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Control of the Mitotic Exit Network during meiosis.

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ABSTRACT

The Mitotic Exit Network (MEN) is an essential GTPase signaling pathway that triggers exit from mitosis in budding yeast. We show here that during meiosis, the MEN is dispensable for exit from meiosis I, but contributes to the timely exit from meiosis II. Consistent with a role for the MEN during meiosis II, we find that the signaling pathway is only active during meiosis II. Our analysis further shows that MEN signaling is modulated during meiosis in several key ways. Whereas binding of MEN components to spindle pole bodies (SPBs) is necessary for MEN signaling during mitosis, during meiosis MEN signaling occurs off SPBs, and does not require the SPB recruitment factor Nud1. Furthermore, unlike during mitosis, MEN signaling is controlled through the regulated interaction between the MEN kinase Dbf20 and its activating subunit Mob1. Our data lead to the conclusion that a pathway essential for vegetative growth is largely dispensable for the specialized meiotic divisions and provide insights into how cell cycle regulatory pathways are modulated to accommodate different modes of cell division.

INTRODUCTION

In the final phase of the cell cycle, after chromosomes segregate in anaphase, cells exit from mitosis. In the budding yeast *Saccharomyces cerevisiae* this cell cycle transition is brought about by the inactivation of mitotic cyclin-dependent kinases, known as Clb-CDKs (Stegmeier and Amon, 2004). Central to the precipitous inactivation of Clb-CDKs at the end of mitosis is the protein phosphatase Cdc14 (Jaspersen *et al.*, 1998; Visintin *et al.*, 1998; Zachariae *et al.*, 1998). Cdc14 triggers the degradation of Clb cyclins, the up-regulation of a Clb-CDK inhibitor and the dephosphorylation of Clb-CDK substrates to rapidly reset the cell to a low CDK state. This resetting in turn causes the events of mitotic exit: mitotic spindle disassembly, chromosome decondensation and cytokinesis.

Given the central importance of Cdc14 to exit from mitosis, it is not surprising that its activity is tightly controlled (Shou *et al.*, 1999; Visintin *et al.*, 1999). Cdc14 is bound to its nucleolar-localized inhibitor Cfi1/Net1 throughout most of the cell cycle. During anaphase, the phosphatase is released from its inhibitor, freeing it to dephosphorylate its targets in the nucleus and cytoplasm. This release occurs in two waves and by two pathways: the Cdc14 early anaphase release (FEAR) network and the Mitotic Exit Network (MEN). The FEAR network is not essential for viability and promotes a transient burst of Cdc14 release early in anaphase (Pereira *et al.*, 2002; Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002; Rock and Amon, 2009), which contributes to the coordination of anaphase events. The MEN is required for sustained Cdc14 release which is essential for exit from mitosis (Stegmeier and Amon, 2004).

The MEN is a Ras-like GTPase signaling cascade in which the GTPase is encoded by *TEM1* (Shirayama *et al.*, 1994). Localization of Tem1 to the spindle pole body (SPB, yeast equivalent of the centrosome), is essential for MEN activation (Valerio-Santiago and Monje-Casas, 2011). Tem1 is recruited to SPBs during metaphase and remains there until the completion of anaphase (Bardin *et al.*, 2000; Pereira *et al.*, 2000). During metaphase, Tem1 is kept inactive at SPBs by the two-component GTPase activating protein (GAP) Bub2-Bfa1 (Geymonat *et al.*, 2002). During anaphase, Tem1 is activated by multiple signals, including spindle position. Tem1-GTP then recruits the kinase Cdc15 to SPBs, which is thought to activate Cdc15 (Asakawa *et al.*, 2001). Cdc15 in turn recruits Dbf2, the founding member of the NDR (nuclear Dbf2-related) protein kinase family, and its regulatory subunit Mob1 to SPBs and activates the Dbf2-Mob1 kinase complex (Mah *et al.*, 2001; Visintin and Amon, 2001). Budding yeast harbor two Dbf2-like kinases, Dbf2 and Dbf20. Dbf2 provides the predominant activity during vegetative growth and is active only during anaphase. Dbf20 is expressed at low-levels during vegetative growth but is induced during sporulation (Chu *et al.*, 1998). Regulation of its activity in mitosis is not understood in detail, but it has been shown that Dbf2 is more active than Dbf20 *in vitro* (Toyn and Johnston, 1994).

