-20A-3V5* (A30611), *NUD1; GAL-nud1-16A-3V5* (A30614), *NUD1; GAL-nud1-12A-3V5* (A30617), *NUD1; GAL-nud1-8A-3V5* (A30620), *NUD1; GAL-nud1-7A-3V5* (A30623), *NUD1; GAL-nud1-6A-3V5* (A30649), and *NUD1; GAL-nud1-4A-3V5* (A30652) cells were spotted on YEP plates containing either galactose and raffinose (YEPRG) or

glucose (YEPD) and incubated 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 pictures shown depict 2 days of growth (YEPD) or 3 days of growth (YEPRG). The cartoon on the left shows the mutations contained in each hybrid *nud1* phosphomutant allele.

(B) Wild-type (A2587), *NUD1; GAL-NUD1-3V5* (A27931), *NUD1; GAL-nud1-27A-3V5* (A27933), *NUD1; GAL-nud1-25A-3V5* (A30917), *NUD1; GAL-nud1-24A-3V5* (A30915), *NUD1; GAL-nud1-23A-3V5* (A31176), and *NUD1; GAL-nud1-21A-3V5* (A30913) cells were spotted on YEPD or YEPRG plates and incubated at 30°C as in Figure 4A. The cartoon on the left shows the mutations contained in each hybrid *nud1* phosphomutant allele.

(C) wild-type (A2587), *NUD1; GAL-NUD1-3V5* (A27931), *NUD1; GAL-nud1-42A-3V5* (A29239), *NUD1; GAL-nud1-S49A,S53A,S63A-3V5* (A30655), *NUD1; GAL-nud1-T78A-3V5* (A30925), *NUD1; GAL-nud1-S53A,T78A-3V5* (A31209), *NUD1; GAL-nud1-S63A,T78A-3V5* (A31211), and *NUD1; GAL-nud1-S53A,S63A,T78A-3V5* (A31215) cells were grown to exponential phase in YEP medium containing raffinose. Galactose was added a t = 0 to induce the *GAL1-10* promoter. Cells were collected and analyzed for spindle morphology as in Figure 3A.

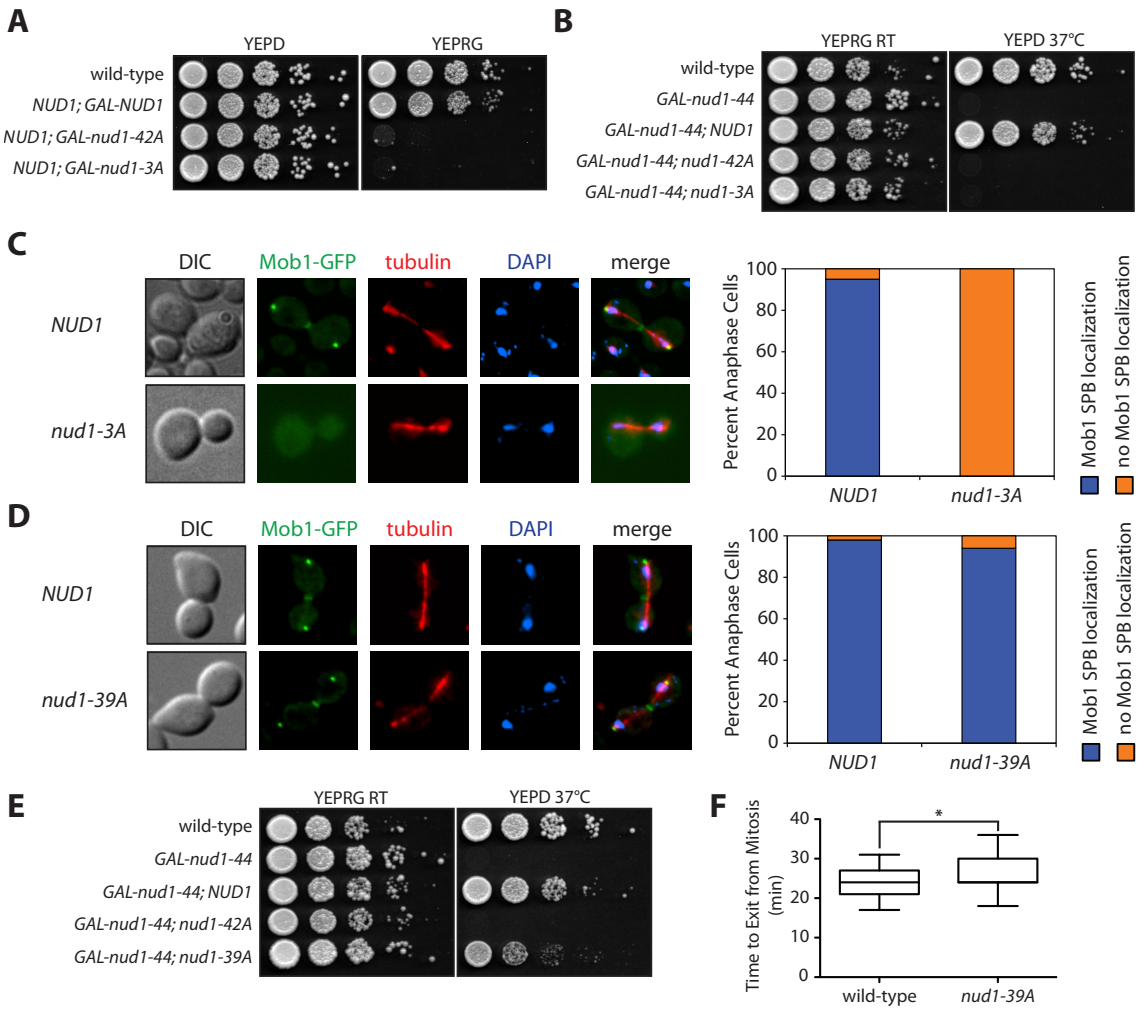

Figure 5: Phosphorylation of Nud1 S53, S63, and T78 is essential for Dbf2-Mob1 recruitment to SPBs and exit from mitosis

(A) Wild-type (A2587), *NUD1; GAL-NUD1-3V5* (A28009), *NUD1; GAL-nud1-42A-3V5* (A29239), and *NUD1; GAL-nud1-3A-3V5* (A31215) cells were spotted on YEPRG or YEPD plates and incubated at 30°C as in Figure 1E.

(B) Wild-type (A2587), *GAL-nud1-44* (A29248), *GAL-nud1-44; NUD1-3V5* (A29685), *GAL-nud1-44; nud1-42A-3V5* (A29500), and *GAL-nud1-44; nud1-3A-3V5* (A32292) cells were spotted on YEPRG or YEPD plates and incubated at room temperature (YEPRG) or 37°C (YEPD) as in Figure 1E.

(C) *nud1Δ; NUD1-3V5 Dbf2-HyA MOB1-eGFP* (A29453) and *nud1Δ; nud1-3A-3V5 Dbf2-HyA MOB1-eGFP* (A31169) cells containing an mCherry-Tub1 fusion protein were grown and scored as in Figure 2C. Representative images of anaphase cells are shown.

(D) *MOB1-eGFP* (A24631) and *nud1Δ; nud1-39A-3V5 MOB1-eGFP* (A31477) cells containing an mCherry-Tub1 fusion protein were grown to log phase in YEPD medium and imaged and scored as in Figure 2C. Representative images of anaphase cells are shown.
(E) Wild-type (A2587), *GAL-nud1-44* (A29248), *GAL-nud1-44; NUD1-3V5* (A29685), and *GAL-nud1-44; nud1-39A-3V5* (A32295) cells were spotted on YEPRG or YEPD plates and incubated at room temperature (YEPRG) or 37°C (YEPD) as in Figure 1E.
(F) Wild-type (A32731) and *nud1Δ; nud1-39A-3V5* (A32730) cells containing GFP-Tub1 and SV40NLS-mCherry fusion proteins were grown to log phase in complete synthetic medium and processed for live cell microscopy as described in Experimental Procedures. The time to exit from mitosis was defined as the time interval between entry of the daughter-bound SPB into the bud and breakdown of the anaphase spindle ($n \geq 30$ cells). Data are summarized in box-and-whisker plots: boxes span between the 25th and 75th percentile with a line at the median; whisker extend from the minimum value to the maximum. *$p < 0.05$, t test.

Like the *nud1-42A* cells, *nud1-3A* cells failed to localize Mob1 to SPBs (Figure 5C). Importantly, cells expressing a *NUD1* allele in which all mitotic phosphorylation sites were mutated to alanine with the exception of S53, S63 and T78 (henceforth the *nud1-39A* allele; Table 1) localized Mob1 to SPBs normally (Figure 5D). The *nud1-39A* allele was also able to complement the lethality of *GAL-nud1-44* cells grown at 37°C on YEPD (Figure 5E). Live cell microscopy-based measurements of the time required to exit from mitosis (quantified as the time interval from entry of the daughter-bound SPB into the bud to the disassembly of the mitotic spindle) did reveal a slight delay in exit from mitosis in *nud1-39A* cells as compared to wild-type cells (26.7 minutes compared to 24.1 minutes, respectively), suggesting that there may be additional Nud1 phosphosites that play a very minor role in the activation of the MEN (Figure 5F). Like *nud1-42A* cells, however, a significant fraction of *nud1-39A* cells showed mispositioned anaphase spindles and nearly all anaphase cells harbored detached astral microtubules (Figure 6A, data not shown). Thus, *nud1-3A* and *nud1-39A* are separation-of-function alleles, with *nud1-3A* specifically defective in MEN activation and *nud1-39A* defective in astral microtubule organization. We conclude that phosphorylation of Nud1 at S53, S63, and T78 is required for Dbf2-Mob1 recruitment to SPBs and exit from mitosis and phosphorylation outside of these sites is required for astral microtubule anchorage. Importantly, S53, S63, and T78 are conserved across fungal orthologs, with S63 and T78 being highly constrained (>80% conserved; Figure 7). Thus, these residues may be playing similarly important roles in other fungal species.

Nud1 phosphorylation is necessary for astral microtubule organization and spindle positioning.

In addition to Dbf2-Mob1 SPB recruitment, Nud1 also serves to organize astral microtubules and thereby coordinate the positioning of the mitotic spindle with MEN activation (Gruneberg

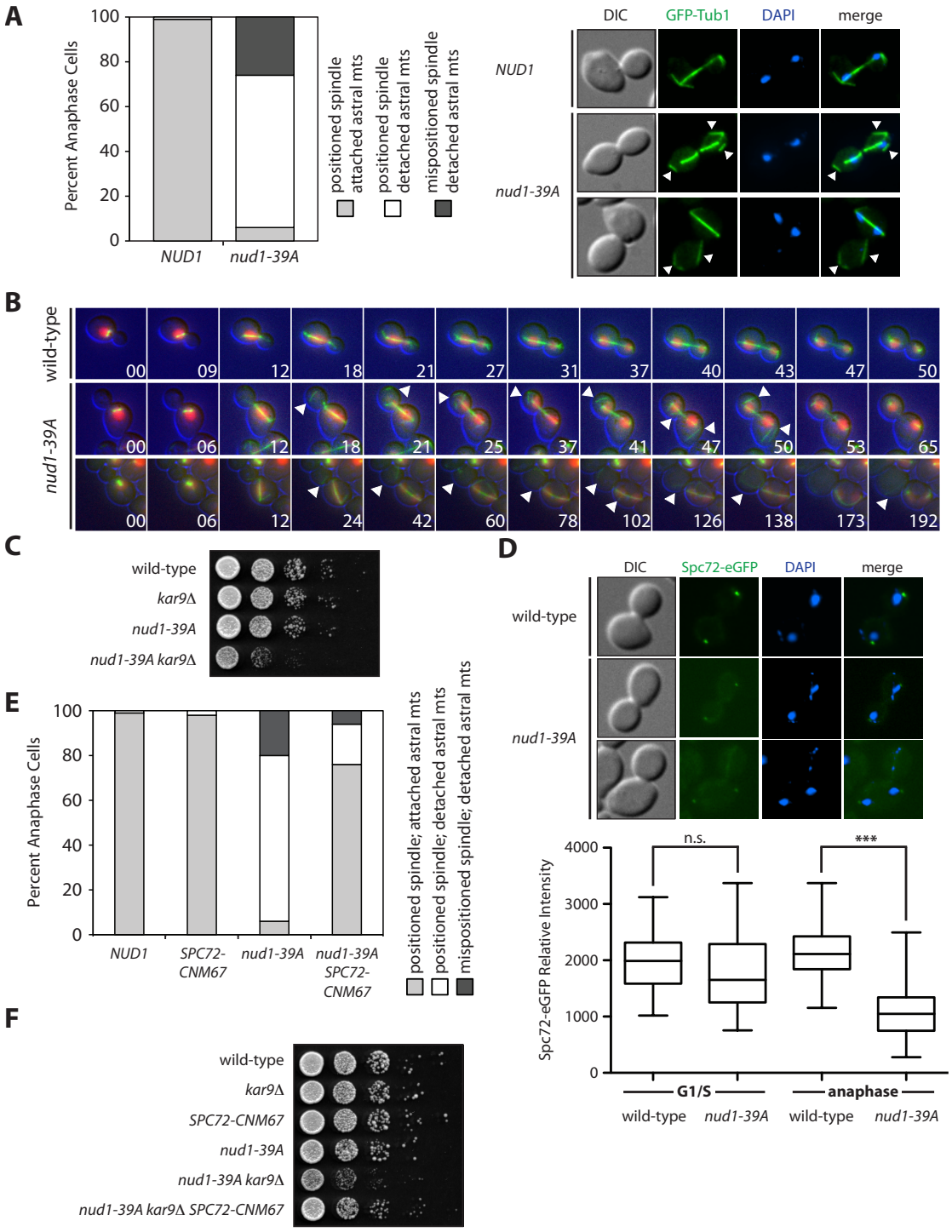

Figure 6: Nud1 phosphorylation is required for anaphase astral microtubule organization and spindle positioning.

(A) *nud1Δ; NUD1-3V5* (A32241) and *nud1Δ; nud1-39A-3V5* (A31472) cells containing a GFP-Tub1 fusion protein were grown to log phase in YEPD medium and imaged after a brief

paraformaldehyde fixation. Cells were scored as in Figure 1F. Representative images of anaphase cells are shown. The arrows point to astral microtubules that detached from SPBs.
(B) Wild-type (A32731) and *nud1Δ; nud1-39A-3V5* (A32730) cells containing GFP-Tub1 and SV40NLS-mCherry fusion proteins were grown to log phase in complete synthetic medium and processed for live cell microscopy as described in Experimental Procedures. Representative montages of a wild-type cell with a correctly positioned anaphase spindle (top row), a *nud1-39A-3V5* cell with a correctly positioned anaphase spindle (middle row), and a *nud1-39A-3V5* cell with a mis-positioned anaphase spindle (bottom row) are shown. White arrowheads mark detached astral microtubules.
(C) Wild-type (A2587), *kar9Δ* (A18274), *nud1Δ; nud1-39A-3V5* (A31388), and *nud1Δ; nud1-39A-3V5 kar9Δ* (A31966) cells were spotted on YEPD plates and incubated at 30°C as in Figure 1E.
(D) Wild-type (A27353) and *nud1Δ; nud1-39A-3V5* (A31466) cells containing a *SPC72-eGFP* fusion protein were grown to log phase YEPD medium and imaged after a brief paraformaldehyde fixation. Representative images of anaphase cells are shown. Fluorescence intensity was quantified with Openlab software. Quantified relative fluorescent intensities are summarized in box-and-whisker plots as in Figure 3F. n.s. = not significant, ***$p < 0.001$, t test.
(E) *nud1Δ; NUD1-3V5* (A32241), *SPC72-CNM67* (A32796), *nud1Δ; nud1-39A-3V5* (A31472), and *nud1Δ; nud1-39A-3V5 SPC72-CNM67* (A32793) cells containing a GFP-Tub1 fusion protein were grown to log phase in YEPD medium and imaged after a brief paraformaldehyde fixation. Cells were scored as in Figure 4A.
(F) Wild-type (A2587), *kar9Δ* (A18274), *SPC72-CNM67* (A28553), *nud1Δ; nud1-39A-3V5* (A31388), *nud1Δ; nud1-39A-3V5 kar9Δ* (A31966), and *nud1Δ; nud1-39A-3V5 kar9Δ SPC72-CNM67* (A32888) cells were spotted on YEPD plates and incubated at 30°C as in Figure 1E.