MEN components are thought to assemble into signaling modules by a scaffold protein Nud1. The Nud1 protein localizes to SPBs and is required for the association of Tem1, Cdc15, and Dbf2-Mob1 with SPBs (Adams and Kilmartin, 1999; Gruneberg *et al.*, 2000;

Visintin and Amon, 2001; Valerio-Santiago and Monje-Casas, 2011). This function is essential for exit from mitosis. Cells harboring a temperature sensitive allele of *NUD1* arrest in anaphase with MEN components not localized to SPBs (Adams and Kilmartin, 1999; Bardin *et al.*, 2000; Gruneberg *et al.*, 2000; Visintin and Amon, 2001). This requirement of *NUD1* for exit from mitosis is at least in part due to its function in recruiting MEN components to SPBs because binding of Tem1 and Cdc15 to SPBs is essential for MEN activity (Rock and Amon, 2011; Valerio-Santiago and Monje-Casas, 2011).

The MEN integrates multiple cellular events, such as onset of chromosome segregation, Polo kinase activity and spindle position. This ensures that exit from mitosis only occurs when chromosome segregation has been completed yielding two daughter cells each containing a complete complement of the genome (Stegmeier and Amon, 2004). Regulation of the MEN by spindle position is understood best. The MEN is only activated when the nucleus has been pulled into the daughter cell and a MEN component-carrying SPB has entered the bud (Yeh *et al.*, 1995; Bardin *et al.*, 2000; Pereira *et al.*, 2000). Spindle position control of the MEN 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 Kin4 inhibitor Lte1 in the bud, and the MEN GTPase Tem1 is localized to the SPB that will migrate into the bud (Bardin *et al.*, 2000; Pereira *et al.*, 2000; D'Aquino *et al.*, 2005; Maekawa *et al.*, 2007; Chan and Amon, 2010; Bertazzi *et al.*, 2011; Falk *et al.*, 2011). Only when the MEN-bearing SPB escapes the zone of the MEN inhibitor Kin4 in the mother cell and moves into the bud where the Kin4 inhibitor (and hence MEN activator) Lte1 resides, can exit from mitosis occur. In this manner, spatial information is sensed and translated to regulate MEN activity.

While the function of the MEN has been characterized in mitosis, it has not been well characterized in meiosis, a specialized cell division. During meiosis, a diploid cell undergoes two rounds of chromosome segregation following one round of DNA replication, and results in the formation of four haploid gametes called spores in yeast (Marston and Amon, 2004). Whereas *S. cerevisiae* divides by budding during vegetative growth, meiosis occurs within the confines of the mother cell with

membranes growing around the four meiotic products after both meiotic divisions have been completed. These changes in the chromosome segregation pattern and in the morphological constraints on chromosome segregation require the basic cell cycle machinery to be changed in fundamental ways.

Here, we investigate the function and regulation of the Mitotic Exit Network during meiosis. Previous studies showed that Cdc14 is essential for progression through meiosis (Sharon and Simchen, 1990). As during mitosis, the phosphatase is released from the nucleolus during anaphase I and anaphase II. However, the FEAR network rather than the MEN appears to be critical for the activation of Cdc14, at least during anaphase I (Buonomo *et al.*, 2003; Marston *et al.*, 2003). The MEN in fact appears to be dispensable for exit from meiosis I (Kamieniecki *et al.*, 2005; Pablo-Hernando *et al.*, 2007). This is perhaps not surprising as one of the major functions of the MEN, coordinating exit from mitosis with spindle position, is less important during meiosis, as both nuclear divisions occur within the confines of a single cell. Together, these observations raise the question of whether a pathway, which is absolutely essential for progression through mitosis, is also required for progression through meiosis and, if it is, which signals regulate it. We find that the MEN is dispensable for exit from meiosis I, and contributes to the timely release of Cdc14 from the nucleolus during anaphase II and exit from meiosis II. Consistent with a role of the MEN only during meiosis II, we find that the signaling pathway is only active during meiosis II. Our analysis further revealed that the MEN pathway is regulated differently during meiosis and mitosis. Meiotic MEN signaling does not require the Nud1 scaffold protein and relies instead on the regulated interaction between Dbf20 and its regulatory subunit Mob1. Our data show that the MEN, a signaling cascade essential for mitotic exit, is dispensable for the meiotic divisions and shed light on how MEN signaling is adapted to function during a symmetric cell division, meiosis.

RESULTS

The Mitotic Exit Network is required for the timely exit from meiosis II.

The MEN is essential for viability and serves the important function of ensuring that exit from mitosis only occurs when the nucleus has been threaded through the bud neck into the daughter cell. In contrast, during meiosis the two nuclear divisions occur within the

mother cell and are followed by membrane growth around the meiotic products (Neiman, 2011). This difference in cell division pattern and morphology raises the question of whether the MEN, a pathway critical to cell division by budding, functions during meiosis and if it does, how its activity is regulated. To address these questions we examined the consequences of loss of MEN function during meiosis and investigated the regulation of MEN signaling.