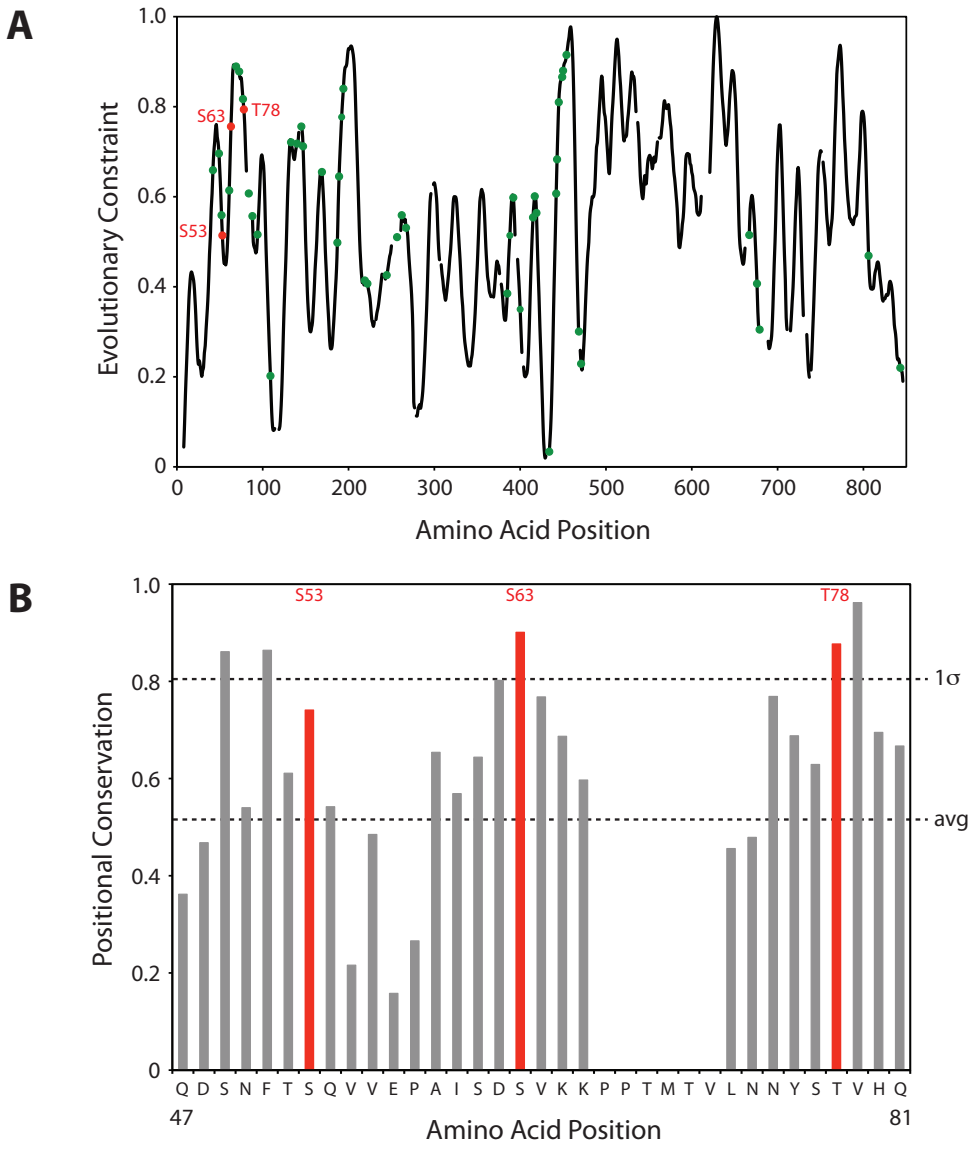

Figure 7: Conservation of Nud1 phosphorylation sites

(A) Regional evolutionary constraints for Nud1 were calculated in (Keck et al., 2011). The *Y* axis represents the Nud1 regional evolutionary constraint value among fungi (17 amino acid

sliding regions) where a value of 1 indicates full conservation across all orthologs; the *X* axis is the amino acid position of Nud1. Green dots and red dots represent identified phosphorylation sites in Nud1 (the red dots mark S53, S63, and T78).
(B) Positional evolutionary constraints for Nud1 were calculated in (Keck et al., 2011). The *Y* axis is the Nud1 positional evolutionary constraint value among fungi where a value of 1 indicates full conservation across all orthologs; the *X* axis is the amino acid position of Nud1. Dashed lines indicate the average Nud1 positional evolutionary constraint value (avg) and 1 standard deviation above this value (1σ). Highlighted in red are amino acids S53, S63, and T78.
(C) BLASTP was used to identify the five closest fungal homologs of the N-terminus of Nud1 (amino acids 1 – 400). These homologs were then aligned with Nud1 using ClustalW2. Nud1 S53, S63, and T78 are highlighted in red.

et al., 2000). The generation of the *nud1-39A* separation-of-function allele allowed us to investigate the role of Nud1 phosphorylation in mitotic spindle positioning. Spindle positioning in budding yeast is controlled by two partially redundant pathways (Pearson and Bloom, 2004). Prior to anaphase, a *KAR9*-dependent pathway aligns the mitotic spindle along the mother-daughter cell axis and positions the nucleus at the bud neck. Upon entry into anaphase, a *DYN1*-dependent pathway translocates the aligned spindle through the bud neck. Both pathways promote spindle positioning and nuclear migration through the action of astral microtubules that are nucleated by the γ–tubulin complex anchored at SPBs through Spc72 and Nud1 (Gruneberg et al., 2000).

nud1-39A cells appear defective in astral microtubule anchorage and spindle positioning. 25% of *nud1-39A* cells failed to align the anaphase spindle along the mother-daughter cell axis (Figure 6A). Furthermore, astral microtubules were detached from SPBs in nearly all *nud1-39A* cells (Figure 6A,B). Live-cell microscopy revealed that detachment of astral microtubules occurred during anaphase. Similar results were seen in *nud1-24A* cells (data not shown), which are identical to *nud1-39A* cells with the exception that only those serine and threonine residues that are specifically phosphorylated in mitosis (i.e. excluding sites that are phosphorylated in both mitosis and G1) were mutated to alanine. The majority (80%) of metaphase *nud1-39A* cells showed proper nuclear migration and spindle alignment and subsequently underwent anaphase with a correctly positioned spindle (regardless of the presence of detached astral microtubules). However, 20% of metaphase *nud1-39A* cells failed to properly position the nucleus and subsequently underwent anaphase in the mother cell with a mispositioned spindle (Figure 6B, data not shown). The majority of these cells arrested in anaphase for approximately 3 hours, after which they exited mitosis. The anaphase-specific spindle alignment defect suggests that dynein-dependent spindle positioning is defective in

nud1-39A cells. Consistent with this idea, we found that deleting *DYN1* did not interfere with proliferation of *nud1-39*A cells, but deleting *KAR9* did (Figure 6C, data not shown). We conclude that Nud1 phosphorylation is critical for dynein-mediated anaphase spindle positioning.

We next asked whether the astral microtubule detachment seen in *nud1-39A* cells was due to defects in Spc72 SPB localization, which has previously been shown to be partially dependent on *NUD1* (Gruneberg et al., 2000). We found that while both wild-type and *nud1-39A* cells showed SPB-localized Spc72-eGFP prior to mitosis, *nud1-39A* cells exhibited a defect in Spc72-eGFP SPB localization specifically in anaphase (Figure 6D). Quantification of the relative intensity of Spc72-eGFP at SPBs showed no significant difference in the amount of Spc72-eGFP at SPBs prior to anaphase in wild-type and *nud1-39A* cells (Figure 6D). However, there was a statistically significant reduction in the amount of Spc72-eGFP at anaphase SPBs in *nud1-39A* cells as compared to wild-type cells (Figure 6D). If the observed defect in astral microtubule organization in *nud1-39A* cells is the result of a defect in the anchoring of Spc72 to the outer plaque of the SPB, then targeting Spc72 to the outer plaque independently of *NUD1* should suppress the astral microtubule anchoring and spindle positioning defects of *nud1-39A* cells. Expression of an *SPC72-CNM67* fusion protein, which targets Spc72 to SPBs in a *NUD1* independent manner (Gruneberg et al., 2000), reduced both the astral microtubule detachment and spindle positioning defects of *nud1-39A* cells (Figure 6E). Furthermore, the constitutive targeting of Spc72 to SPBs ameliorated the growth defect of *nud1-39A kar9*Δ cells (Figure 6F). Taken together, our data indicate that the phosphorylation of Nud1 in mitosis is required for targeting the γ–tubulin complex binding protein Spc72 to Nud1, which in turn is necessary for the anchoring of astral microtubules to SPBs and proper positioning of the

anaphase spindle. Thus, phosphorylation of Nud1 serves to coordinate the positioning of the mitotic spindle with MEN activation.

Cdc15 phosphorylation of Nud1 T78 induces Nud1-Mob1 binding.

We next sought to further refine our understanding of how Nud1 contributes to Dbf2-Mob1 SPB recruitment. First, we sought to determine the identity of the kinase that phosphorylates Nud1 S53, S63, and T78. Cdc15 is an excellent candidate because it, like Nud1 phosphorylation, is essential for Dbf2-Mob1 SPB localization. Dbf2-Mob1 fails to localize to SPBs in cells harboring a temperature sensitive *cdc15-2* allele (Luca et al., 2001; Visintin and Amon, 2001) or cells in which an analog sensitive *CDC15* allele, *cdc15-as1*, are grown in the presence of inhibitor (Figure 8A, (Konig et al., 2010)). Importantly, treatment of *cdc15-as1* cells with inhibitor arrests cells in anaphase with Cdc15 localized to both SPBs (Figure 8A, (Konig et al., 2010)), thus Cdc15 kinase activity at SPBs, and not simply Cdc15 protein, is necessary for Dbf2-Mob1 SPB localization. Consistent with the localization data, Nud1 was found to co-immunoprecipitate Mob1 in anaphase-arrested cells in which *CDC15* is active (*cdc14-3* cells) but not in cells in which *CDC15* is inactive (*cdc14-3 cdc15-2* cells; Figure 8B). Thus, Cdc15 kinase activity is necessary for Dbf2-Mob1 localization to SPBs and Nud1-Mob1 complex formation.

We next asked if Cdc15 kinase activity is sufficient for Dbf2-Mob1 SPB localization. Constitutive targeting of Cdc15 to SPBs by fusing *CDC15* to the SPB outer plaque component *CNM67* (henceforth *CDC15-SPB*; (Rock and Amon, 2011) led to Dbf2-Mob1 recruitment to SPBs in all cell cycle stages soon after induction of the *CDC15-SPB* fusion (Figure 8C). Thus, Cdc15 kinase activity is necessary and sufficient for Dbf2-Mob1 SPB recruitment.

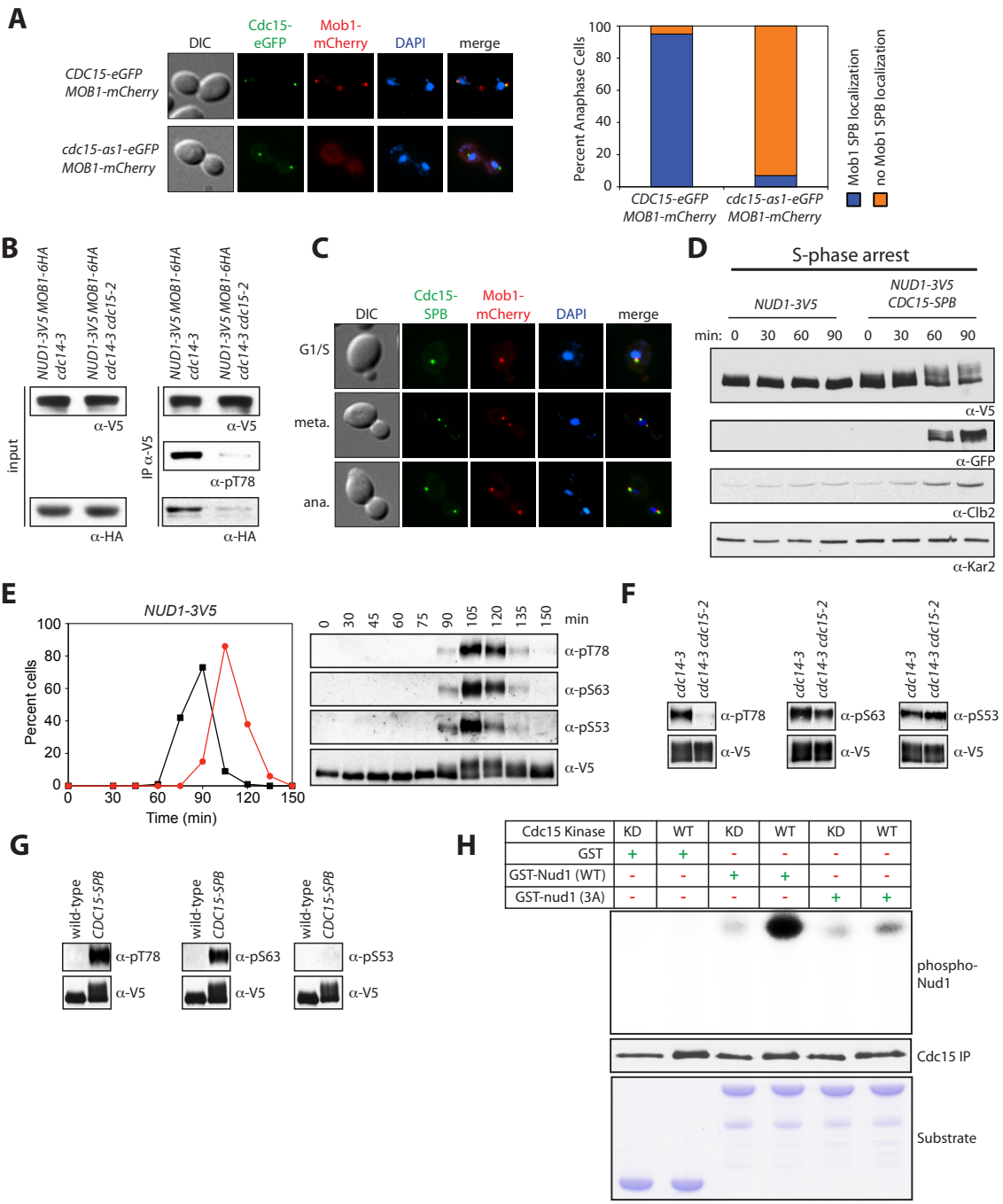

Figure 8: Cdc15 phosphorylates Nud1 in anaphase to promote Mob1 recruitment to SPBs.

(A) *CDC15-eGFP MOB1-mCherry* (A31089) and *cdc15-as1-eGFP MOB1-mCherry* (A31355) cells were grown for 2 hours in YEPD supplemented with 10 μM 1-NA-PP1 and imaged after a brief paraformaldehyde fixation. Representative images of anaphase cells are shown.

(B) *MOB1-6HA NUD1-3V5 cdc14-3* (A31602) and *MOB1-6HA NUD1-3V5 cdc14-3 cdc15-2* (A31661) cells were grown to log phase in YEPD at room temperature and then arrested in anaphase by incubating them at 37°C for 2 hours. Nud1-3V5 was immunoprecipitated and bound Mob1 (IP α-HA), bound Nud1 (IP α-V5), Nud1 phosphorylated at T78 (IP α-pT78), total Mob1 (input α-HA), and total Nud1 (input α-V5) was determined by Western blot analysis.
(C) *MET3-CDC15-SPB MOB1-mCherry* (A28499) cells were grown to log phase in YEPD supplemented with 8 mM methioinine. Cells were then shifted to CSM-methionine medium for 60 minutes to induce expression of the *CDC15-SPB* fusion and imaged after a brief paraformaldehyde fixation. Representative images of G1/S, metaphase, and anaphase cells are shown.
(D) *NUD1-3V5* (A24513) and *NUD1-3V5 MET3-CDC15-SPB* (A31422) cells were arrested in S phase with hydroxyurea (200 mM) in YEPD supplemented with 8 mM methionine. When the arrests were complete (after 2 hours), cells were transferred into CSM-methionine medium containing glucose supplemented with hydroxyurea to induce Cdc15-SPB expression. Nud1-3V5, Cdc15-SPB, Clb2, and Kar2 levels were monitored by Western blotting at the indicated time points.
(E) *NUD1-3V5* (A24513) cells were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium. When the arrest was complete (after 150 minutes), cells were released into pheromone free YEPD 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 (black closed squares) and anaphase spindles (red closed circles) was determined at the indicated times. Nud1-3V5 was immunoprecipitated under denaturing conditions and phosphorylation of Nud1 T78, S63, S53, and total Nud1 was monitored by Western blot analysis.
(F) *NUD1-3V5 cdc14-3* (A29851) and *NUD1-3V5 cdc14-3 cdc15-2* (A31661) cells were grown to log phase in YEPD medium at room temperature and then arrested in anaphase in YEPD medium at 37°C for 2 hours. Nud1-3V5 was immunoprecipitated under denaturing conditions and phosphorylation of Nud1 T78, S63, S53, and total Nud1 protein levels were monitored by Western blot analysis.
(G) *NUD1-3V5* (A24513) and *NUD1-3V5 MET3-CDC15-SPB* (A31422) cells were arrested in S phase with hydroxyurea (200 mM) in YEPD supplemented with 8 mM methionine. When the arrest was complete (after 2 hours), cells were transferred into CSM-methionine medium

containing glucose supplemented with the hydroxyurea to induce Cdc15-SPB expression. Nud1-3V5 was immunoprecipitated under denaturing conditions and phosphorylation of Nud1 T78, S63, S53, and total Nud1 protein levels were determined by Western blot analysis. (H) GST, GST-Nud1 (amino acids 1-150), and GST-nud1-S53A,S63A,T78A (amino acids 1-150) were purified from *E. coli*. *cdc14-3 GAL-CDC15-3HA* (WT; A24957) and *cdc14-3 GAL-cdc15(K54L)-3HA* (KD; A30371) cells were arrested in YEPRG at 37°C. Phosphorylated Nud1 (top, phospho-Nud1), immunoprecipitated Cdc15-3HA (second row, Cdc15 IP), and the amount of GST fusion protein substrate (as monitored by Coomassie stain) added to the kinase reaction (bottom, Substrate) are shown.

Expression of *CDC15-SPB* not only recruited Dbf2-Mob1 to SPBs outside of anaphase, it also induced premature Nud1 phosphorylation. When cells were arrested in S phase followed by a brief induction of the *CDC15-SPB* fusion, slower migrating phospho-forms of Nud1 became apparent (Figure 8D). Importantly, the Cdc15-SPB-dependent shift in the S phase arrest was not a result of the cells escaping the cell cycle block as levels of the mitotic cyclin Clb2 remain low at all time points (Figure 8D).

To directly test whether Cdc15 phosphorylated Nud1 *in vivo* we raised antibodies to phosphopeptides encompassing S53, S63 and T78. Each antibody recognized Nud1 in a phospho-specific manner (Figure 9). Importantly, the antibodies recognized wild-type Nud1 only in anaphase cells (Figure 8E), indicating that Nud1 phosphorylation at these sites occurred only during anaphase. To determine whether S53, S63 and T78 phosphorylation depended on *CDC15* we examined phosphorylation of these sites in anaphase with either active (*cdc14-3*) or inactive *CDC15* (*cdc14-3 cdc15-2*). T78 phosphorylation depended on *CDC15* (Figure 8B,F). In contrast, S53 phosphorylation did not depend on *CDC15* function (Figure 8F). S63 phosphorylation was reduced in *cdc14-3 cdc15-2* mutants (Figure 8F), suggesting that *CDC15* is one of several kinases that can phosphorylate this residue *in vivo*. Consistent with this interpretation, we find that premature targeting of Cdc15 to SPBs was sufficient to cause phosphorylation of Nud1 T78 and S63, but not S53 in S-phase arrested cells (Figure 8G). Our data indicate that Cdc15 is the relevant kinase for Nud1 T78 and a relevant kinase for S63 *in vivo*.

Finally, we tested the ability of Cdc15 to directly phosphorylate Nud1 *in vitro*. We purified an N-terminal fragment of Nud1 (amino acids 1-150) as a GST fusion protein and assayed its phosphorylation by either wild-type Cdc15 or kinase-dead Cdc15 immunopurified from yeast

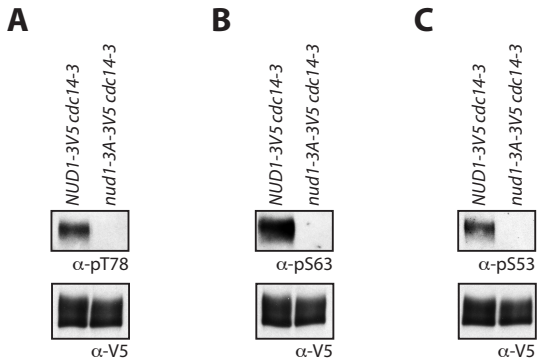

Figure 9: Nud1 phosphoantibodies are phospho-specific

(A-C) *nud1*Δ; *NUD1-3V5 cdc14-3 DBF2-HyA* (A32483) and *nud1*Δ; *nud1-S53A,S63A,T78A-3V5 cdc14-3 DBF2-HyA* (A32507) cells were grown to log phase in SC-Leu medium at room temperature and then arrested in anaphase in SC-Leu medium at 37°C for 2 hours. Nud1-3V5 and nud1-S53A,S63A,T78A-3V5 were immunoprecipitated under denaturing conditions and phosphorylation of Nud1 T78, S63, S53, and total Nud1 were monitored by Western blot analysis.

extracts. Cdc15 but not kinase-dead Cdc15 was able to phosphorylate the Nud1 N-terminal fragment *in vitro* (Figure 8H). This phosphorylation occurred on either S53, S63 or T78 or a combination of the sites as Cdc15 was not able to phosphorylate a GST-Nud1 fusion in which these three sites were mutated to alanine (Figure 8H). We conclude that Cdc15 phosphorylates Nud1 on residues T78 and S63. Our data furthermore show that this activity is restricted to anaphase.