To generate cell cultures that progress through the meiotic divisions in a synchronous manner we employed a previously developed synchronization protocol termed the “Ndt80 block-release” system (Carlile and Amon, 2008). Briefly, *NDT80* encodes a transcription factor that is essential for entry into the meiotic divisions. Cells in which *NDT80* is expressed from the *GAL1-10* promoter and that also contain a fusion between the *GAL1-10* transcription factor Gal4 and the estrogen receptor (*GAL4-ER*) arrest prior to entry into meiosis I (in pachytene) in the absence of estrogen, but progress synchronously through the meiotic divisions upon estrogen addition to the medium. To determine whether the MEN is required for the meiotic divisions we examined the consequences of inactivating various MEN components. We employed an allele of *CDC15* (*cdc15-as1*) (Bishop *et al.*, 2000; D'Aquino *et al.*, 2005) that can be specifically inhibited upon addition of an ATP analog, 1-NA-PP1, while leaving other cellular kinase activities intact. Treatment of vegetatively growing *cdc15-as1* cells with inhibitor prevented exit from mitosis causing an anaphase arrest (see Figure 6F). In contrast, exit from meiosis I was not delayed in *cdc15-as1* cells and exit from meiosis II was only subtly delayed (Figure 1A–C). This is best seen when time spent in anaphase II is measured by integrating the area under the line, representing the number of cells harboring anaphase II spindles. In the presence of inhibitor, *cdc15-as1* cultures harbored 1.75 fold more cells with anaphase II spindles than wild-type cultures (Figure 1D; n=6 independent experiments). These findings are consistent with previous reports analyzing the effect of depleting Cdc15 and Tem1 in meiosis (Kamieniecki *et al.*, 2005; Pablo-Hernando *et al.*, 2007).

Similar results were obtained in cells lacking the MEN component Mob1. Mob1 was selectively depleted during meiosis by placing the *MOB1* gene under the control of the mitosis-specific *CLB2* promoter (*MOB1-mn*). Lack of transcription during the meiotic

divisions effectively depletes the Mob1 protein (Figure S1A). *MOB1-mn* cells progressed through the meiosis I-meiosis II transition with wild-type kinetics, but were delayed in exit from meiosis II (Figure S1B-D). We were not able to deplete the essential component Dbf2 (data not shown), preventing us from examining the consequences of losing both Dbf2 and Dbf20 activity on meiotic progression. However, deleting *DBF20* alone did not affect progression through meiosis (data not shown), presumably because low-levels of Dbf2 present in meiotic cells support the timely exit from meiosis I. We also have not been able to deplete the essential component Tem1 during meiosis. However, other studies have shown that like other MEN components, *TEM1* is not required for the meiosis I-meiosis II transition (Kamieniecki *et al.*, 2005). We conclude that the Mitotic Exit Network, essential for cell cycle progression during vegetative growth, is dispensable for progression through meiosis.

The essential function of the MEN during mitosis is to release Cdc14 from the nucleolus (Shou *et al.*, 1999; Visintin *et al.*, 1999). Cdc14 is also released from the nucleolus during exit from meiosis I and meiosis II and is essential for meiosis (Buonomo *et al.*, 2003; Marston *et al.*, 2003). The observation that the MEN was dispensable for the meiotic divisions therefore predicts that Cdc14 release from the nucleolus during exit from meiosis I and II must occur independently of MEN function. This is indeed what we observe. Cdc14 was released with wild type kinetics during exit from meiosis I in *cdc15-as1* and *mob1-mn* mutants. However, we observed a slight impairment in Cdc14 release during exit from meiosis II, consistent with a minor contribution of the MEN to this cell cycle transition (Figure 1E-F; Figure S1E; (Pablo-Hernando *et al.*, 2007)). This requirement for Cdc14 release was particularly evident in later time points raising the possibility that the MEN is needed to maintain Cdc14 in the released state during exit from meiosis II. We conclude that while the MEN is essential for Cdc14 release from the nucleolus during exit from mitosis, other pathways must be responsible for bringing about this event during the meiotic divisions. The FEAR network likely plays this role. Meiotic cells lacking FEAR network components exhibit a severe defect in releasing Cdc14 from the nucleolus during meiosis I and exhibit a phenotype remarkably similar to that of cells lacking *CDC14* function (Buonomo *et al.*, 2003; Marston *et al.*, 2003).

MEN activity is restricted to meiosis II.

During mitosis, MEN activity is controlled by nuclear position. The pathway is only activated when the nucleus moves into the bud (Bardin *et al.*, 2000; Pereira *et al.*, 2000). In contrast, the meiotic nuclear divisions occur within the mother cell, raising the question of how, if at all, the MEN is controlled during the meiotic divisions. To address this question we first determined whether MEN components are expressed during the meiotic divisions. Consistent with the observation that the MEN is needed for the timely exit from meiosis II, we found that all MEN components analyzed were expressed during meiosis. The GTPase Tem1 and the effector kinase Cdc15 were present at constant levels throughout meiosis and the proteins exhibited no noticeable mobility shifts (Figure S2A-D). Mob1 and Nud1 were also found throughout meiosis but their mobility changed. Slower migrating forms of the proteins appeared concomitantly with meiosis I entry (Figure S2E-H). This change in mobility is likely due to phosphorylation (Luca and Winey, 1998; Gruneberg *et al.*, 2000; Keck *et al.*, 2011) but the significance of this mobility shift is at present unclear.

Of the two Dbf2-like kinases present in *S. cerevisiae* only Dbf20 was expressed at appreciable levels during meiosis (Figure S2I-L). Dbf20 mobility also changed during meiosis. Concomitant with entry into meiosis II, slower migrating forms of the protein became apparent. The appearance of this form of Dbf20 correlated well with Dbf20 activity. Dbf20 immunoprecipitated from cells progressing through meiosis in a synchronous manner exhibited low activity during meiosis I but showed robust activity during anaphase of meiosis II (Figure 2A-B, Figure S3A; note that slower migrating forms of Dbf20 can only be detected with the Dbf20-Myc fusion and not with the Dbf20-ProA fusion).

As during mitosis where Dbf2 activity depends on *CDC15* (Visintin and Amon, 2001), we found that Dbf20 activity depended on Cdc15 kinase activity during meiosis. Dbf20 kinase activity was greatly reduced in *cdc15-as1* cells progressing through meiosis (Figure 2C; Figure S3B-C). We conclude that the MEN is only active during exit from meiosis II. This observation is consistent with the finding that the MEN is required for the timely exit from meiosis II but not for exit from meiosis I. We have thus far not been able to determine the mechanisms that restrict MEN activity to exit from meiosis II, but

as described below, our analyses of MEN regulation during meiosis II revealed that the pathway is regulated in fundamentally different ways during meiosis II than during mitosis.

MEN components are not detected on spindle pole bodies in meiosis

During mitosis, association of MEN components with SPBs is essential for MEN activity (Rock and Amon, 2011; Valerio-Santiago and Monje-Casas, 2011). The MEN components Bub2-Bfa1, Tem1, Cdc15 and Dbf2-Mob1 localize to SPBs in a manner that depends on the spindle pole body component Nud1 (Bardin *et al.*, 2000; Gruneberg *et al.*, 2000; Visintin and Amon, 2001). We examined the ability of these MEN components to bind to SPBs during meiosis. The MEN components Tem1, Cdc15, or Mob1, whether tagged with GFP, Myc or HA, were never detected at SPBs in any stage of meiosis (Figure S4A-D). Additionally, despite the role of Cdc15 in spore wall formation (Kamieniecki *et al.*, 2005; Pablo-Hernando *et al.*, 2007), Cdc15-eGFP was not detected on SPBs during spore formation (Figure S4B, bottom panels). However, the protein required for tethering these proteins to SPBs during mitosis, Nud1, was present at all SPBs throughout meiosis (Figure S5A-B).

Bfa1 localized to both SPBs during anaphase I as judged by co-localization with the SPB component Spc42. During anaphase II Bfa1 localized to only two of the four SPBs (Figure S5C-D). This anaphase II localization pattern is reminiscent of that found in mitotically dividing cells: Bub2-Bfa1 is concentrated at the SPB that migrates into the bud during anaphase (Pereira *et al.*, 2000; Molk *et al.*, 2004). This asymmetric localization is brought about by bud-specific cell cortex proteins (Monje-Casas and Amon, 2009). It will be interesting to determine whether these cell cortex proteins are also asymmetrically localized in symmetrically shaped meiotic cells. Taken together, these results indicate that although the MEN is active during anaphase II, most MEN components are not detected at spindle pole bodies.

***NUD1* is not required for Dbf20 kinase activity in meiosis II**

The absence of MEN components from SPBs during meiosis raises the interesting possibility that MEN signaling does not require localization of its components to spindle pole bodies during meiosis II. A prediction of this hypothesis is that *NUD1* is not required for MEN activity during anaphase II. To test this, we analyzed meiotic Dbf20