Mob1 is novel class of phosphoserine/threonine binding domains

Cdc15 kinase activity is necessary and sufficient to recruit Dbf2-Mob1 to SPBs by phosphorylating Nud1 on residues S63 and T78. How does Dbf2-Mob1 bind to SPBs? Previous studies showed that Mob1 is not only an activator of Dbf2 kinase activity but also a Dbf2 targeting subunit. Dbf2 cannot localize to SPBs in the absence of Mob1 (Figure 10A). However, Mob1 can localize to SPBs in the absence of *DBF2* (Figure 6B, (Luca et al., 2001)). Furthermore, co-immunoprecipitation experiments indicate that Mob1 and Nud1 form a complex in a *CDC15*-dependent manner (Figure 8B). These observations raise the interesting possibility that Mob1 directly binds Nud1 in a phosphorylation dependent manner. To test this, we assayed the ability of recombinant Mob1 to interact with Nud1 phosphopeptides in a peptide pull-down assay. GST-Mob1 (amino acids 79-314) that contains the conserved Mob1 core but not the extreme N-terminus unique to yeast (Mrkobrada et al., 2006) bound phosphorylated T78 Nud1 peptides (pT78) but not the unphosphorylated control peptides (T78, Figure 10C). We did not observe binding of Mob1 to Nud1 pS53 and pS63 peptides in this assay (Figure 10C). Given the additive phenotype of mutation of Nud1 S53 or S63 with T78 observed *in vivo*, it is likely that Mob1 also interacts with pS53 and/or pS63 peptides, but that this interaction is of insufficient affinity to be seen in this assay. We conclude that Dbf2-Mob1 localization to SPBs is likely mediated by the direct binding of Mob1 to Nud1 phosphopeptides.

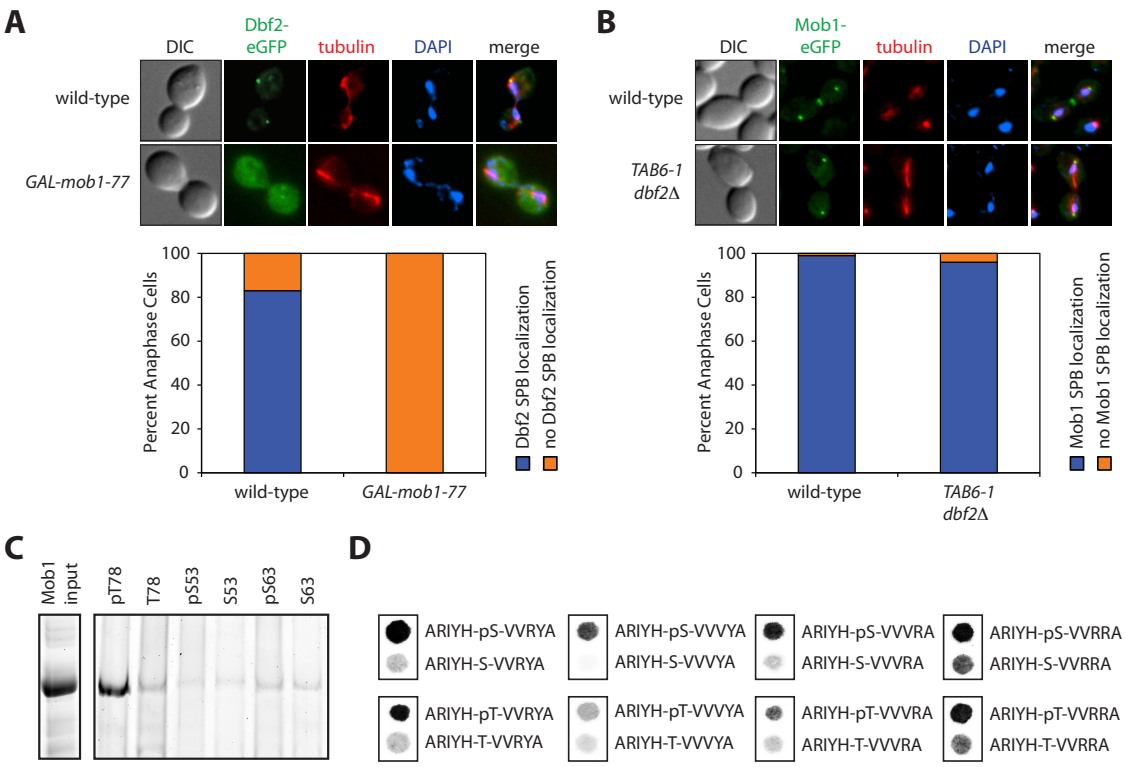

Figure 10: Mob1 is a Phosphopeptide Binding protein that interacts with Phosphorylated Nud1.

(A) *DBF2-eGFP* (A29921) and *DBF2-eGFP GAL-mob1-77* (A32654) cells containing an mCherry-Tub1 fusion protein were grown in YEPRG at room temperature, transferred to YEPD medium for 2 hours at room temperature, followed by incubation at 37°C for 2 hours. Samples were imaged and scored as in Figure 2C. Representative images of anaphase cells are shown.

(B) *MOB1-eGFP* (A24631) and *MOB1-eGFP dbf2Δ TAB6-1* (A32656) cells containing an mCherry-Tub1 fusion protein were grown to log phase in YEPD and imaged and scored as in Figure 2C. Representative images of anaphase cells are shown.

(C) Phosphorylated (pT78,pS53,pS63) or non-phosphorylated (T78,S53,S63) Nud1 peptides were loaded on Streptavidin beads and incubated with recombinant GST-Mob1 (aa79-314). Input and eluates were analyzed by SDS-PAGE followed by Coomassie staining.

(D) Binding of GST-Mob1 (aa79-314) to a filter array of peptide spots. Bound GST-Mob1 (aa79-314) was detected by blotting with α-GST-IRDye800 and scanned with a LiCOR.

To further explore the phosphopeptide binding activity of Mob1, we constructed two synthetic phosphopeptide libraries to determine the optimal sequence for Mob1 binding. These libraries had either a fixed phosphothreonine or phosphoserine flanked by four degenerate residues on each side. GST-Mob1 (aa79-314) was then incubated with the peptide libraries and bound peptides were eluted and sequenced. Mob1 selected for peptides containing specific amino acids N- and C-terminal to the orienting phosphoamino acid (Figure 11A,B). Mob1 displayed a striking preference for Y or F in the -2 position and preferred aliphatic, hydrophobic, or R residues in the +1 to +4 positions. Interestingly, Mob1 also showed a preference for P in the +1 position following a phosphoserine (Figure 11A,B). The Nud1 protein sequence surronding T78 and S63 shows features consistent with these results (Figure 11C). To confirm the results of the peptide library screening, we measured the binding of GST-Mob1 (aa79-314) to individual filter-bound peptides. These peptides are predicted to be optimal ligands based on the peptide library screening. Mob1 bound to the optimal peptides in a phospho-specific manner (Figure 10D). Interestingly, Mob1 appears to prefer phosphoserine over phosphothreonine, at least *in vitro* (Figure 10D). Furthermore, the inclusion of positively charged amino acids C-terminal to the orienting phospho-amino acid led to less phosphospecific binding (Figure 10D), the significance of which is at present unclear.

Mob1 interaction with Nud1 must be dynamic.

In order to promote exit from mitosis, Dbf2-Mob1 activated at SPBs must act on the phosphatase Cdc14, likely in the nucleolus and/or nucleus. This implies that Dbf2-Mob1 must be able to bind Nud1 in a dynamic fashion. While the dynamics of Dbf2-Mob1 association with SPBs have not been measured, the association of the *S. pombe* Dbf2 homologue Sid2 with the SPB was shown to be highly dynamic (Morrell et al., 2004). To directly test whether a dynamic

A

Phospho-Threonine Peptide Motif Selection by Mob1

-4	-3	-2	-1		+1	+2	+3	+4
R (1.8)	X	Y (1.7) F (1.5)	H (1.2)	pT	H (1.2)	V (2.0) I (1.5)	V (1.9) I (1.6) Y (1.3) R (1.2)	V (1.9) I (1.5) Y (1.4) R (1.3)

B

Phospho-Serine Peptide Motif Selection by Mob1

-4	-3	-2	-1		+1	+2	+3	+4
M (2.2) R (1.5)	A (2.5) I (1.2) F (1.2)	G (2.4) F (1.2) Y (1.2)	G (3.0) Y (1.2)	pS	G (2.1) P (1.2)	I (1.3) F (1.2) V (1.2)	I (1.4) V (1.3) R (1.2) Y (1.2)	I (1.4) V (1.3) F (1.2) Y (1.2) R (1.2) L (1.2)

C

Nud1 phosphosites

	-4	-3	-2	-1		+1	+2	+3	+4
pS53	A	I	S	D	pS	V	K	K	P
pS63	S	N	F	T	pS	Q	V	V	E
pT78	N	N	Y	S	pT	V	H	Q	K

Figure 11: Oriented peptide library screening of Mob1

(A,B) Mob1 specificities identified using the phospho-threonine and phospho-serine oriented degenerate peptide libraries. GST-Mob1 (aa79-314) was screened with peptide libraries containing the sequence MAXXXXpTXXXXAKKK or MAXXXXXpSXXXXXAKKK where X indicates all amino acids except Cys. Enrichment values, shown in parentheses, indicate a quantitative measure of the preference of Mob1 for that particular amino acid at that particular position. A value of 1 indicates neither preference nor discrimination. Amino acids are denoted in-single letter code.

(C) Sequence context of Nud1 S53, S63, and T78. Highlighted in yellow are residues that conform to the Mob1 sequence preferences identified in the oriented peptide library screening.

association between Mob1 and Nud1 was a requirement for MEN signaling, we examined the consequences of constitutively targeting Mob1 to SPBs. We fused the open reading frame of *MOB1* to that of *NUD1* via a flexible linker to generate a Mob1-eGFP-Nud1 fusion protein. Expression of the fusion protein from the endogenous *MOB1* promoter is lethal (data not shown) so we placed *MOB1-NUD1* under the control of the low-strength conditional *MET3* promoter. Mob1-Nud1 mirrored Nud1 localization, constitutively localizing to SPBs throughout the cell cycle (Figure 12A). Consistent with the Mob1-Nud1 fusion protein being functional, we found that induction of Mob1-Nud1 resulted in the constitutive localization of Dbf2 to SPBs (Figure 12B). However, despite the proper localization pattern and ability to recruit Dbf2 to SPBs, *MOB1-NUD1* did not complement the temperature sensitive lethality of a *mob1-77* allele (Figure 12C). The *MOB1-NUD1* fusion protein is not a simple loss-of-function allele, however, as the constitutive expression of the *MOB1-NUD1* fusion protein is lethal in otherwise wild-type cells, causing cells to arrest in anaphase with Cdc14 sequestered in the nucleolus (Figure 12D,E). Consistent with a defect in Cdc14 release causing lethality in Mob1-Nud1-expressing cells, we found that the Mob1-Nud1-induced lethality was suppressed by *TAB6-1*^{CDC14}, an allele of *CDC14* that has a reduced affinity for its nucleolar-localized inhibitor (Figure 12F, (Shou and Deshaies, 2002)).

Attempts to determine whether Dbf2-Mob1 was active in *MOB1-NUD1* expressing cells were unsuccessful, presumably because the Mob1-Nud1 protein is tightly bound to the SPB and thus is not amenable to standard Dbf2 IP-kinase assays (data not shown). However, it is interesting to note that the anaphase arrest of *MOB1-NUD1* cells was not suppressed by a constitutively active Dbf2 allele (*DBF2-HyA*), arguing that even constitutively active Dbf2-Mob1, if not able to dissociate from Nud1, cannot promote exit from mitosis (Figure 12G. Together, our data are consistent with the hypothesis that the constitutive association of

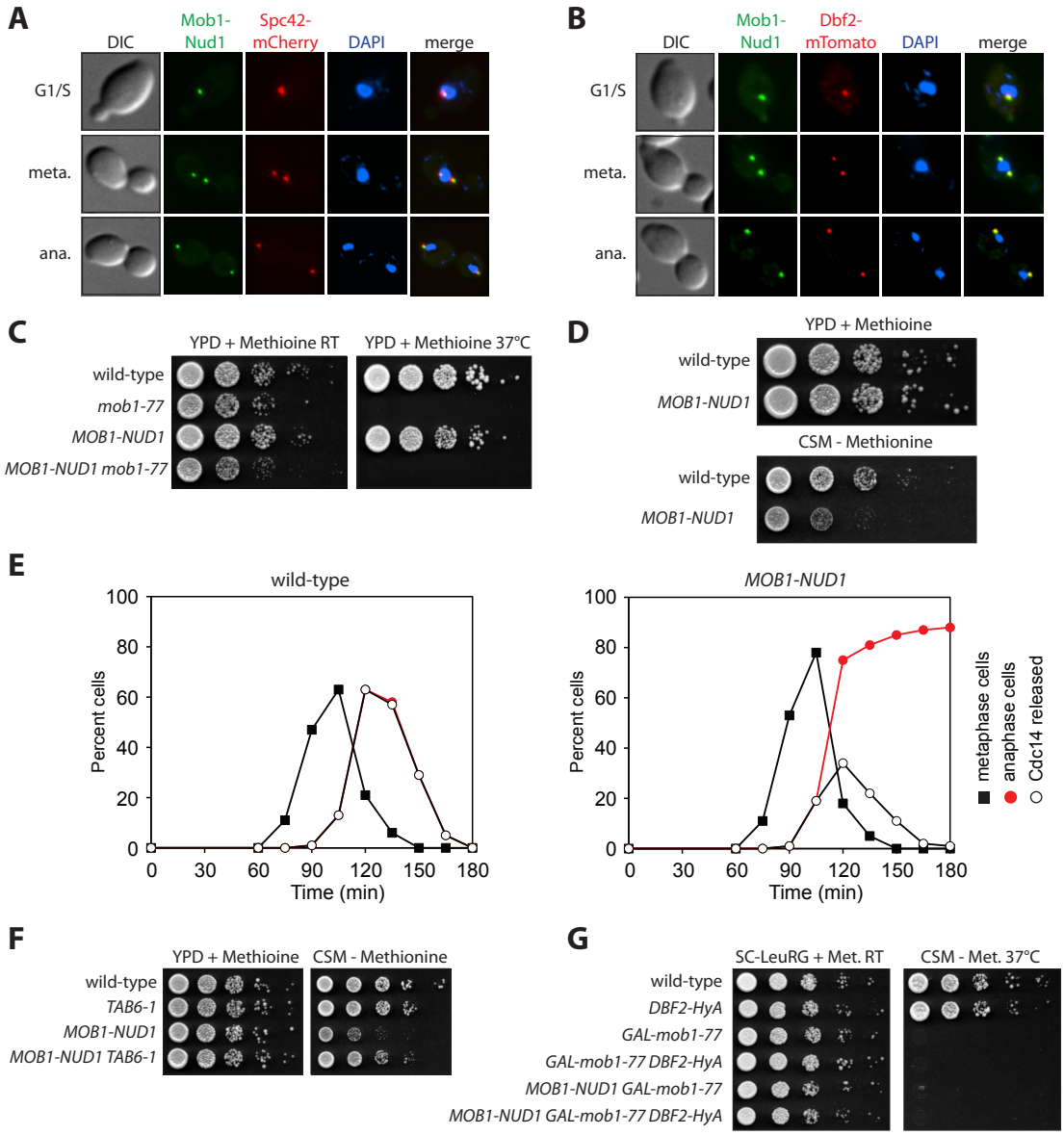

Figure 12: The interaction between Dbf2-Mob1 and Nud1 must be dynamic.

(A) *MET3-MOB1-eGFP-NUD1 SPC42-mCherry* (hereafter referred to as *MOB1-NUD1*; A32624) cells were grown to log phase in YEPD supplemented with 8 mM methioinine. Cells were then shifted to CSM-methionine medium containing glucose for 60 minutes to induce expression of the Mob1-Nud1 fusion and imaged after a brief paraformaldehyde fixation. Representative images of G1/S, metaphase, and anaphase cells are shown.

(B) *MOB1-NUD1 DBF2-tdTomato* (A32797) cells were grown to log phase in YEPD supplemented with 8 mM methioinine. Cells were then shifted to CSM-methionine medium

containing glucose for 60 minutes to induce expression of the Mob1-Nud1 fusion and imaged after a brief paraformaldehyde fixation. Representative images of G1/S, metaphase, and anaphase cells are shown.

(C) Wild-type (A2587), *mob1-77* (A31479), *MOB1-NUD1* (A32336), and *MOB1-NUD1 mob1-77* (A32635) cells were spotted on YEPD plates supplemented with 8 mM methioinine incubated at 30°C or 37°C as in Figure 4A.

(D) Wild-type (A2587) and *MOB1-NUD1* (A32336) cells were spotted on YEPD plates supplemented with 8 mM methionine or CSM-methionine medium containing glucose and incubated at 30°C as in Figure 4A.

(E) Wild-type (A2747) and *MOB1-NUD1* (A32667) cells were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium. One hour prior to release, the cells were shifted to CSM-methionine medium containing glucose supplemented with α-factor pheromone (5 μg/ml) to induce expression of the Mob1-Nud1 fusion. When the arrest was complete (after 170 minutes), cells were released into pheromone free CSM-methionine containing glucose. 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 (black closed squares), anaphase spindles (red closed circles), and 3HA-Cdc14 released from the nucleolus (open circles) was determined at the indicated times.

(F) Wild-type (A2587), *TAB6-1* (A28436), *MOB1-NUD1* (A32336), and *MOB1-NUD1 TAB6-1* (A32661) cells were spotted on YEPD plates supplemented with 8 mM methioinine or CSM-methionine medium containing glucose and incubated at 30°C as in Figure 4A.

(G) Wild-type (A32979), *DBF2-HyA* (A28022), *GAL-mob1-77* (A32986), *GAL-mob1-77 DBF2-HyA* (A32551), *MOB1-NUD1 GAL-mob1-77* (A32992), and *MOB1-NUD1 GAL-mob1-77 DBF2-HyA* (A32881) cells were spotted on SC-Leu plates containing raffinose, galactose, and methionine (SC-LeuRG+Met. RT) or CSM-methionine containing glucose (CSM-Met. 37°C) and incubated at room temperature (SC-LeuRG+Met. RT) or 37°C (CSM-Met. 37°C) as in Figure 4A.