kinase activity in cells carrying the temperature sensitive *nud1-44* allele (Adams and Kilmartin, 1999). We first established 34°C as a restrictive temperature for the *nud1-44* allele, which is a temperature that is still permissive for progression through meiosis. To analyze cells progressing through the mitotic cell cycle in a synchronous manner, *nud1-44* cells were arrested in G1 with pheromone. Upon release from the G1 arrest, *nud1-44* cells arrested in anaphase and failed to activate Dbf2 kinase at this temperature (Figure 3A-C). Thus, *nud1-44* cells are defective in MEN signaling at 34°C during vegetative growth. In contrast to mitosis, *nud1-44* cells progressed through the meiotic divisions at 34°C and activated Dbf20 kinase activity with kinetics indistinguishable from that of wild-type cells (Figure 3D-F). To address the possibility that Dbf2 and Dbf20 exhibit a differential requirement for Nud1 in their activation we expressed *DBF2* in meiosis from the copper inducible *CUP1* promoter (to induce sufficient amounts of Dbf2) and examined the effects of inactivating *NUD1* on Dbf2 kinase activity. *nud1-44* cells progressing through meiosis at 34°C harbored wild-type levels of Dbf2 kinase activity (Figure S6A-C).

We were unable to tag *nud1-44* in order to examine the fate of the *nud1-44* protein at elevated temperatures during meiosis. We therefore cannot exclude the formal possibility that 34°C is not a restrictive temperature for the *nud1-44* allele during meiosis. However, given that Dbf2 kinase activity is completely abolished in *nud1-44* cells at 34°C during vegetative growth, yet Dbf20 or Dbf2 exhibit wild-type levels of kinase activity in *nud1-44* cells progressing through meiosis at 34°C, we consider it more likely that during meiosis Dbf2 and Dbf20 kinase activity do not require *NUD1*. We were also not able to assess the requirement for *NUD1* in spore formation because spore formation is greatly impaired at 34°C even in wild type cells (data not shown). We conclude that an SPB component essential for MEN activation in mitosis is dispensable for MEN activation in meiosis. Furthermore, unlike in mitosis, when SPB binding is essential for MEN signaling, SPB binding is not a prerequisite for MEN activity during meiosis.

Dbf2 and Dbf20 are differentially regulated.

Our results indicate that SPB association is not important for MEN regulation during meiosis. Are other aspects of MEN signaling also differentially regulated between

mitosis and meiosis? One obvious difference between the two types of divisions is the use of different Dbf2 family members, Dbf2 in mitosis and Dbf20 during meiosis. Are additional differential controls operative on Dbf2 and Dbf20 during mitosis and meiosis? To address this possibility, we placed both *DBF2* and *DBF20* under the control of the *CUP1* promoter, allowing us to express equal levels of both proteins during mitosis and meiosis (Figure 4A, E).

We first compared Dbf2- and Dbf20-associated kinase activity in cells progressing through the mitotic cell cycle in a synchronous manner. Whereas Dbf2 kinase activity peaked during anaphase, Dbf20-associated kinase activity remained at low levels throughout the cell cycle (Figure 4A-D). Expression of Dbf2 during the meiotic divisions led to Dbf2 kinase activity that is regulated similarly to that of Dbf20 (Figure 4E-H). Our results indicate that, when expressed, both Dbf2 and Dbf20 are active during meiosis and the activity of both kinases is restricted to exit from meiosis II. In contrast, during mitosis, the kinases are differentially regulated. Dbf2 is active during exit from mitosis (Figure 4A-D, (Visintin and Amon, 2001)), but Dbf20 is largely inactive throughout the mitotic cell cycle (Figure 4A-D). The basis for this phenomenon is unknown.

Furthermore, we note that expression of *DBF2* has no adverse effects on progression through meiosis and spore formation. Thus, it is unclear why Dbf2 expression is down-regulated during the meiotic divisions.

The Dbf20 - Mob1 interaction peaks at exit from meiosis II and depends on *CDC15*.

During mitosis, Cdc15 is activated by Tem1 and Cdc5 at SPBs and phosphorylates Dbf2 to activate the Dbf2-Mob1 complex (Mah *et al.*, 2001; Rock and Amon, 2011). The observation that MEN activation does not require *NUD1* during meiosis II raised the question of whether MEN signaling was differently wired during meiosis II than during mitosis. We first examined the association of Dbf2 and Dbf20 with its activating subunit Mob1 by co-immunoprecipitation in cells progressing through mitosis in a synchronous manner. The interaction between Dbf2 and Mob1 was low during G1 but steadily increased as cells progressed through the cell cycle reaching peak levels during mitosis (Figure 5A-B), indicating that the interaction between Dbf2 and Mob1 is subtly cell cycle regulated, being higher during mitosis.

To facilitate the comparison of the Dbf20-Mob1 interaction between meiosis and mitosis we expressed *DBF20* from the *CUP1* promoter. We found that Dbf20 and Mob1 bind to each other throughout the cell cycle during vegetative growth, as judged by co-immunoprecipitation (Figure 5C-D). This result shows that the binding between Dbf20 and Mob1 is not regulated during the mitotic cell cycle. Furthermore, since we do not detect Dbf20-associated kinase activity in mitosis (Figure 4A), we also conclude that the Dbf20-Mob1 interaction is not sufficient for Dbf20 kinase activity during vegetative growth. During the meiotic divisions the Dbf20-Mob1 interaction fluctuated. The interaction between the two proteins was low from pachytene until metaphase I, somewhat increased during anaphase I and significantly increased during anaphase II (Figure 5E-F).

Given that the Dbf20-Mob1 interaction was especially high during anaphase II we next asked whether the interaction between the two proteins was regulated by *CDC15*. Interestingly, the interaction between Dbf20 and Mob1 was dramatically reduced in *cdc15-as1* cells treated with inhibitor in meiosis (Figure 6A-C). In contrast, the Dbf20-Mob1 interaction occurred independently of *CDC15* during mitosis (Figure 6D-F). These results indicate that during mitosis, Dbf20-Mob1 and Dbf2-Mob1 complexes form before MEN activation and that Cdc15 activates the kinase complex specifically during anaphase. During meiosis, Cdc15 activation during anaphase II is required for Dbf20-Mob1 complex formation and kinase activation. Our results indicate that MEN signaling is regulated in a substantially different manner during meiosis than during mitosis. In both cell division types the two pathways regulate Cdc14 activity, but signal transmission through the pathway has evolved perhaps so that the pathway can respond to different cellular signals.

DISCUSSION

Our studies of the MEN during meiosis led to two remarkable conclusions. First, the MEN, a signaling pathway essential for vegetative growth contributes little to meiotic cell cycle regulation. Second, MEN regulation changes dramatically during meiosis. Thus, MEN signaling serves as a paradigm for understanding how signaling pathways are rewired to serve different functions in different biological contexts.

MEN functions in meiosis.

In mitosis, the MEN is essential for the release of Cdc14 from the nucleolus and hence its activation during anaphase in mitosis. In contrast, during meiosis, release of Cdc14 from the nucleolus during anaphase is also essential (Buonomo *et al.*, 2003; Marston *et al.*, 2003), but surprisingly, the MEN components Cdc15 and Mob1 do not appear to contribute to the release of Cdc14 during anaphase I at all and only plays a minor maintenance role in Cdc14 release during anaphase II. Instead, it appears that the FEAR network, which plays only a minor role in promoting Cdc14 activation during mitosis, is essential for Cdc14 activation during anaphase I (Pereira *et al.*, 2002; Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002; Buonomo *et al.*, 2003; Marston *et al.*, 2003). A role for the FEAR network in anaphase II activation of Cdc14 has not been described yet, but given that the MEN has only a minor function in Cdc14 activation, it is likely that the FEAR network also functions during meiosis II to bring about Cdc14 release from the nucleolus.

Why does the FEAR network rather than the MEN bring about the activation of Cdc14 during meiosis? Cdc14 regulation must be responsive to different cellular signals during gametogenesis than during vegetative growth. During vegetative growth Cdc14 activity is controlled by spindle position, which is essential because of the inherently asymmetric manner by which budding yeast divides (Fraschini *et al.*, 2008). The MEN couples Cdc14 activation to spindle position (Bardin *et al.*, 2000; Pereira *et al.*, 2000). In contrast, meiosis is a symmetric division, as both meiotic divisions occur within the confines of a single cell (Neiman, 2011), rendering the control of Cdc14 activation during meiosis I and II by spindle position unimportant. Consistent with this idea is our finding that a gene essential for the regulation of the MEN by spindle position, *KIN4*, (D'Aquino *et al.*, 2005; Pereira and Schiebel, 2005; Chan and Amon, 2010) does not function during the meiotic divisions. Cells harboring a deletion of *KIN4* undergo meiosis with wild-type kinetics (M.A.A., unpublished observations). However, exit from meiosis I and II as well as spore wall formation must still be coordinated with chromosome segregation. This dependence is established by the FEAR network (Stegmeier and Amon, 2004) and the Polo kinase Cdc5 (Rock and Amon, 2011). Indeed their activity is

essential for meiotic progression (Buonomo *et al.*, 2003; Clyne *et al.*, 2003; Lee and Amon, 2003; Marston *et al.*, 2003).

During meiosis, the MEN has lost its essential function to promote the final stages of chromosome segregation, but at least *CDC15* has been co-opted to fulfill a novel meiosis-specific function, spore-wall morphogenesis ((Kamieniecki *et al.*, 2005; Pablo-Hernando *et al.*, 2007); M.A.A. unpublished observations). As spore wall formation must occur only after the completion of the two meiotic divisions it is perhaps not surprising that the MEN is only active during meiosis II. What keeps the MEN inactive during exit from meiosis I is thus an important question that remains to be addressed. Despite intense efforts we have not been able to prematurely activate the MEN during meiosis I. Genetic alterations known to hyper-activate the MEN during mitosis failed to do so in meiosis (M.A.A., unpublished observations). This suggests that it is important to keep the MEN inactive in meiosis I, possibly to ensure that cells do not initiate spore wall formation prematurely.