Mob1 with Nud1 prevents Dbf2-Mob1 from promoting the release of Cdc14 from the nucleolus. We conclude that the site of Dbf2-Mob1 activation is almost certainly not the essential site of action and that Dbf2-Mob1 must be able to interact with Nud1 in a dynamic manner.

Mob1 phosphopeptide binding mediates SPB localization, MEN activation, and exit from mitosis.

As the crystal structure of *S. cereivisiae* Mob1 has been solved, we next sought to determine if there are any Mob1 structural elements consistent with Nud1 phosphopeptide binding (Mrkobrada et al., 2006). Examination of the electrostatic surface potential of Mob1 reveals a positively charged pocket that is located on the opposite face to the Dbf2-interacting surface (Figure 13A). This positively charged pocket is conserved in the structures of both *X. laevis* and *H. sapiens* Mob1 proteins (Ponchon et al., 2004; Stavridi et al., 2003). Interestingly, a sulfate ion co-crystallized in the basic pocket of *S. cerevisiae* Mob1 (the sulfate ion derives from ammonium sulfate used in the crystallization experiments, Figure 13A). Since sulfate was not used in the structural determination experiments of *X. laevis* nor *H. sapiens* Mob1, neither of these structures shows a coordinated sulfate ion.

The basic pocket of budding yeast Mob1 is composed of three arginine residues, R253, R254, and R257, that coordinate the sulfate ion (Figure 13B). These basic residues exist in a block of conserved sequence from yeast to human (Figure 13C). The presence of a sulfate ion in a basic pocket has previously been used to predict the location of a phosphopeptide binding domain (Yaffe et al., 1997). Thus, the structural studies are consistent with Mob1 being a phosphopeptide binding domain and suggest that the three basic residues coordinating the sulfate ion, R253, R254, and R257, mediate coordination of a phospho-amino acid. To test

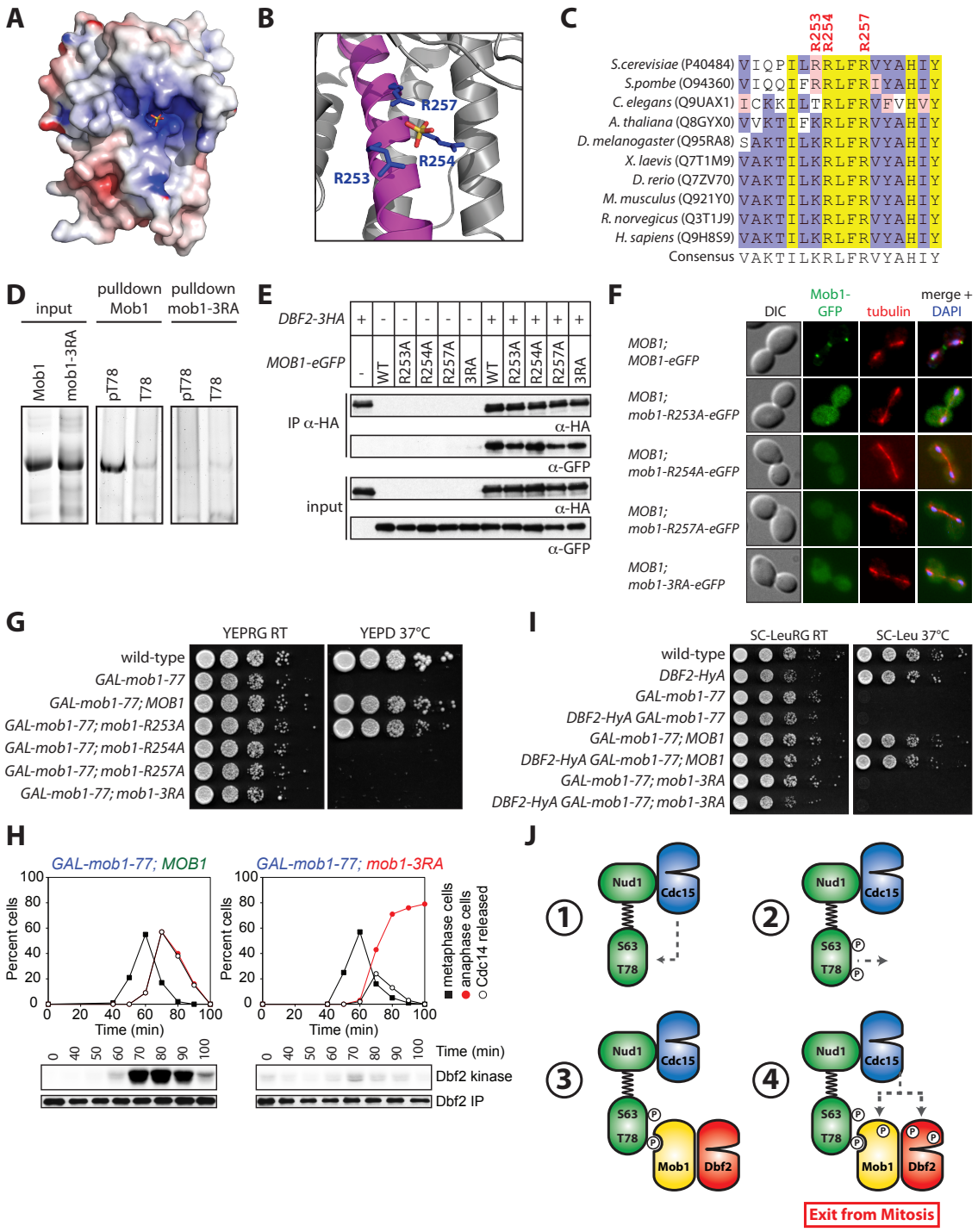

Figure 13: Mob1 binds to phospho Nud1 through a conserved basic pocket.

(A) Surface electrostatic potential between -10 kT (red) and +10 kT (blue) were calculated with APBS using the default parameters. Note the basic pocket harbors a co-crystallized SO_4^{2-} ion.

(B) Zoom of the basic pocket of Mob1, highlighting the coordination of the SO_4^{2-} ion by Mob1 R253, R254, and R257.
(C) Sequence comparison of the residues surrounding *S. cerevisiae* Mob1 R253-R257 with this Mob1 region from other species. Invariant residues are boxed in yellow, conserved blocks are boxed in purple, and similar residues are boxed in pink.
(D) Phosphorylated (pT78) or non-phosphorylated (T78) Nud1 peptides were loaded on Streptavidin beads and incubated with recombinant GST-Mob1 (aa79-314) or GST-mob1-3RA (aa79-314). Inputs and eluates were analyzed by SDS-PAGE followed by Coomassie staining.
(E) *DBF2-3HA* (A3911), *MOB1-eGFP* (A31548), *mob1-R253A-eGFP* (A31728), *mob1-R254A-eGFP* (A31730), *mob1-R257A-eGFP* (A31732), *mob1-R253A,R254A,R257A-eGFP* (*3RA*; A31550), *MOB1-eGFP DBF2-3HA* (A31502), *mob1-R253A-eGFP DBF2-3HA* (A31901), *mob1-R254A-eGFP DBF2-3HA* (A31905), *mob1-R257A-eGFP DBF2-3HA* (A31909), and *mob1-R253A,R254A,R257A-eGFP DBF2-3HA* (A31506) cells were grown to log phase in YEPD. Dbf2-3HA was immunoprecipitated and bound Mob1 (IP α-GFP), bound Dbf2 (IP α-HA), total Mob1 (input α-GFP), and total Dbf2 (input α-HA) was monitored by Western blot analysis.
(F) *MOB1; MOB1-eGFP* (A31500), *MOB1; mob1-R253A-eGFP* (A31893), *MOB1; mob1-R254A-eGFP* (A31895), *MOB1; mob1-R257A-eGFP* (A31897), and *MOB1; mob1-R253,R254A,R257A-eGFP* (*3RA*, A31504) cells containing an mCherry-Tub1 fusion protein were grown to log phase in YEPD and imaged after a brief paraformaldehyde fixation. Representative images of anaphase cells are shown.
(G) Wild-type (A2587), *GAL-mob1-77* (A32452), *GAL-mob1-77; MOB1-eGFP* (A32586), *GAL-mob1-77; mob1-R253A-eGFP* (A32592), *GAL-mob1-77; mob1-R254A-eGFP* (A32595), *GAL-mob1-77; mob1-R257A-eGFP* (A32598), and *GAL-mob1-77; mob1-R253A,R254A,R257A-eGFP* (*3RA*, A32589) cells were spotted on YEPRG or YEPD plates and incubated at room temperature (YEPRG) or 37°C (YEPD) as in Figure 1E.
(H) *MOB1-eGFP; GAL-mob1-77* (A32818) and *mob1-R253A,R254A,R257A-eGFP; GAL-mob1-77* (3RA, A32823) cells containing 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium at room temperature. 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 YEPD 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 (black closed squares), anaphase spindles (red closed circles), Cdc14

released from the nucleolus (open circles), and the amount of Dbf2-associated kinase activity (Dbf2 kinase) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B) was determined at the indicated times.

(I) Wild-type (A32979), *DBF2-HyA* (A28022), *GAL-mob1-77* (A32986), *DBF2-HyA GAL-mob1-77* (A32551), *MOB1-eGFP; GAL-mob1-77* (A32988), *DBF2-HyA MOB1-eGFP; GAL-mob1-77* (A32674), *mob1-R253A,R254A,R257A-eGFP; GAL-mob1-77* (*3RA*, A32990), and *DBF2-HyA mob1-R253A,R254A,R257A-eGFP; GAL-mob1-77* (*3RA*, A32676) cells were spotted on SC-Leu plates containing raffinose and galactose (SC-LeuRG) or SC-Leu plates containing glucose (SC-Leu) and incubated at room temperature (SC-LeuRG) and 37°C (SC-Leu) as in Figure 1E.

(J) Model for the two-step mechanism of Dbf2-Mob1 activation *in vivo*.

whether these three residues are important for Mob1 binding to phospho-Nud1, we first assayed the ability of GST-mob1-R253A,R254A,R257A (aa79-314) to interact with phosphopeptides. Consistent with these Arg residues mediating phosphopeptide binding, we find that whereas GST-Mob1(aa79-314) could interact with Nud1 pT78 peptides, GST-mob1-R253A,R254A,R257A (hereafter referred to as 3RA; aa79-314) could not (Figure 13D). Thus, the basic pocket of Mob1 mediates phosphopeptide binding.

To determine the importance of phosphopeptide binding for Mob1 function *in vivo* we mutated these Arg residues to alanine, either individually or in combination. When introduced into yeast, all the mob1-R>A proteins were expressed at wild-type levels and were able to interact with Dbf2 as judged by co-immunoprecipitation assays (Figure 13E). However, mutation of these residues severely compromised localization of Mob1 to SPBs (Figure 13F). While mutation of R253 alone showed residual Mob1 SPB localization, the mutation of either R254 or R257 abolished Mob1 localization to the SPB (Figure 13F). Consistent with the localization data, these residues were essential for *MOB1* function. The *mob1-R254A* and *mob1-R257A* containing mutant alleles could not complement the temperature sensitive lethality of *GAL-mob1-77* cells grown in glucose-containing medium at 37°C (Figure 13G).

Analysis of the terminal phenotype of *mob1-3RA GAL-mob1-77* cells grown at 37°C in YEPD medium revealed that the *mob1-3RA* allele did not support Dbf2 kinase activity, Cdc14 release from the nucleolus, nor exit from mitosis (Figure 13H). Interestingly, this late anaphase arrest was not complemented by a constitutively active Dbf2 allele (*DBF2-HyA*) (Figure 13I). Thus, even if the defect in Dbf2 activation is corrected in *mob1-3RA* cells, the inability of mob1-3RA to bind phosphopeptides and presumably target Dbf2 to its relevant substrates results in an inability to exit from mitosis. Taken together, our results indicate that the conserved, basic

pocket of Mob1 mediates phosphopeptide binding, a property essential for Dbf2-Mob1 SPB localization, MEN activation, and exit from mitosis. The conservation of these three basic residues in Mob1 homologs from yeast to human suggests that the Mob1 protein family is a novel class of phosphoserine/threonine binding domains.

Discussion

The scaffold Nud1 is essential for MEN signal transduction.

The MEN integrates multiple signals with exit from mitosis. The GTPase Tem1 is regulated by spindle position and anaphase onset; Polo kinase Cdc5 is regulated by the phase of the cell cycle, with kinase activity peaking at the metaphase to anaphase transition (Bardin et al., 2000; Charles et al., 1998; Pereira et al., 2000; Yeh et al., 1995). The kinase Cdc15 integrates the signal inputs from Tem1 and Polo kinase. Here we describe how this signal integration is propagated through the MEN kinase cascade. MEN signaling requires an unusual two-step process (Figure 13J). Rather than directly activating its downstream kinase Dbf2-Mob1, Cdc15 first creates phospho-docking sites on the MEN scaffold Nud1 by phosphorylating S63 and T78. Nud1 phosphorylation at these sites then recruits Dbf2-Mob1 to SPBs, followed by Cdc15-dependent phosphorylation and activation of Dbf2-Mob1.

Our data indicate that Nud1 is not simply a passive platform onto which signaling components assemble, but rather is a dynamic participant in signal transmission. These results serve to highlight the increasing appreciation of scaffold proteins as direct targets of regulation. It is interesting to note that Nud1 Y76 is also phosphorylated (Keck et al., 2011). Given the striking preference of Mob1 for Y or F in the -2 position, it is tempting to speculate that phosphorylation of Nud1 Y76 may prevent Mob1 binding to Nud1 pT78. Y76

phosphorylation, in addition to pT78 dephosphorylation, could provide a novel regulatory mechanism for the precise spatiotemporal control of phosphopeptide binding domains.

Cdc15 phosphorylates Nud1 at S63 and T78, but our data indicate that additional kinase(s) phosphorylate S63 and S53. Phosphorylation of both sites is restricted to anaphase, suggesting that cell cycle regulated kinases are responsible for the phosphorylation of these two sites. Nud1 has been shown to be phosphorylated by Polo kinase Cdc5 and CDKs (Maekawa et al., 2007; Park et al., 2008). Some Polo kinase substrates are only phosphorylated during anaphase (Hu et al., 2001; Meitinger et al., 2011; Shou et al., 2002), raising the possibility that this kinase could contribute to Nud1 phosphorylation at these two critical residues. Phosphatases acting on S53, S63 and T78 could also contribute to restricting phosphorylation of these sites to anaphase. The $PP2A^{Cdc55}$ phosphatase is a negative regulator of the MEN and its activity is downregulated at the metaphase to anaphase transition (Queralt et al., 2006; Wang and Ng, 2006). Downregulation of $PP2A^{Cdc55}$ could contribute to restricting S53, S63 and T78 to anaphase. Indeed, PP2A homologs in fission yeast have been implicated in the dephosphorylation of the *S. pombe NUD1* homolog, *cdc11+* (Krapp et al., 2003; Singh et al., 2012).

Nud1 phosphorylation is essential for astral microtubule anchorage during anaphase.

Our data show that in addition to MEN activation, mitotic Nud1 phosphorylation is also important for astral microtubule anchorage and spindle positioning. Phosphorylation of Nud1 in mitosis is necessary for the proper recruitment of the γ–tubulin complex binding protein Spc72 to the outer plaque of the SPB, which in turn is necessary for the anchoring of astral microtubules to SPBs and proper positioning of the anaphase spindle. It is interesting to note

that Polo kinase plays an essential role in localizing the γ–tubulin complex to centrosomes in higher eukaryotes (Zhang et al., 2009). As Cdc5 phosphorylates Nud1 (Maekawa et al., 2007; Park et al., 2008), Cdc5 may play a conserved role in the phosphorylation of a centrosome/SPB-localized scaffold to promote the recruitment of the γ–tubulin complex.

Mob1 is a novel phosphoserine/threonine binding domain.

Phosphoserine/threonine binding domains nucleate signal transduction events by forming macromolecular complexes with substrates of protein serine/threonine kinases. The known families of phosphoserine/threonine binding domains include 14-3-3 proteins, FHA domains, WW domains, and Polo-box domains (Yaffe and Smerdon, 2004). We show here that *S. cerevisiae* Mob1 is a novel class of phosphoserine/threonine binding domains and that this phoshopeptide binding property is essential for its function. Abrogation of phosphopeptide binding results in an inability of Mob1 to target Dbf2 to SPBs, an inability to activate the MEN, and a terminal arrest in anaphase. Mob1 proteins are conserved across eukaryotes and can be divided into 4 subgroups, Mob1-Mob4 (Vitulo et al., 2007). The basic pocket that mediates phosphopeptide binding is conserved in the Mob1, Mob3, and Mob4 subgroups, indicating that phophopeptide binding may be a conserved feature of these three subfamilies of proteins. Interestingly, the basic pocket has been lost in the Mob2-like subgroup. Thus, we predict that Mob2-like proteins are unlikely to be phosphopeptide binding domains.

The phosphopeptide binding property of Mob1 is likely to be critical not only for binding to Nud1 during Dbf2-Mob1 activation, but may also play an important role in targeting Dbf2-Mob1 to its substrates. Consistent with such a mechanism, it was recently shown that Cdc5 phosphorylates the F-BAR protein Hof1 to facilitate Hof1-Mob1 binding and subsequent Hof1 phosphorylation by Dbf2 (Meitinger et al., 2011). We propose that at least some Dbf2-Mob1

substrates require phosphorylation by a priming kinase in order to be recognized and phosphorylated by Dbf2-Mob1. The need for priming phosphorylation may also explain the puzzling observation that premature activation of Dbf2-Mob1 early in the cell cycle is not sufficient to bring about premature release of Cdc14 from the nucleolus (Rock and Amon, 2011; Visintin and Amon, 2001). Perhaps the Dbf2-Mob1 targets that trigger Cdc14 release from the nucleolus require priming phosphorylation to recruit Dbf2-Mob1, and such priming phosphorylation does not occur early in the cell cycle. The combined premature activation of Cdc5 and Dbf2-Mob1 is sufficient for Cdc14 release from the nucleolus in any stage of the cell cycle (Visintin et al., 2008). This observation, together with the findings of Meitinger et al. (2011), is consistent with the idea that Polo kinase Cdc5 is a priming kinase for Dbf2-Mob1. We propose that the reliance on priming phosphorylation establishes an order of dependency in which the priming kinase must function before Dbf2-Mob1. It is interesting to note that Meitinger et al. (2011) found that Dbf2-Mob1 could also phosphorylate Hof1 sites important for Mob1-Hof1 interaction, suggesting that Dbf2-Mob1 may be capable of self-priming some substrates. It will be important to determine if priming phosphorylation is a general mechanism for Dbf2-Mob1 substrate targeting and, if so, the identities of the priming kinases and phosphatases.