Signaling through the MEN differs between mitosis and meiosis II in multiple ways.

Our analyses indicate that signal transmission through the MEN is modified in at least three ways during meiosis II: (1) Dbf2 is replaced by Dbf20, (2) Dbf20 binding to its activating subunit Mob1 is regulated differently and (3) MEN signaling does not occur at SPBs, nor does it require the SPB scaffolding subunit Nud1.

During mitosis, Dbf2 is the predominant kinase required for MEN signaling. In fact in several strain backgrounds including SK1, Dbf2 is essential. Even in S288C, where Dbf2 is not essential, Dbf2 is the predominant kinase (Toyn and Johnston, 1994). In contrast, during meiosis II Dbf20 takes over this role. This differential requirement for the two homologs is in part due to their expression levels, with Dbf2 levels being low during the meiotic divisions (Figure S2I). When we expressed Dbf2 from an inducible promoter in meiosis, the protein was readily expressed and was active as a kinase (Figure 4E). Curiously, this was not the case for Dbf20. Dbf20 expressed from the *CUP1* promoter readily accumulated in vegetative cells, bound to Mob1 but did not exhibit kinase activity. These results indicate that Dbf20 is differentially regulated from Dbf2 at least during mitosis. The mechanism for this differential regulation remains to be

discovered. Perhaps an activator in addition to Cdc15 is needed for Dbf20 to be active, and this activator is absent during mitosis. Alternatively, Dbf20 may be inhibited during vegetative growth. Why cells use *DBF20* rather than *DBF2* in meiosis II remains unclear. However we note that even though Dbf2 is active as a kinase when expressed during meiosis its activity level is lower than that of Dbf20 (Figure 4E). Perhaps Dbf2 cannot be as effectively activated off SPBs during meiosis II as Dbf20.

Dbf2 and Dbf20 not only show differential expression during mitosis and meiosis, their association with their common activator Mob1 appears to be differentially regulated between mitosis and meiosis. Whereas Dbf2 and Dbf20 bind to Mob1 throughout the cell cycle during vegetative growth, Dbf20 binding to Mob1 fluctuated during the meiotic divisions. Complex formation was significantly increased during meiosis II. Furthermore, whereas Dbf20 binding to Mob1 did not require *CDC15* during vegetative growth, it depended on the upstream kinase during meiosis II. These results suggest that during mitosis a mechanism exists that can promote Dbf20 – Mob1 binding that is absent during meiosis II. During meiosis II, this mechanism is either replaced by a *CDC15*-dependent mechanism or, more likely, *CDC15* can also promote the interaction between Dbf2 and Mob1 during mitosis, but is not required for this, because of a second redundant mechanism that appears operative throughout the cell cycle. Taken together, our data are consistent with a model where Cdc15 is activated during meiosis II, which then promotes the interaction and activity of the Dbf20-Mob1 kinase complex, and maintaining Cdc14 in its released form. These events may require posttranslational modifications on Dbf20 that are restricted to meiosis II and independent of *CDC15* (Figure S2K; M.A.A., unpublished observations).

In addition to multiple forms of differential regulation of the Dbf2-Mob1 family of protein kinases during meiosis, signal transmission as a whole appears very different between meiosis II and mitosis. In mitosis, loading of MEN components onto SPBs is critical for MEN activation (Valerio-Santiago and Monje-Casas, 2011). Furthermore, MEN signaling in mitosis requires the SPB component Nud1, the putative scaffold for MEN signaling (Gruneberg *et al.*, 2000; Visintin and Amon, 2001). In contrast, MEN components are not found at SPBs during meiosis II. The spindle pole body undergoes a major restructuring of its outer plaque during meiosis II to initiate spore wall formation

(Neiman, 2011). It is possible that the altered structure of the outer plaque of the SPB during meiosis II precludes detection of MEN components. However, the fact that Bfa1 can be detected at SPBs during meiosis and that Nud1 is dispensable for MEN signaling during meiosis II indicates that MEN signaling does not occur in the context of the SPB during meiosis II. Nud1 is present at SPBs during meiosis II, but for reasons that we do not understand does not recruit MEN components to SPBs at detectable levels. The altered architecture of this organelle may impede Nud1-dependent recruitment of MEN components to SPBs. How MEN signaling modules are assembled during meiosis II off SPBs is not known. There is no obvious Nud1 homolog encoded in the *S. cerevisiae* genome, but functional homologs could exist. Proteomic-based screens could address this possibility in the future.