Assembly of MEN-like signaling modules in higher eukaryotes

The MEN is conserved across eukaryotes and in metazoans is known as the Hippo tumor-suppressor pathway (Hergovich and Hemmings, 2012; Zhao et al., 2011). The Hippo pathway plays conserved roles in the regulation of cell growth, proliferation, and apoptosis. Mutation in Hippo pathway components have been found to directly contribute to tumorigenesis in model organisms and are found mutated in primary human cancers or cancer cell lines (Hisaoka et al., 2002; Lai et al., 2005; St John et al., 1999; Tapon et al., 2002). In addition to these

tumor suppressive properties, Hippo-like signaling cascades play essential roles in centrosome duplication, cytokinesis, neuronal dendritic tiling, and many other processes (Hergovich et al., 2006; Zhao et al., 2011).

In higher eukaryotes, Cdc15-like kinases are known as Hippo or MST kinases, Dbf2-like kinases are known as LATS or NDR kinases, and Mob1-like coactivators are known as Mats or Mob1. While there is no clear Hippo pathway homolog of Nud1 based on sequence conservation, the proteins Salvador (*D. melanogaster*) and WW45 and RASSF1A (*H. sapiens*) play an analogous scaffolding role. The C-terminus of Nud1 is homologous to Centriolin (*H. sapiens*), a centriole-localized scaffold important for cytokinesis and cell cycle progression (Gromley et al., 2003). Based on the high degree of conservation between MEN and Hippo pathway components, it is tempting to speculate that Hippo signaling complexes may be assembled in manner analogous to that of the MEN. Indeed, just as in budding yeast, Hippo-like kinases phosphorylate their scaffolding proteins (Callus et al., 2006; Pantalacci et al., 2003; Vichalkovski et al., 2008; Wu et al., 2003). Furthermore, just as Cdc15-dependent phosphorylation of Nud1 is essential for MEN activation, Mst2-dependent phosphorylation of WW45 is essential for Hippo-pathway induced cell death (Park and Lee, 2011). Finally, Mob1 coactivators and scaffolding proteins are necessary for the assembly of Hippo signaling complexes (Guo et al., 2007; Hirabayashi et al., 2008; Vichalkovski et al., 2008).

Phospho-dependent recruitment of LATS/NDR kinases to scaffolds has thus far not been reported in higher eukaryotes. LATS/NDR kinases have previously been shown to be recruited to their scaffold proteins via PPXY-WW domain mediated interactions (Tapon et al., 2002). However, mutation of these PPXY motifs in human LATS1 does not interfere with Hippo pathway function (Chan et al., 2005). Thus, additional modes of interaction may exist. We

propose that MST kinases may phosphorylate their scaffolding proteins to recruit and activate LATS/NDR-Mob1 complexes. Furthermore, given the conservation of the Mob1 basic pocket from yeast to humans, it is tempting to speculate human Mob1 may also be a phosphopeptide binding domain and that this phosphopeptide binding property may be critical for the function of Hippo-like signaling pathways.

Logic of signaling through scaffold-assisted kinase cascades.

Cdc15 is sufficient to activate Dbf2-Mob1 *in vitro* (Mah et al., 2001). Activation of Dbf2-like kinases requires the phosphorylation of a conserved threonine in a hydrophobic motif in the C-terminus of the protein (Hergovich, 2011). In addition, phosphorylation of the Mob1 activating subunit by Cdc15-like kinases facilitates the formation of LATS-Mob1 complexes in humans and likely other organisms (Hirabayashi et al., 2008).

If Cdc15 can directly activate Dbf2-Mob1, why do cells employ a two-step mechanism for Dbf2-Mob1 activation? Why must Cdc15 first localize to SPBs, phosphorylate the scaffold Nud1 to recruit Dbf2-Mob1, and only then activate the terminal MEN kinase?

We propose that this two-step scaffold-assisted mechanism of activating downstream kinases affords many advantages. First, such a mechanism allows for greater regulatory power over pathway output. Docking site phosphorylation depends on the balance of kinase and phosphatase activity, which can be tuned to meet the needs of the cell. Second, this two-step mechanism mitigates the so called "biphasic effect" in which scaffold proteins can exhibit concentration-dependent titration effects (Levchenko et al., 2000). Increasing concentrations of scaffold proteins initially favor increased interaction of partner proteins but, at higher concentrations, will titrate partner proteins into separate complexes. The dependency of terminal kinase localization on scaffold phosphorylation ensures that terminal kinases engage

in complexes in which their upstream kinases are bound and active. Perhaps most importantly, we propose that this two-step mechanism of activating downstream kinases allows for signal transmission and substrate phosphorylation to occur at distinct sites in the cell. The localization of the MEN components to SPBs allows for the spatial and temporal coordination of MEN signaling with the completion of chromosome segregation. The translocation of the daughter bound SPB into the bud simultaneously delivers the daughter-bound genome and activates the MEN. However, while Dbf2-Mob1 is activated at SPBs by Cdc15, it must phosphorylate Cdc14 in the nucleolus and/or nucleus. Thus, Dbf2-Mob1 must associate with Nud1 in a dynamic fashion. Dynamicity could be achieved by Cdc15 first generating a high-affinity SPB docking site for Dbf2-Mob1. Phosphorylation of Dbf2-Mob1 by Cdc15 could then, in addition to activating the kinase, decrease SPB affinity, thereby promoting the dissociation of Dbf2-Mob1 from SPBs. The dynamic association of Dbf2-Mob1 with SPBs could also provide a means of signal amplification. Dbf2 and Mob1 are present at 15 – 21 fold higher concentrations *in vivo* than their activator Cdc15 (Ghaemmaghami et al., 2003). Thus, the dynamic association of Dbf2-Mob1 with Nud1 allows few molecules of Cdc15 to activate many molecules of Dbf2-Mob1.

Evolution of signaling pathways

This two-step mode of kinase activation- phosphorylation-dependent recruitment of the downstream kinase to a scaffold protein followed by activation- provides an ideal substrate for the evolution of novel regulatory mechanisms in signaling pathways. For example, signal integration of two distinct upstream kinases could be achieved by having one kinase perform the priming phosphorylation of the scaffold while the other kinase phosphorylates and activates the downstream kinase. Thus, activation of the downstream kinase would depend on the coincident action of both upstream kinases. Alternatively, positive feedback could be

generated if the downstream kinase itself phosphorylates the scaffold protein. Initial priming phosphorylation of the scaffold by the downstream kinase would lead to additional downstream kinase recruitment, additional scaffold phosphorylation, and rapid pathway activation.

In summary, we have uncovered a distinct mechanism of scaffold-assisted kinase cascade activation. Here, an upstream kinase directly regulates the dynamic association of the downstream kinase with the scaffold protein. This dynamic association is mediated by a novel phosphopeptide binding domain, which in turn directs the activation of its effector kinase in trans. We propose that two-step activation of terminal kinases may be a general feature of scaffold-assisted signaling cascades. Further, we posit that this mechanism provides a potentially general means to organize signaling pathways in which the site of pathway activation differs from the essential site of action.

Experimental Procedures

Yeast Strains and Growth Conditions

All strains are derivatives of W303 (A2587) and are listed in Table S2. Growth conditions are described in the figure legends.

Plasmid Construction

All plasmids used in this study are listed in Table S3.

Immunoblot Analysis

Immunoblot analysis of Nud1-3V5, 3myc-Dbf2, Cdc15-eGFP-Cnm67, Clb2, Kar2, Mob1-6HA, and Cdc15-3HA were performed as in (Rock and Amon, 2011). Nud1-3V5 was detected using an anti-V5 antibody (Invitrogen) at a 1:2000 dilution. Cdc15-3HA was detected using an anti-HA antibody (HA.11, Covance) at a 1:2000 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 (Rock and Amon, 2011).

Fluorescence Microscopy

Indirect *in situ* immunofluorescence methods to detect Tub1 were performed as previously described (Kilmartin and Adams, 1984). V5 tagged proteins were detected with anti-V5 antibody (Invitrogen) at a 1:1000 dilution. HA tagged proteins were detected with anti-HA antibody (Covance) at a 1:150 dilution. Imaging of Bfa1-GFP, Tem1-GFP, Cdc15-eGFP, Cdc15-eGFP-Cnm67, Dbf2-eGFP, Dbf2-tdTomato, Mob1-eGFP, Mob1-mCherry, Mob1-eGFP-Cnm67,

GFP-Tub1, mCherry-Tub1, Spc42-mCherry, and Spc72-eGFP cells were performed as described previously (Rock and Amon, 2011).

Live cell imaging

Asynchronous cells were grown overnight in complete synthetic medium and transferred to a microfluidic chamber (CellASIC Corp. Hayward, CA). Cells were imaged using a Zeiss Axio Observer-Z1 with a 100X objective (NA=1.45), equipped with a Hamamatsu ORCA-ER digital camera. 11 Z-stacks (1 micron apart) were acquired and maximally projected. Metamorph software was used for image acquisition and processing.

Peptide pulldown assays

Peptides used in pull-down experiments were N-terminally tagged with a long-chain biotin group (30.5 Å spacer) and C-terminally amidated. The sequences TVLNNYS-[T or pT]-VHQKVPS, FQDSNFT-[S or pS]-QVVEPAI, and VEPAISD-[S or pS]-VKKPPTM were used for the Nud1 T78, S53 and S63 sites, respectively. The peptides (at ~1 mM) were batch loaded on streptavidin beads (Pierce) in 10 mM Tris pH 8, 150 mM NaCl. The beads were washed twice with 9 mM Tris pH 8, 135 mM NaCl, 10% DMSO and three times with 10 mM Tris pH 8, 150 mM NaCl. Recombinant MOB1 constructs (residues 79-314) were overexpressed as His_6-GST-tagged proteins from *Escherichia coli*, and batch purified on NiNTA beads (Qiagen). Purified proteins were batch loaded on streptavidin beads (preloaded with peptides) at ~0.4 µg/µL in 10 mM Tris pH 8, 150 mM NaCl, 2 mM DTT. The beads were subsequently washed three times with 10 mM Tris pH 8, 150 mM NaCl, 2 mM DTT. Bound proteins were then eluted by heating the beads in 6X sample loading buffer at 95°C for 3 min 33s and analyzed by 12% SDS PAGE. Peptide library screening was performed as in (Yaffe et al., 1997).

Peptide library screening

Peptide library screening was performed as in (Durocher et al., 2000).

Table S1. Nud1 phosphosites and *nud1* phosphomutant alleles

SPB protein	Amino Acid	Asynchronous	Mitotic	Covered	G1	Covered	*nud1-42A*	*nud1-39A*	*nud1-27A*	*nud1-3A*
Nud1	S42	S42	S42	+	S42	+	A	A	S	S
	S49		S49	+		+	A	A	A	S
	T52		T52	+	T52	+	A	A	T	T
	S53		S53	+		+	A	S	A	A
	S61		S61	+	S61	+	A	A	S	S
	S63		S63	+		+	A	S	A	A
	T71		T71	+		-	A	A	A	T
	Y76	Y76		+		-	Y	Y	Y	Y
	S77	?	S77	+		-	A	A	A	S
	T78	?	T78	+		-	A	T	A	A
	S85			+	S85	+	S	S	S	S
	S88	S88		+		+	S	S	S	S
	S94		S94	+		+	A	A	A	S
	S109	S109	S109	+		+	A	A	A	S
	S133		S133	+		+	A	A	A	S
	S140	S140	S140	+	S140	+	A	A	A	S
	T145		T145	+		+	A	A	A	T
	T147	T147		+		+	T	T	T	T
	T169		T169	+		+	A	A	A	T
	S187		S187	+		-	A	A	A	S
	S189	S189	S189	+		-	A	A	A	S
	S192	S192	S192	+		-	A	A	A	S
	S194	S194		+		-	A	A	A	S
	S219		S219	+		-	S	S	S	S
	S222	S222	S222	+		-	A	A	A	S
	S240	S240	S240	+		+	A	A	A	S
	T259		T259	+		-	A	A	A	T
	S262	S262		+		-	S	S	S	S
	S267		S267	+		-	A	A	A	S
	S385	S385	S385	+	S385	+	A	A	S	S
	T388	T388	T388	+	T388	+	A	A	T	T
	T392	T392	T392	+	T392	+	A	A	T	T
	T400		T400	+		+	A	A	A	T
	T415	T415		+		+	T	T	T	T
	S417	S417		+		+	A	A	A	S
	S419		S419	+		+	A	A	A	S
	T432	T432		+		+	T	T	T	T
	T442	T442	T442	+		+	A	A	A	T
	S443	S443	S443	+	S443	+	A	A	S	S
	S445	S445	S445	+	S445	+	A	A	S	S
	T449	T449	T449	+		+	A	A	T	T
	S450	S450	S450	+	S450	+	A	A	S	S
	T454	T454	T454	+		+	A	A	A	T
	S469	S469	S469	+	S469	+	A	A	S	S
	S471		S471	+		+	A	A	A	S
	S667			+	S667	+	S	S	S	S
	T676	T676		+		+	T	T	T	T
	S681	T681		+		+	T	T	T	T
	T806		T806	+		+	A	A	T	T
	T843		T843	+		+	A	A	T	T
							S467A	S467A	S467A	
Ambiguous Assignment	*Y359 or S363*		Y359 or S363	+		+	T805A	T805A		
Ambiguous Assignment	*S813 or S815*		S813 or S815	+		+	S815A	S815A		
Potential phosphorylation site	*S33*		S33 or T52	+		+				
Potential phosphorylation site	*Y40*		Y40 or T52	+		+				
Potential phosphorylation site	*T93*		T93 or S94	+		+				
Potential phosphorylation site	*S224*	S222 or S224		+		-				
Potential phosphorylation site	*T404*		T392 or T404	+		+				
Potential phosphorylation site	S467	S467 or S469		+		+				
Potential phosphorylation site	*T805*		T805 or T806	+		+				
Possible cell cycle observation	S77	S77 or T78								
Possible cell cycle observation	S77	S77 or T78								

Table 2. Table of Yeast Strains

A2587	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+*
A2747	*MATa, CDC14-3HA, DBF2-3MYC*
A3911	*MATa, DBF2-3HA::HIS3*
A18274	*MATa, kar9::HIS5*
A24513	*MATa, NUD1-3V5::KanMX6*
A24631	*MATa, MOB1-eGFP::KanMx6, ura3:pRS306-mCherry-TUB1:URA3*
A24957	*MATa, cdc14-3, GAL-CDC15-3HA::URA3*
A27463	*MATa, NUD1-3V5::TRP1 (Ylplac204)*
A27931	*MATa, HIS3Mx6::GAL-NUD1-3V5::TRP1 (Ylplac204)*
A27933	*MATa, HIS3Mx6::GAL-nud1-27A-3V5::TRP1 (Ylplac204)*
A28009	*MATa, HIS3Mx6::GAL-NUD1-3V5::TRP1 (Ylplac204)*
A28022	*MATa, Dbf2-HyA (LEU2;pRS315)*
A28436	*MATa, TAB6-1::TRP1*
A28499	*MATa, PMET3-CDC15-eGFP-CNM67::LEU2, Mob1-mCherry::NatMX6*
A28553	*MATa, pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67)*
A28650	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), CDC15-eGFP::KanMX6, pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3*
A29128	*MATa, nud1-42A-3V5::TRP1 (Ylplac204)*
A29239	*MATa, His3MX6::GAL-nud1-42A-3V5::TRP1 (Ylplac204)*
A29248	*MATa, KanMX6::GAL-3HA-nud1-44::TRP1, nud1::HIS5*
A29412	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67)*
A29450	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3, MOB1-eGFP::KanMx6*
A29453	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (Ylplac204), ura3::pRS306-mCherry-TUB1::URA3, MOB1-eGFP::KanMx6*
A29500	*MATa, KanMX6::GAL-3HA-nud1-44::TRP1, nud1::HIS5, nud1-42A-3V5::LEU2 (Ylplac128)*
A29506	*MATa, nud1::KanMX6, nud1-42A-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), CDC15-eGFP::KanMX6, pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3*
A29508	*MATa, nud1::KanMX6, nud1-42A-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3*
A29679	*MATa, nud1::KanMX6, nud1-42A-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3, Tem1-GFP:HisMx6*
A29682	*MATa, nud1::KanMX6, nud1-42A-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3, DBF2-eGFP::His3MX6*
A29685	*MATa, KanMX6::GAL-3HA-nud1-44::TRP1, nud1::HIS5, NUD1-3V5::LEU2 (Ylplac128)*

A29711	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (YIplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3, DBF2-eGFP::His3MX6*
A29722	*MATa, nud1::KanMX6, nud1-42A-3V5::TRP1 (YIplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3, MOB1-eGFP::KanMx6*
A29730	*MATa, nud1::KanMX6, nud1-42A-3V5::TRP1 (YIplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3, BFA1-yEGFP::KanMX*
A29733	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (YIplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3, BFA1-yEGFP::KanMX*
A29851	*MATa, MOB1-3HA::His3MX6, NUD1-3V5::KanMX6, cdc14-3*
A29878	*MATa, NUD1-3V5::LEU2 (YIplac128), nud1::HIS5, KanMX6::GAL-3HA-nud1-44::TRP1, CDC14-3HA, DBF2-3MYC*
A29881	*MATa, nud1-42A-3V5::LEU2 (YIplac128), nud1::HIS5, KanMX6::GAL-3HA-nud1-44::TRP1, CDC14-3HA, DBF2-3MYC*
A29899	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (YIplac204), Dbf2-HyA (LEU2;pRS315), pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pRS306-mCherry-TUB1::URA3, Tem1-GFP:HisMx6*
A29921	*MATa, DBF2-eGFP::His3MX6, ura3::pRS306-mCherry-TUB1::URA3*
A30371	*MATa, GAL-cdc15-K54L::URA3, cdc14-3*
A30611	*MATa, HIS3MX6::GAL-nud1-20A-3V5::TRP1 (YIplac204)*
A30614	*MATa, HIS3MX6::GAL-nud1-16A; NUD1-3V5::TRP1 (YIplac204)*
A30617	*MATa, HIS3MX6::GAL-nud1-12A-3V5::TRP1 (YIplac204)*
A30620	*MATa, HIS3MX6::GAL-nud1-8A; NUD1-3V5::TRP1 (YIplac204)*
A30623	*MATa, HIS3MX6::GAL-nud1-7A-3V5::TRP1 (YIplac204)*
A30649	*MATa, HIS3MX6::GAL-nud1-6A-3V5::TRP1 (YIplac204)*
A30652	*MATa, HIS3MX6::GAL-nud1-4A-3V5::TRP1 (YIplac204)*
A30655	*MATa, HIS3MX6::GAL-nud1-S49A,S53A,S63A-3V5::TRP1 (YIplac204)*
A30913	*MATa, HIS3MX6::GAL-nud1-21A-3V5::TRP1 (YIplac204)*
A30915	*MATa, HIS3MX6::GAL-nud1-24A-3V5::TRP1 (YIplac204)*
A30917	*MATa, HIS3MX6::GAL-nud1-25A-3V5::TRP1 (YIplac204)*
A30925	*MATa, HIS3Mx6::GAL-nud1-T78A-3V5::TRP1 (YIplac204)*
A31089	*MATa, Mob1-mCherry::NatMX6, CDC15-eGFP::KanMX6*
A31169	*MATa, nud1::KanMX6, nud1-S53A,S63A,T78A-3V5::TRP1 (YIplac204), Dbf2-HyA (LEU2;pRS315), ura3::pRS306-mCherry-TUB1::URA3, MOB1-eGFP::KanMx6*
A31176	*MATa, HIS3MX6::GAL-nud1-23A-3V5::TRP1 (YIplac204)*
A31209	*MATa, HIS3Mx6::GAL-nud1-S53A,T78A-3V5::TRP1 (YIplac204)*
A31211	*MATa, HIS3Mx6::GAL-nud1-S63A,T78A-3V5::TRP1 (YIplac204)*
A31215	*MATa, HIS3Mx6::GAL-nud1-S53A,S63A,T78A-3V5::TRP1 (YIplac204)*
A31355	*MATa, cdc15::CDC15-as1 (L99G)-eGFP::KANMX6, Mob1-mCherry::NatMX6*
A31388	*MATa, nud1::KanMX6, nud1-39A-3V5::TRP1 (YIplac204)*
A31422	*MATa, NUD1-3V5::KanMX6, PMET3-CDC15-eGFP-CNM67::LEU2*
A31472	*MATa, ura3::pAFS125-TUB1p-GFPTUB1::URA3, nud1::KanMX6, nud1-39A-3V5::TRP1 (YIplac204)*
A31477	*MATa, MOB1-eGFP::KanMx6, ura3:pRS306-mCherry-TUB1:URA3, nud1::KanMX6, nud1-39A-3V5::TRP1 (YIplac204)*
A31479	*MATa, mob1-77*

A31500	*MATa, MOB1-eGFP (Ylplac204), ura3::pRS306-mCherry-TUB1::URA3*
A31502	*MATa, MOB1-eGFP (Ylplac204), DBF2-3HA::HIS3*
A31504	*MATa, mob1-R253A,R254A,R257A-eGFP (Ylplac204), ura3::pRS306-mCherry-TUB1::URA3*
A31506	*MATa, mob1-R253A,R254A,R257A-eGFP (Ylplac204), DBF2-3HA::HIS3*
A31548	*MATa, MOB1-eGFP (Ylplac204)*
A31550	*MATa, mob1-R253A,R254A,R257A-eGFP (Ylplac204)*
A31602	*MATalpha, MOB1-6HA::His3MX6, NUD1-3V5::KanMX6, cdc14-3*
A31661	*MATa, NUD1-3V5::KanMX6, MOB1-6HA::His3MX6, cdc14-3, cdc15-2*
A31728	*MATa, mob1-R253A-eGFP (Ylplac204)*
A31730	*MATa, mob1-R254A-eGFP (Ylplac204)*
A31732	*MATa, mob1-R257A-eGFP (Ylplac204)*
A31893	*MATa, mob1-R253A-eGFP (Ylplac204), ura3::pRS306-mCherry-TUB1::URA3*
A31895	*MATa, mob1-R254A-eGFP (Ylplac204), ura3::pRS306-mCherry-TUB1::URA3*
A31897	*MATa, mob1-R257A-eGFP (Ylplac204), ura3::pRS306-mCherry-TUB1::URA3*
A31901	*MATa, mob1-R253A-eGFP (Ylplac204), DBF2-3HA::HIS3*
A31905	*MATa, mob1-R254A-eGFP (Ylplac204), DBF2-3HA::HIS3*
A31909	*MATa, mob1-R257A-eGFP (Ylplac204), DBF2-3HA::HIS3*
A31966	*MATa, nud1::KanMX6, nud1-39A-3V5::TRP1 (Ylplac204), kar9::HIS5*
A32241	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (Ylplac204), ura3::pAFS125-TUB1p-GFPTUB1::URA3*
A32292	*MATa, nud1-S53A,S63A,T78A-3V5::LEU2 (Ylplac128), KanMX6::GAL-3HA-nud1-44::TRP1, nud1::HIS5*
A32295	*MATa, nud1-39A-3V5::LEU2 (Ylplac128), KanMX6::GAL-3HA-nud1-44::TRP1, nud1::HIS5*
A32336	*MATa, PMET3-MOB1-eGFP-NUD1::URA3*
A32452	*MATa, KANMX6::GAL-mob1-77*
A32483	*MATa, nud1::KanMX6, NUD1-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), cdc14-3*
A32507	*MATa, nud1::KanMX6, nud1-S53A,S63A,T78A-3V5::TRP1 (Ylplac204), Dbf2-HyA (LEU2;pRS315), cdc14-3*
A32551	*MATa, KANMX6::GAL-mob1-77, Dbf2-HyA (LEU2;pRS315)*
A32586	*MATa, KANMX6::GAL-mob1-77, MOB1-eGFP (Ylplac204)*
A32589	*MATa, KANMX6::GAL-mob1-77, mob1-R253A,R254A,R257A-eGFP (Ylplac204)*
A32592	*MATa, KANMX6::GAL-mob1-77, mob1-R253A-eGFP (Ylplac204)*
A32595	*MATa, KANMX6::GAL-mob1-77, mob1-R254A-eGFP (Ylplac204)*
A32598	*MATa, KANMX6::GAL-mob1-77, mob1-R257A-eGFP (Ylplac204)*
A32624	*MATa, PMET3-MOB1-eGFP-NUD1::URA3, SPC42-mCherry:KanMx6*
A32635	*MATa, PMET3-MOB1-eGFP-NUD1::URA3, mob1-77*
A32654	*MATa, DBF2-eGFP::His3MX6, ura3::pRS306-mCherry-TUB1::URA3, KANMX6::GAL-mob1-77*
A32661	*MATa, PMET3-MOB1-eGFP-CNM67::URA3, TAB6-1::TRP1*
A32667	*MATa, PMET3-MOB1-eGFP-NUD1::URA3, CDC14-3HA, DBF2-3MYC*
A32674	*MATa, KANMX6::GAL-mob1-77, MOB1-eGFP (Ylplac204), Dbf2-HyA (pRS315)*
A32676	*MATa, KANMX6::GAL-mob1-77, mob1-R253A,R254A,R257A-eGFP (Ylplac204), Dbf2-HyA (pRS315)*

A32715	*MATa, PMET3-MOB1-eGFP-NUD1::URA3, Dbf2-HyA (LEU2;pRS315)*
A32730	*MATa, pRS306-pCTS1-2xmCherry-SV40NLS::URA3 (integrated at CTS1), ura3::pAFS125-TUB1p-GFPTUB1::URA3 (integrated at URA3), nud1::KanMX6, nud1-39A-3V5::TRP1 (Ylplac204)*
A32731	*MATa, pRS306-pCTS1-2xmCherry-SV40NLS::URA3 (integrated at CTS1), ura3::pAFS125-TUB1p-GFPTUB1::URA3 (integrated at URA3)*
A32793	*MATa, pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pAFS125-TUB1p-GFPTUB1::URA3, nud1::KanMX6, nud1-39A-3V5::TRP1 (Ylplac204)*
A32796	*MATa, pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), ura3::pAFS125-TUB1p-GFPTUB1::URA3*
A32797	*MATa, Dbf2-tdTomato::KanMX6, PMET3-MOB1-eGFP-NUD1::URA3*
A32818	*MATa, KANMX6::GAL-mob1-77, MOB1-eGFP (Ylplac204), DBF2-3MYC*
A32823	*MATa, KANMX6::GAL-mob1-77, mob1-R253A,R254A,R257A-eGFP (Ylplac204), DBF2-3MYC*
A32881	*MATa, KANMX6::GAL-mob1-77, Dbf2-HyA (LEU2;pRS315), PMET3-MOB1-eGFP-NUD1::URA3*
A32888	*MATalpha, pSPC72-SPC72(1-276)-NotI-CNM67::URA3 (integrated at CNM67), nud1::KanMX6, nud1-39A-3V5::TRP1 (Ylplac204), kar9::HIS5*
A32979	*MATa, pRS315 (CEN, LEU2)*
A32986	*MATa, KANMX6::GAL-mob1-77, pRS315 (CEN, LEU2)*
A32988	*MATa, KANMX6::GAL-mob1-77, MOB1-eGFP (Ylplac204), pRS315 (CEN, LEU2)*
A32990	*MATa, KANMX6::GAL-mob1-77, mob1-R253A,R254A,R257A-eGFP (Ylplac204), pRS315 (CEN, LEU2)*
A32992	*MATa, PMET3-MOB1-eGFP-NUD1::URA3, KANMX6::GAL-mob1-77, pRS315 (CEN, LEU2)*

Table 3. Table of Plasmids

pA1358	pRS306-*GFP-TUB1*
pA1420	pRS306-*CTS1-2xmCherry-SV40NLS*
pA1469	pRS306-*mCherry-TUB1*
pA1842	pRS315-*Dbf2-HyA*
pA1880	YIplac204-*PMET3-CDC15-eGFP-CNM67*
pA1921	YIplac204-*NUD1-3V5*
pA1931	YIplac204-*nud1-27A-3V5*
pA1998	YIplac204-*nud1-42A-3V5*
pA2056	YIplac204-*nud1-T78A-3V5*
pA2086	YIplac204-*nud1-S53A,T78A-3V5*
pA2088	YIplac204-*nud1-S63A,T78A-3V5*
pA2096	YIplac204-*nud1-S53A,S63A,T78A-3V5*
pA2111	pGEX6p1-*GST-NUD1(aa1-150)*
pA2117	YIplac204-*nud1-39A-3V5*
pA2122	pGEX6p1-*GST-nud1-S53A,S63A,T78A (aa1-150)*
pA2124	YIplac204-*MOB1-eGFP*
pA2126	YIplac204-*mob1-R253A,R254A,R257A-eGFP*
pA2165	YIplac204-*mob1-R253A-eGFP*
pA2167	YIplac204-*mob1-R254A-eGFP*
pA2169	YIplac204-*mob1-R257A-eGFP*
pA2186	YIplac128-*nud1-S53A,S63A,T78A-3V5*
pA2192	YIplac211-*MET3-MOB1-eGFP-NUD1*
pA2248	pET28a-*His6-GST-mob1-R253A,R254A,R257A (aa79-314)*

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Chapter IV: Discussion

Key conclusions of this thesis

The Hippo-like kinase Cdc15 integrates spatial (Tem1) and temporal (Cdc5) signals to activate the MEN

The MEN GTPase Tem1 has been assumed to be the central switch in MEN regulation. Our work identifies a novel role for Polo kinase 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 Hippo-like 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.

Cdc15 phosphorylates the scaffold Nud1 to create a docking site for Dbf2-Mob1 at SPBs

To understand the regulatory mechanisms by which MEN activity is coordinated with genome segregation, we must understand how active MEN signaling complexes are assembled at SPBs. We find that phosphorylation of Nud1 in mitosis is essential for MEN activation. Failure to activate the MEN in the absence of Nud1 phosphorylation is the result of a defect in Dbf2-Mob1 SPB recruitment.

Surprisingly, Dbf2-Mob1 SPB recruitment requires only three of the 50 identified Nud1 phosphorylation sites. The MEN kinase Cdc15 is responsible for the phosphorylation of these three Nud1 sites. Furthermore, we present preliminary evidence that the Mob1 protein family is a novel class of phosphoserine/threonine binding domains and show that this phosphopeptide binding property may allow for the phosphorylation-dependent recruitment of Dbf2-Mob1 to SPBs. Thus, Cdc15-dependent phosphorylation of the scaffold Nud1 may create a docking site for the Mob1 coactivator, thereby resulting in the recruitment of Dbf2 to SPBs and firing of the MEN. This work thus defines the order of activation of the MEN *in vivo*. Importantly, the dependency of this Nud1-Cdc15-Dbf2-Mob1 complex on the actions of the GTPase Tem1 and Polo kinase Cdc5 allows the MEN to integrate both spatial (spindle position) and temporal (cell cycle phase) cues with mitotic exit, thereby ensuring that exit from mitosis only occurs after proper genome partitioning.

Unanswered questions and future directions

While the field has made great strides in understanding the regulatory mechanisms by which MEN activity is coordinated with genome segregation, much work remains to be done. Discussed below are key unanswered questions that follow from the work in this thesis.

How is Tem1 activity restricted to anaphase?

The active form of Tem1 is assumed to be Tem1-GTP, although this has never been experimentally verified. There is no known guanine nucleotide exchange factor (GEF) for Tem1. While the MEN activator Lte1 contains putative (GEF) domains, purified Lte1 does not exhibit GEF activity towards Tem1 (Geymonat et al. 2009). Rather, Lte1 is thought to activate the MEN by negatively regulating the MEN inhibitor Kin4 (Bertazzi et al. 2011; Falk et al. 2011). Biochemical evidence suggests that Tem1 is an unusual GTPase in that it has a very high basal rate of GDP exchange. GDP exchange for Tem1 is estimated to be approximately 3.3/min at 37°C (Geymonat et al. 2002). Similar results were reported for the Tem1 homolog in *S. pombe* (Furge et al. 1998). For the sake of comparison, the GDP exchange rate for $p21^{Ras}$ has been reported to be 0.025/min at 37°C (Huang et al. 1990). Thus, it has been proposed that the high GDP dissociation rate of Tem1 obviates the requirement for external GEF activity (Geymonat et al. 2002). However, while no data was presented, it has recently been hypothesized that Nud1 may act as a Tem1 GEF (Hotz et al. 2012).

Our data indicate that MEN activity is regulated by multiple inputs. The dependence of MEN activity on *CDC5* ensures that the MEN can only be activated during 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 (Toyn and Johnston 1994; Visintin and Amon 2001). In *tem1Δ CDC15-UP* cells arrested in metaphase, however, Dbf2-Mob1 is activated (Rock and Amon 2011). Thus, an unknown anaphase event, likely under

the control of APC/C^{Cdc20}, must be responsible for activating Tem1 at anaphase onset or keeping Tem1 inactive in earlier cell cycle stages.

If the biochemical data are to be believed, then the mechanism(s) restricting Tem1 activity to anaphase likely function by keeping Tem1 inactive in earlier stages of the cell cycle. 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. It is interesting to note that deletion of *bub2Δ* results in the activation of the MEN in metaphase (Toyn and Johnston 1994; Visintin and Amon 2001). Thus, inactivation of the Tem1 GAP results in the activation of Tem1 prior to anaphase. These data are consistent with Tem1 activity in metaphase, but Tem1 may in fact be active well before this stage of the cell cycle in *bub2Δ* cells. As Cdc5 is essential for MEN activation in both wild-type and *bub2Δ* cells and Cdc5 activity is only present in mitosis, the restriction of MEN activity to metaphase in *bub2Δ* cells is likely the result of the restriction of Cdc5 activity to mitosis (Cheng et al. 1998; Rock and Amon 2011). Taken as a whole, these data argue that whatever the identity of the alternative pathway that restricts Tem1 activity to anaphase, it most likely functions through the regulation of the Bub2-Bfa1 GAP complex.

What target does Cdc5 phosphorylate to promote Cdc15 SPB localization?

While much remains to be learned about the mechanisms by which Cdc15 localizes to SPBs, we have some understanding of how this association occurs. Tem1 binds

to and recruits Cdc15 to SPBs via a region in Cdc15 immediately adjacent to its kinase domain (Asakawa et al. 2001). It has also been demonstrated that Cdc15 contains an additional domain immediately C-terminal to this Tem1-association domain that mediates Tem1-independent Cdc15 localization to SPBs. This SPB association domain presumable mediates Cdc5-dependent Cdc15 SPB association. Finally, by unknown mechanisms, the C-terminus of Cdc15 negatively regulates Cdc15 SPB localization (Bardin et al. 2003).

The sole essential MEN activating function of the Polo kinase Cdc5 is targeting Cdc15 to SPBs. The molecular mechanism by which this occurs remains unknown and is complicated by the fact that we do not yet know the receptor for Cdc15 at the SPB. The two simplest mechanisms by which Cdc5 might target Cdc15 to SPBs are: (1) Cdc5 phosphorylates Cdc15 which then allows for SPB association; or (2) Cdc5 phosphorylates a SPB resident protein which then recruits Cdc15 to SPBs.

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).

Nud1 is an attractive potential receptor for Cdc15 as it is required for Cdc15 localization to SPBs (Visintin and Amon 2001). Furthermore, Nud1 is a substrate of

Cdc5 both *in vitro* and *in vivo* (Maekawa et al. 2007; Park et al. 2008). However, mutation of all known mitotic Nud1 phosphorylation sites (the *nud1(42A)* allele) did not affect Cdc15 localization to the SPB (J. M. R., manuscript in preparation), thus arguing against Nud1 being the essential Cdc5 target in MEN activation.

An interesting alternative receptor for Cdc15 is the SPB protein Cnm67. Nud1 is recruited to SPBs by way of a direct physical interaction with Cnm67, thus Cnm67 is localized to the correct region of the SPB to participate in MEN signaling (Elliott et al. 1999). Localization of MEN components to SPBs is dependent on Cnm67, although this is likely due a lack of Nud1 SPB localization *cnm67Δ* cells. It has been reported, however, that Cdc15 may be able to localize to SPBs in the absence of Nud1 (Gruneberg et al. 2000). Cnm67 is extensively phosphorylated in mitosis and has numerous sites that match the Cdc5 consensus phosphorylation motif (Keck et al. 2011). Furthermore, deletion of *cnm67Δ* is synthetic lethal with the *tem1-3* temperature sensitive allele (J. M. R., unpublished observations), thus arguing that Cnm67 plays a role in activating the MEN. However, the simplest interpretation of the fact that Cdc5 is essential for MEN activation would be that the Cdc5 substrate would also be essential for MEN activation. As *CNM67* is not essential, a more complicated interpretation of the role for Cnm67 in MEN activation would be necessary.

Dbf2-Mob1 kinase activity is regulated by mechanisms other than Cdc15 SPB association

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 (Rock and Amon 2011). 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. A simple test of this hypothesis would be to perturb CDK activity in a synchronous mitosis in *CDC15-SPB* cells, either by prematurely increasing CDK activity with a *GAL-CLB2* allele or decreasing CDK activity with the *clb1*Δ, *clb2-IV* (temperature sensitive allele) allele combination, and monitoring the effects on Dbf2-Mob1 kinase activity.

What phosphatase(s) disassembles the Nud1-Dbf2-Mob1 complex?

Upon completion of exit from mitosis, the MEN is deactivated and Cdc14 returns to the nucleolus. Failure to return Cdc14 to the nucleolus results in a severe defect in Clb-CDK activation in the following cell cycle (Visintin et al. 1998; Shou et al. 1999; Visintin et al. 1999).

Two primary mechanisms contribute to the inactivation of the MEN: Cdc5-dependent phosphorylation of the Tem1 GAP Bub2-Bfa1 is reversed, potentially by the phosphatase Cdc14, thereby stimulating GAP activity and inactivating Tem1 (Hu et al. 2001; Pereira et al. 2002; Geymonat et al. 2003); and the APC/C bound to its specificity factor Cdh1 targets the Polo kinase Cdc5 for ubiquitin-mediated proteolysis (Visintin et al. 2008).

Not only must the MEN be deactivated after exit from mitosis, the MEN must be re-directed at telophase. In addition to their essential role in exit from mitosis, the MEN also regulates cytokinesis (reviewed in (Meitinger et al. 2012)). The MEN components Cdc14, Cdc15, Dbf2-Mob1, and Cdc5 all localize to the bud neck during telophase. At the bud neck, both Cdc5 and Dbf2-Mob1 phosphorylate the F-BAR protein Hof1, which then promotes actomyosin ring contraction and membrane ingression (Meitinger et al. 2011).

The Nud1-Dbf2-Mob1 complex is assembled as a result of Cdc15-dependent phosphorylation of Nud1. The disassembly of this complex is presumably necessary for both the ultimate inactivation of the MEN as well as relocalization of Dbf2-Mob1 to the bud neck. Interestingly, when expressed from their endogenous promoters, the constitutive targeting of both Cdc15 and Mob1 to SPBs (*CDC15-SPB* and *MOB1-SPB* fusion proteins) is lethal (J. M. R., unpublished observations). It will be important to determine both how this complex is disassembled in telophase as well as the mechanism of lethality in constitutive *CDC15-SPB* and *MOB1-SPB* cells.

As described above, mechanisms exist to inactivate both Cdc5 and Tem1, thereby resulting in the loss of Cdc15 from SPBs and the cessation of Cdc15-dependent Nud1 phosphorylation. What might be the identity of the phosphatase that then dephosphorylates Nud1? It is tempting to speculate that this phosphatase may be Cdc14. Cdc14 localizes to SPBs in anaphase and is thought to assist in the downregulation of the MEN by dephosphorylating and activating the Bub2-Bfa1 GAP complex. Interestingly, localization of Cdc14 to SPBs is largely dependent on Bub2-Bfa1 (Pereira et al. 2002). Furthermore, it was demonstrated that Cdc14 activity is necessary for Dbf2-Mob1 relocalization from SPBs to the bud neck (Frenz et al. 2000; Yoshida and Toh-e 2001). Thus, Bub2-Bfa1-dependent Cdc14 localization to SPBs may then allow Cdc14 to dephosphorylate Nud1, thus contributing to the inactivation of the MEN in late telophase and the relocalization of MEN components to bud necks to accomplish cytokinesis.

What kinase(s) prime Dbf2-Mob1 substrates? What phosphatase(s) remove Dbf2-Mob1 substrate priming phosphorylation?

Our data indicate that Dbf2-Mob1 activity 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 (Rock and Amon 2011). 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. The mechanisms that restrict Dbf2-Mob1-dependent Cdc14 release to anaphase are unknown.

The requirement for priming phosphorylation of Dbf2-Mob1 substrates is a possible explanation for the fact that Dbf2 kinase activity is necessary but potentially not sufficient for phosphorylation of its targets. The basis for this model presumes that the mechanism by which Mob1 interacts with Nud1 is generalizable to the mechanism by which Dbf2-Mob1 interacts with its substrates. By this priming model, phosphorylation of Dbf2-Mob1 substrates by an upstream kinase(s) results in the recruitment of Dbf2-Mob1 by virtue of the putative phosphopeptide binding pocket of Mob1. Priming phosphorylation thus establishes an order of dependency in which Dbf2-Mob1 cannot act on its substrates unless they have been previously phosphorylated by an upstream kinase. Consistent with this notion, disruption of the putative Mob1 phosphopeptide binding pocket abrogates both Mob1 SPB localization as well as localization to Dbf2-Mob1 substrates at the bud neck.

What might be the identity of the priming kinase(s) for Dbf2-Mob1? One might expect that the combined premature activation of a Dbf2-Mob1 priming kinase

with the premature activation of Dbf2-Mob1 would result in the premature phosphorylation of Dfb2-Mob1 substrates. It is interesting to note that overexpression of Cdc5 in combination with the premature activation of the Dbf2-Mob1 is sufficient to drive Cdc14 out of the nucleolus in any cell cycle stage (Manzoni et al. 2010). Furthermore, the inactivation of Cdc5 results in a significant defect in Cdc14 release even in the presence of the premature activation of Dbf2-Mob1 (Visintin et al. 2003). Moreover, it has recently been proposed that Dbf2-Mob1-dependent phosphorylation of the bud neck protein Hof1 is facilitated by prior phosphorylation by Cdc5 (Meitinger et al. 2011). Thus, it is tempting to speculate that a priming kinase for Dbf2-Mob1 substrates is the Polo kinase Cdc5. However, as Cdc5 also acts on its substrates in a priming-phosphorylation dependent manner, this data is also consistent with a model in which Dbf2-Mob1 is in fact a priming kinase for Cdc5 (Elia et al. 2003a; Elia et al. 2003b).

Finally, what might be the identity of the phosphatase(s) that remove priming phosphorylation of Dbf2-Mob1 substrates? One might expect that the combined inactivation of a Dbf2-Mob1 priming phosphatase with the premature activation of Dbf2-Mob1 would result in the premature phosphorylation of Dfb2-Mob1 substrates. In this light, it is interesting to note that deletion of the PP2A targeting subunit *CDC55* results in synthetic lethality with the premature activation of Dbf2-Mob1 (J. M. R. unpublished observations). Thus, it is tempting to speculate that $PP2A^{Cdc55}$ may remove priming phosphorylation events from Dbf2-Mob1 substrates.

Does Nud1 play additional roles in MEN activation?

We have presented data that is consistent with Nud1 playing an essential role in MEN activation by serving as a phosphorylation-dependent docking site for Dbf2-Mob1 at SPBs. Nud1 has also been shown to interact with the GTPase Tem1 and the Tem1 GAP Bub2-Bfa1 and is essential for their localization to the SPB (Gruneberg et al. 2000; Valerio-Santiago and Monje-Casas 2011). Might Nud1 play additional roles in MEN activation? While no data was shown, it was recently proposed that Nud1 may be a Tem1 GEF (Hotz et al. 2012). In light of this, it is interesting to note that a Nud1 point mutant (G585E) has been shown genetically to hyperactivate the MEN (Harper et al. 2008). For unknown reasons, the deletion of the nucleoporin *nup1Δ* is synthetic lethal with the deletion of *bub2Δ* or *bfa1Δ*. The *NUD1(G585E)* allele also exhibited synthetic lethality with a *nup1Δ*. Importantly, Nud1(G585E) did not abolish localization of Bub2-Bfa1 to SPBs (Harper et al. 2008). Thus, these data are consistent with the hypothesis that the *NUD1(G585E)* allele hyperactivates the MEN. It will be very interesting to explore the mechanism by which this allele functions.

The *S. pombe* homolog of *NUD1* is called *CDC11*. Cells harboring the *cdc11-136* temperature sensitive allele fail to activate the SIN. Interestingly, these cells retain the Tem1 homolog Spg1 at SPBs but fail to localize the Cdc15 homolog Cdc7 to SPBs (Krapp et al. 2001). As the mutation in cdc11-136 (N768M) lies well outside the predicted binding site for Cdc7, it is tempting to speculate that the cdc11-136 protein may have a defect in Spg1 activaiton at the restrictive temperature

(Feoktistova et al. 2012). Given that a GDP-specific antibody exists for Spg1, this is a simple hypothesis to test.

Finally, it would be interesting to attempt to generate novel hyperactive Nud1 alleles. Such a screen might be performed by error-prone PCR mutagensis of Nud1 followed by screening for alleles that suppress the synthetic lethality of a *spo12Δ lte1Δ*, *lte1Δ* lethality at 16°C, or some other hypoactive MEN phenotype.

How does phosphorylation of Nud1 contribute to spindle positioning?

Nud1 phosphorylation is essential for MEN activation. Mitotic phosphorylation of Nud1 also appears to play an important role in the positioning of the mitotic spindle. When the defect in Dbf2-Mob1 SPB recruitment is corrected, approximately 30% of *nud1* phosphomutant anaphase cells display a mispositioned anaphase spindle (*nud1(39A)* allele; this allele is the *nud1(42A)* allele in which S53, S63, and T78 are reverted to S, S, and T, respectively; J. M. R., unpublished observations). It is interesting to note that the observed frequency of mispositioned anaphase spindles in *nud1(39A)* cells is very similar to that observed in *cnm67Δ* cells, in which the entire outer plaque does not localize to the SPB (Hoepfner et al. 2000).

The defects in spindle positioning in *nud1(39A)* cells correlates with the appearance of astral microtubules that are no longer connected to the SPB. Astral microtubules are anchored to the cytoplasmic face of the SPB by the γ–tubulin receptor Spc72 (Knop and Schiebel 1998). Spc72 in turn is anchored to the outer plaque of the SPB

by Nud1 (Gruneberg et al. 2000). Given that the constitutive targeting of Spc72 to the SPB (*SPC72-CNM67* fusion allele) ameliorates the phenotype of *nud1(42A)* cells, it seems plausible that the nuclear positioning problem in the absence of Nud1 phosphorylation is a result of a defect in Spc72 SPB localization. Preliminary evidence suggests that *nud1(39A)* cells do not have a striking defect in the recruitment of Spc72 to the SPB. However, this result may be misleading as the half-bridge protein Kar1 can also recruit Spc72 to SPBs, at least in the G1 phase of the cell cycle (Pereira et al. 1999). It is interesting to note that Polo kinase plays an essential role in localizing the γ–tubulin complex to centrosomes in higher eukaryotes (Zhang et al. 2009). Thus, a simple possible model by which Nud1 could promote spindle positioning is that phosphorylation of Nud1 results in the recruitment of Spc72 to the outer plaque of the SPB. Spc72 in turn recruits the γ–tubulin complex which results in astral microtubule nucleation and proper spindle alignment. It will be important to explore the molecular mechanism of Nud1-mediated spindle positioning.

Concluding remarks

The work presented in this thesis has shed light on the mechanisms by which MEN activity is coordinated with genome segregation. This work highlights the importance of the ordered assembly of active signaling complexes, and details how the regulated assembly of these complexes can integrate both spatial and temporal information. While the MEN has been studied extensively for 14 years and many significant findings have been made, it is clear that much remains to be

discovered in our efforts to understand how exit from mitosis is coordinated with the faithful segregation of the genome.

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Appendix I: The FEAR network

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

Summary

Mitosis is governed by the oscillation of cyclin dependent kinase (CDK) activity and ubiquitin-dependent proteolysis. Entry into mitosis is initiated by mitotic cyclin-CDK activation. Anaphase onset occurs upon activation of the Anaphase Promoting Complex/Cyclosome (APC/C), a ubiquitin ligase that promotes the destruction of the anaphase inhibitor Securin. Destruction of Securin initiates chromosome segregation by activation of the protease Separase, allowing it to cleave a subunit of the cohesin complexes that hold the duplicated sister chromatids together. Upon completion of nuclear division cells exit from mitosis, a process defined by the inactivation of CDKs, disassembly of the mitotic spindle, and cytokinesis. In the budding yeast *S. cerevisiae*, a signaling network known as the FEAR network is critical to ensure accurate anaphase chromosome segregation and the integration of this process with other anaphase events. Here, we summarize what is known about the regulation and function of the FEAR network in budding yeast and discuss the potential for conserved FEAR network functions in other eukaryotes.

Control of late mitotic events in budding yeast

In budding yeast, cyclin B-CDK (Clb-CDK) activity drives the entry and progression through mitosis. Inactivation of Clb-CDKs is essential for exit from mitosis. In budding yeast, Clb-CDK inactivation is controlled by the essential phosphatase Cdc14. Cdc14 promotes Clb-CDK downregulation by two primary mechanisms: (1) Cdc14 dephosphorylates the APC/C specificity factor Cdh1 thereby stimulating the APC/C-dependent destruction of Clb cyclins. (2) Cdc14 dephosphorylates the Clb-CDK inhibitor Sic1 and the transcription factor Swi5, resulting in the stabilization of Sic1 and Swi5-dependent activation of *SIC1* transcription. Furthermore, Cdc14 counteracts CDK activity by dephosphorylating a host of CDK substrates, allowing for the rapid resetting of cells to the G1 state. Cdc14 activation in late anaphase is controlled by the Mitotic Exit Network (MEN), a GTPase signaling cascade, and results in exit from mitosis (Box 1).

In addition to the essential role Cdc14 plays in Clb-CDK inactivation at the end of anaphase, there is growing appreciation for the ability of Cdc14 to modulate CDK activity in early anaphase. This important function of Cdc14 is mediated by the FEAR network (Cdc **F**ourteen **E**arly **A**naphase **R**elease network).

Cdc14 regulation

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 (Figure 1). Upon Separase activation and entry into

Box 1. The Mitotic Exit Network

The MEN resembles a Ras-like GTPase signal transduction cascade, with the G protein Tem1 functioning at the top of the pathway (Figure 1). Tem1 is positively regulated by its putative guanine nucleotide exchange factor (GEF) Lte1 and negatively regulated by its two component GTPase activating protein (GAP) Bub2-Bfa1. As a regulator of the MEN, Cdc5 phosphorylates Bfa1 which reduces Bub2-Bfa1 GAP activity. 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. Nud1 is thought to function as a scaffold for the core MEN components Tem1, Bub2-Bfa1, Cdc15, and Dbf2-Mob1 at spindle pole bodies. Activation of Dbf2-Mob1 results, at least in part, in the phosphorylation of the Cdc14 nuclear localization sequence, thereby resulting in the retention of Cdc14 in the cytoplasm where it can act on its substrates. Activation of the MEN in late anaphase is essential for the sustained release of Cdc14 from the nucleolus and results in exit from mitosis.

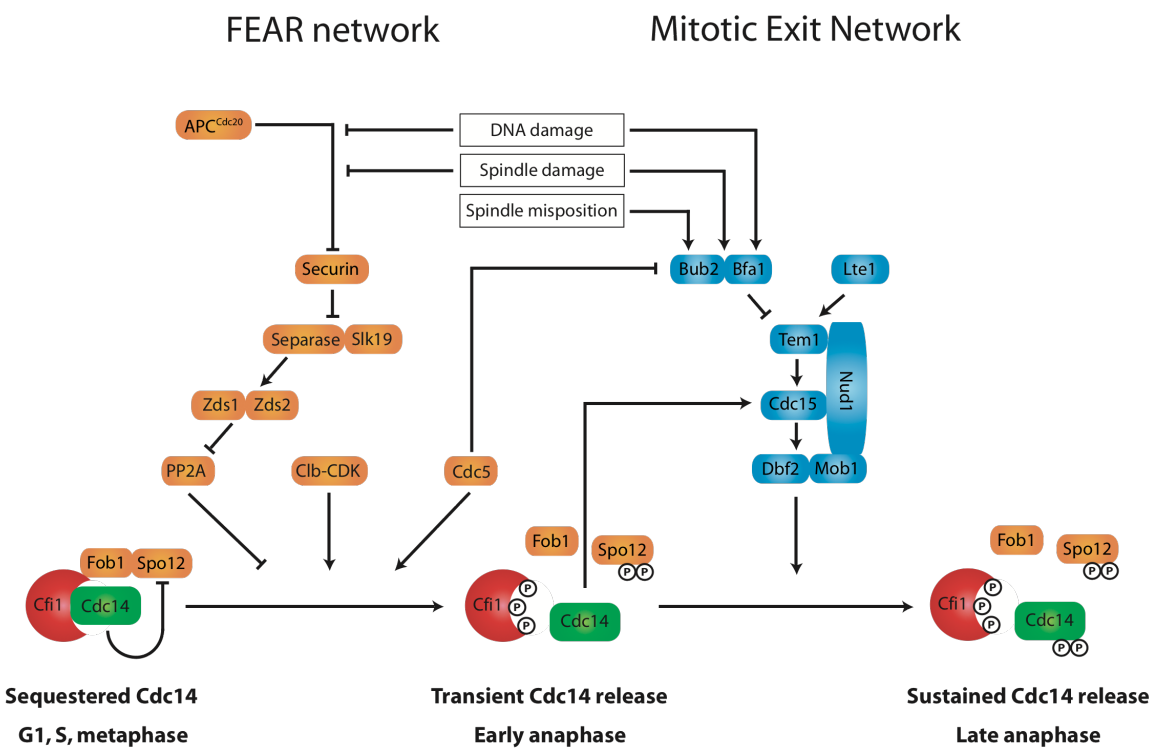

Figure 1. Cdc14 regulation

Cdc14 release from the nucleolus during early anaphase is mediated by the FEAR network and results in a pulse of Cdc14 activity that coordinates multiple anaphase events. Cdc14 release from the nucleolus in late anaphase is mediated by the MEN and results in sustained Cdc14 activity and exit from mitosis.

anaphase, 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 that coordinates many of the anaphase events to be discussed below. FEAR network promoted Cdc14 release from the nucleolus is transient: in the absence of a functional MEN, Cdc14 becomes re-sequestered in the nucleolus during late anaphase and cells fail to exit from mitosis. While not essential, the FEAR network is crucial for the faithful execution of anaphase, as illustrated by the significant loss of viability of cells undergoing anaphase in the absence of FEAR network function. Cdc14 release from the nucleolus during late anaphase is promoted by the MEN (Box 1), which drives the sustained release of Cdc14 in both the nucleus and the cytoplasm and results in exit from mitosis.

The FEAR network

Work over the last eight years has identified a number of proteins that together function to regulate the release of Cdc14 from the nucleolus during early anaphase (Figure 1). These proteins, collectively known as the FEAR network, include Separase, the Separase binding protein Slk19, polo-like kinase Cdc5, Spo12, the replication fork block protein Fob1, PP2A phosphatase bound to its regulatory subunit Cdc55, Zds1 and Zds2, histone modifications such as H2B ubiquitylation and H3 methylation, and CDKs associated with the cyclins Clb1 and Clb2. We do not fully understand how these proteins function together to bring about the release of Cdc14 from the nucleolus but a mechanism is beginning to emerge.

The association between Cdc14 and its nucleolar-localized inhibitor Cfi1/Net1 appears to be regulated by phosphorylation. Prior to anaphase, Cdc14 and Cfi1/Net1 are in an unphosphorylated or hypophosphorylated state. During this stage of the cell cycle, Fob1 stabilizes the Cdc14 - Cfi1/Net1 interaction (Figure 1). At anaphase onset, APC/C activation results in the degradation of the Separase-inhibitor Securin, thereby resulting in the activation of the Separase – Slk19 complex. By a poorly understood mechanism that involves the proteins Zds1 and Zds2, the Separase – Slk19 complex is thought to downregulate $PP2A^{Cdc55}$ phosphatase activity. This downregulation of $PP2A^{Cdc55}$ phosphatase activity allows Clb1-CDK and Clb2-CDK to phosphorylate Cfi1/Net1. Clb1-CDK and Clb2-CDK are also responsible for the phosphorylation of Spo12. It appears, however, that it is Cdc14, rather than PP2A, that restrains Spo12 phosphorylation in cell cycle stages prior to anaphase.

Cdc5 is essential for the release of Cdc14 from the nucleolus. The mechanism by which this polo-like kinase functions in the FEAR network is not yet understood and is clouded by the fact that Cdc5 acts positively in both the FEAR network and the MEN. Epistasis analysis places Cdc5 downstream of and/or in parallel to Separase - Slk19. Cdc5 promotes the phosphorylation of Cdc14 and Cfi1/Net1 *in vivo*, which is at least in part due to Cdc5 dependent degradation of the CDK inhibitory kinase Swe1. Furthermore, Cdc5 can dissociate the Cdc14 – Cfi1/Net1 complex *in vitro*. Ultimately, phosphorylation of Cfi1/Net1 is thought to decrease its

affinity for Cdc14 while phosphorylation of Spo12 is thought to promote, in part, the dissociation of Fob1 from the Cdc14 - Cfi1/Net1 complex.

The FEAR network harbors two regulators of chromosome segregation, Separase and Cdc5. This allows for the coordination of FEAR network activation and chromosome segregation. Activation of Separase at the metaphase – anaphase transition both initiates chromosome segregation and FEAR network activation, thereby ensuring that Cdc14 activation does not occur prior to genome partitioning. Importantly, the essential nature of Separase in FEAR network activation also ensures that FEAR network activation is responsive to insults such as mitotic spindle damage and DNA damage, both of which stabilize Securin.

FEAR network functions

Although not essential for progression through mitosis, Cdc14 released by the FEAR network regulates multiple aspects of chromosome segregation. Interestingly, during early anaphase Cdc14 dephosphorylates its targets when overall Clb-CDK activity is high, raising the possibility that local high concentrations of active Cdc14 or locally repressed Clb-CDK activity is critical to regulate and coordinate anaphase events.

FEAR network promoted Cdc14 activation ensures that the segregation of all chromosomes is initiated simultaneously, it is required for the efficient segregation of the rDNA, positioning of the anaphase nucleus, proper regulation of mitotic spindle dynamics, localization of spindle proteins, and initiation of the MEN. In a

sense, the FEAR network can be viewed as the “fine tuner of anaphase events,” bringing accuracy to the system (Figure 2).

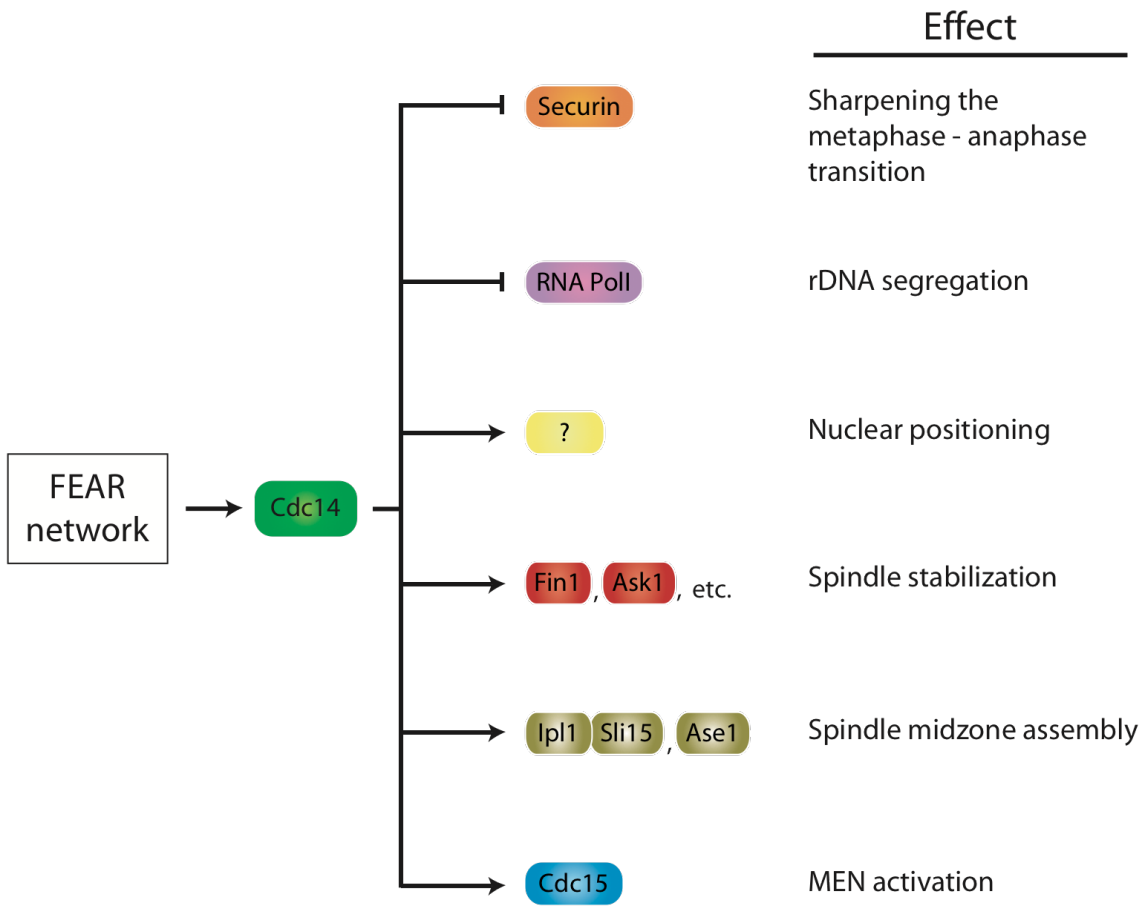

Figure 2. FEAR network functions

Cdc14 released by the FEAR network coordinates multiple mitotic events.

Sharpening the metaphase – anaphase transition

The onset of anaphase is marked by the rapid loss of sister chromatid cohesion, resulting in the synchronous segregation of sister chromatids. This rapid loss in sister chromatid cohesion is driven, in large part, by the activation of Separase, which then cleaves a subunit of the cohesin complex. Separase activation at the metaphase-anaphase transition occurs as a result of APC/C dependent destruction of Securin. The mechanisms by which the switch-like activation of Separase is made are beginning to be understood. Securin is phosphorylated by Clb-CDKs and two of these phosphorylation events decrease the efficiency of Securin ubiquitination by the APC/C, at least *in vitro*. Cdc14 dephosphorylates these CDK marks on Securin, thus suggesting the presence of a positive feedback loop. The initial degradation of Securin activates the Separase – Slk19 complex, which then promotes the early anaphase release of Cdc14. Cdc14 in turn dephosphorylates Securin, resulting in enhanced APC/C dependent Securin degradation (Figure 2). When this positive feedback loop is disrupted, anaphase chromosome segregation is less abrupt and occurs with a significantly increased error rate.

Segregation of the rDNA

Activation of Separase at the metaphase-anaphase transition results in cohesin cleavage and initiates chromosome segregation. While cleavage of the cohesin complexes is sufficient for segregation of most parts of the genome, the rDNA repeats, which encode the rRNA and that nucleate the nucleolus, are late to

segregate during anaphase and are linked through additional cohesin independent mechanisms. The physical nature of these cohesin independent chromosome linkages remains a mystery. What is known, however, is that the resolution of these linkages is dependent on Cdc14 activated by the FEAR network. Inactivation of either results in the defective segregation of the rDNA but not other parts of the genome. Activation of Cdc14 in early anaphase results in the silencing of transcription of rDNA by RNA PolI and the exclusion of RNA PolI subunits from the nucleolus (Figure 2). This anaphase-specific silencing of rDNA transcription is thought to allow for the recruitment of condensins, cohesin-like complexes that mediate the condensation of chromosomes, to the rDNA and facilitate both the compaction and segregation of this genomic region.

Nuclear positioning

In budding yeast, the nucleus is positioned at the bud neck during metaphase. At the onset of anaphase, microtubule motors associated with the cytoplasmic microtubules emanating from the daughter bound spindle pole body (SPB; the yeast equivalent of the centrosome) pull the nucleus into the bud. Concomitantly, microtubule motor proteins associated with the mitotic spindle push the two SPBs apart. The end result of anaphase-specific microtubule associated forces is the equal distribution of the genetic material between the mother and daughter cells.

In cells lacking Separase function the cohesin complexes are not cleaved and sister chromatids fail to segregate. Interestingly, in the majority of these Separase

deficient cells the undivided nucleus is pulled into the bud, the future daughter cell. The reason for this inheritance pattern is that cells that lack Separase function, while proficient in generating a daughter directed cytoplasmic microtubule pulling force, fail to generate or maintain a mother directed cytoplasmic microtubule pulling force (Figure 2). This asymmetric force pattern, coupled with the inability to cleave cohesin complexes and thus properly elongate the mitotic spindle, results in the undivided nucleus being pulled into the daughter cell. While the mechanism by which this mother directed pulling force is generated remains unknown, its activation is dependent on FEAR network components and Cdc14. Thus, during a normal cell cycle, Cdc14 released by the FEAR network directs the establishment or maintenance of a mother directed pulling force that, together with the daughter directed pulling force and elongating spindle, faithfully segregates the genetic material.

Spindle dynamics, stability, and midzone assembly

Microtubules, the basic components of the microtubule cytoskeleton, are highly dynamic structures. Microtubule dynamics increases as cells enter mitosis. This increase is brought about by mitotic CDK activity and is thought to promote both the formation of the mitotic spindle and the attachment of chromosomes to the assembling spindle. At the onset of anaphase, microtubule dynamics decreases dramatically and results in the stabilization of the anaphase spindle and chromosome segregation. This is the result, at least in part, of the activation of

Cdc14 by the FEAR network (Figure 2). Cdc14 dephosphorylates a number of microtubule binding proteins, e.g. Ask1 and Fin1, which then allows these proteins to interact with and stabilize the elongating spindle.

In addition to regulating microtubule dynamics, FEAR network mediated Cdc14 activation also controls the assembly of the spindle midzone. The spindle midzone is the site of overlap between the interpolar microtubules. Numerous proteins are recruited to this site to stabilize this fragile region of the elongating anaphase spindle. The spindle midzone also plays an important role in cytokinesis, particularly in higher eukaryotes. FEAR network driven Cdc14 activation results in the dephosphorylation of a host of proteins, including the microtubule bundling protein Ase1. Ase1 dephosphorylation then results in the proper localization of this protein to the spindle midzone (Figure 2). Cdc14 also dephosphorylates Sli15, which leads to the targeting of the Ipl1 - Sli15 - Bir1 complex, the conserved Aurora B – INCENP - Survivin chromosomal passenger complex in higher eukaryotes, to the spindle midzone (Figure 2). Together, Ase1 and Sli15 - Ipl1 - Bir1 recruit additional factors that stabilize the anaphase spindle, critical among them being the Separase – Slk19 complex. In the absence of proper targeting of these components to the spindle midzone, the elongating anaphase spindle frequently breaks.

Activation of the MEN

FEAR network mutants display a 10 - 20 minute delay in exit from mitosis (an approximately 10 - 20% increase in the length of the *S. cerevisiae* cell cycle). The reason for this delay is that the lack of FEAR network dependent Cdc14 release delays the activation of a positive feedback loop that stimulates Cdc14 release. The MEN kinase Cdc15 is phosphorylated during cell cycle stages prior to anaphase and these phosphorylation events have been shown genetically to inhibit the mitotic exit promoting role of Cdc15. The early anaphase release of Cdc14 dephosphorylates Cdc15, thereby stimulating MEN activity and further promoting Cdc14 release and mitotic exit. As all of the core MEN components are phospho-proteins, it is possible that Cdc14 has multiple targets that collectively function in this positive feedback loop.

The FEAR network - a key coordinator of the meiotic divisions

Meiosis is a specialized gamete-producing cell division in which a single round of DNA replication is followed by two sequential rounds of chromosome segregation, termed meiosis I and meiosis II. During meiosis I, homologous chromosomes are segregated from each other and during meiosis II sister chromatids segregate. The transition from meiosis I to meiosis II represents an unusual form of cell cycle exit in that there is not an intervening DNA synthesis phase.

The FEAR network and Cdc14 play the same role in meiosis as they do in mitosis, that is accomplish Clb-CDK down-regulation and exit from the chromosome

segregation phase. However, while the FEAR network is important but not essential for Clb-CDK downregulation during mitosis, it is absolutely essential to bring about the proper transition from meiosis I to meiosis II. In the absence of FEAR network or Cdc14 function, meiotic cells fail to downregulate Clb-CDK activity at the end of meiosis I which leads to a severe delay in meiosis I spindle disassembly. In addition, Cdc14 and FEAR network mutants exhibit a "mixed" chromosome segregation pattern, which is characterized by some chromosomes segregating in a meiosis I-like pattern and others segregating in a meiosis II-like pattern. This unusual chromosome segregation pattern appears to result from meiotic events being uncoupled. Despite cells arresting in anaphase I, meiosis II chromosome segregation events continue to occur, leading to some chromosomes undergoing both meiotic divisions on the same anaphase I spindle. These findings suggest that Cdc14 and the FEAR network ensure that the two meiotic divisions occur on two sequentially built spindles.

It is interesting to note that unlike in mitosis, the MEN is dispensable for exit from meiosis I. The meiosis I to meiosis II transition is unique in that Clb-CDK activity needs to be lowered to a sufficient extent such that the meiosis I spindle disassembles but kept sufficiently high to prevent the formation of pre-replicative complexes (preRCs) and any subsequent DNA replication. MEN-mediated Cdc14 activation leads to complete inactivation of Clb-CDKs and resetting of the cell to the G1 state. Cdc14 released by the FEAR network lowers Clb-CDK activity, perhaps in a localized manner and/or for certain critical substrates, which may just

accomplish this balancing act. Employing the FEAR network for Cdc14 activation may lower CDK activity sufficiently for meiosis I spindle disassembly to occur but not low enough to allow assembly of preRCs onto origins of replication.

Does a FEAR network operate in higher eukaryotes?

Cdc14 homologues are found across eukaryotic species and play a conserved role in the regulation of CDK activity, although it appears that in many species this regulation may not be restricted to anaphase. It is a long-standing question as to whether or not a FEAR-like network functions in other organisms. All eukaryotes appear to have homologues of many of the budding yeast FEAR network components (Separase, polo-like kinase Cdc5, Clb-CDK, PP2A). In *S. pombe*, it was recently demonstrated that fission yeast FEAR network homologues do not regulate the localization of the Cdc14 homologue Clp1p/Flp1p. The signal transduction pathways that regulate Cdc14 homologue activation in metazoans are not yet known and there has yet to be any assessment for FEAR network contribution to Cdc14 activation in any other organism. It is possible that fission yeast and mammals do not require a FEAR network that brings about localized antagonism of mitotic CDK activity as, unlike in budding yeast, the bulk of Clb cyclin degradation and hence mitotic CDK downregulation occurs globally and abruptly at the metaphase – anaphase transition.

While a conserved role for a FEAR-like network has not yet been implicated in Cdc14 activation in other eukaryotes, it appears that at least some aspects of a FEAR

network-mediated Cdc14 function, that of central spindle assembly and stability, may in fact be conserved. In *C. elegans*, the Cdc14 homolog CeCDC-14 localizes to the spindle midzone in anaphase and to the midbody in telophase. As a result of RNAi depletion of CeCDC-14, the mitotic kinesin ZEN-4 as well as other proteins fail to localize to the spindle midzone, resulting in the complete loss of the central spindle. These CeCDC-14 depleted embryos ultimately die, likely as a failure of cytokinesis. In fission yeast, the Cdc14 homolog Clp1/Flp1 localizes to the spindle midzone in anaphase and regulates the ability of Ase1 to recruit the kinesin Klp9 to the spindle midzone. Mammals contain two Cdc14 homologues, with one isoform (Cdc14B) residing in the nucleolus during interphase but not mitosis and one isoform (Cdc14A) located at centrosomes. Recent work has demonstrated that homozygous deletion of Cdc14B in tissue culture cells resulted in no defects in spindle assembly, anaphase progression, mitotic exit, and cytokinesis. siRNA depletion of the Cdc14A homologue, however, resulted in defects in cytokinesis, perhaps as a result of defective spindle midzone assembly. Furthermore, while roles for Cdc14 homologues have not yet been implicated in species other than budding yeast, the translocation of Ipl1-Sli15 homologs Aurora B-INCENP from kinetochores to the spindle midzone appears to be a conserved feature of anaphase in eukaryotes. Abrogation of this translocation is believed to result in defects in cytokinesis.

Conclusions and Outlook

In the eight years since the discovery of the FEAR network the field has made great strides towards understanding the mechanisms by which the FEAR network operates and, particularly, about how Cdc14 activated by the FEAR network functions during anaphase. Activation of Separase at the metaphase to anaphase transition triggers a cascade of events that ultimately leads to the phosphorylation of Cfi1/Net1 as well as additional proteins, thereby promoting the release of Cdc14 from the nucleolus. This early anaphase activation of Cdc14 is critical for the coordination of multiple anaphase processes including the promotion of the switch-like transition to anaphase, rDNA segregation, positioning of the anaphase nucleus, quelling of microtubule dynamics, promotion of spindle stability and spindle midzone formation, and activation of the MEN.

While much has been learned, many important questions remain. Our molecular understanding of FEAR network driven Cdc14 release is far from complete. In the absence of MEN activity, Cdc14 becomes resequestered after only a brief release from the nucleolus (~ 10 minutes) indicating that FEAR network function is restricted to a brief period during early anaphase. The mechanisms by which this pulse of FEAR network function is generated are unknown. In addition, we do not yet understand the essential FEAR network role of polo-like kinase Cdc5, nor have we determined the mechanism by which Separase – Slk19 downregulates $PP2A^{Cdc55}$. Furthermore, recent data suggest that Cdc14 may in fact be active in the nucleolus in cell cycle phases prior to anaphase and may be a potent negative regulator of its own FEAR network and MEN dependent release. Finally, identification of additional

Cdc14 substrates will reveal novel early anaphase functions for Cdc14. In conclusion, as progression through mitosis in the absence of FEAR network function is associated with a loss of genomic integrity and viability in *S. cerevisiaie*, it will be important to further determine the broader significance of the findings in budding yeast in higher eukaryotes.

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