Why signaling through the MEN is rewired during meiosis II is an important question that remains to be addressed. *CDC15* fulfills a novel spore-wall formation function (Kamieniecki *et al.*, 2005; Pablo-Hernando *et al.*, 2007) which may necessitate employing Dbf20 rather than Dbf2 in signal transmission. Signaling without the MEN scaffold and outer plaque component Nud1 may be necessary as to not interfere with Nud1's function in spore wall formation (Gordon *et al.*, 2006).

Parallels in other organisms

The MEN is a conserved signaling pathway. In *Schizosaccharomyces pombe*, the pathway is known as the septation initiation network (SIN) and regulates cytokinesis. There are several similarities between MEN and SIN function during the meiotic divisions. Like MEN mutants, SIN mutants do not exhibit defects in meiotic progression, but fail to form spore walls (Krapp *et al.*, 2006). This indicates that co-opting of a pathway essential for the mitotic but not meiotic divisions to perform a novel function, spore wall formation, has occurred early during fungal evolution. *S. pombe* also contains two Dbf2 homologs, Sid2 and Slk1. During mitosis, Sid2 functions in SIN signaling (Sparks *et al.*, 1999), but as in budding yeast, the other Dbf2 homolog, Slk1 is up-regulated and active in meiosis (Ohtaka *et al.*, 2008; Perez-Hidalgo *et al.*, 2008; Yan *et al.*, 2008). It thus appears that the utilization of MEN/SIN signaling in spore wall formation is conserved between budding and fission yeast.

The core Cdc15-Dbf2-Mob1 signaling module of the MEN is conserved in mammals. In mammalian cells these components are MST1/2 (mammalian sterile 20-like kinase 1/2), NDR kinase (nuclear Dbf2-related kinase), and hMob1 respectively and function together to control a number of cellular processes including cell proliferation (Hergovich *et al.*, 2006; Pan, 2010). The core signaling module may be regulated similarly between mammalian cells and the MEN in meiosis. The interaction between Mob1 and NDR kinase family members is promoted when Mob1 is phosphorylated (Hirabayashi *et al.*, 2008; Praskova *et al.*, 2008), suggesting that regulation of the NDR kinase-Mob1 interaction may be a common mechanism for regulating the activity of this pathway in the absence of spatial control. Finally, it will be interesting to determine whether meiosis-specific modules also exist in mammals and whether they regulate late stages of germ cell formation.

MATERIALS AND METHODS

Yeast strains

All yeast strains used in this study are derivatives of SK1 and listed in Table S1. *TEM1-9MYC*, *CDC15-9MYC*, *MOB1-13MYC*, *LTE1-13MYC*, *NUD1-13MYC*, *pCLB2-3HA-MOB1*, *BFA1-GFP*, *TEM1-GFP*, *CDC15-eGFP*, *MOB1-GFP*, *DBF20-ProA*, *DBF2-ProA*, *pCUP1-DBF20-ProA*, *pCUP1-DBF2-ProA*, and *MOB1-3V5* strains were made using a PCR-based method (Longtine *et al.*, 1998). *3MYC-DBF2* and *3MYC-DBF20* were created using a URA3-popout based approach (Schneider *et al.*, 1995). The *nud1-44* allele from strain A1920 was backcrossed 5X to SK1.

Sporulation

Strains were grown overnight on YEPG plates (3% glycerol) and then transferred to 4% YEPD (4% glucose) plates in the morning. Cells were cultured in YEPD medium to saturation (approximately 24 hours), and then diluted into buffered YTA medium (1% yeast extract, 2% tryptone, 1% potassium acetate, 50mM potassium phthalate) to $OD_{600}=0.35$. Cells were grown overnight and then resuspended in sporulation medium (1% potassium acetate pH 7) at an $OD_{600}=1.9$. Sporulation experiments were performed at 30°C unless otherwise noted. Meiotic divisions were synchronized with the Ndt80 block-release protocol. Cells contain *NDT80* under the *GAL1-10* promoter and a Gal4-estrogen receptor fusion. Cells were transferred to sporulation medium at

time=0 hours. Owing to the lack of *NDT80*, *GAL-NDT80* cells will arrest in pachytene. At t=6 hours, when most cells had reached pachytene, 1 μ M β -estradiol was added to the medium allowing cells to progress through the meiotic divisions in a synchronous manner. For *cdc15-as1* experiments, 10 μ M 1-NA-PP1 (4-Amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine; Toronto Research Chemicals, North York ON) was added to the medium at the same time as β -estradiol.

Fluorescence microscopy

Indirect in situ immunofluorescence of tubulin was performed as described previously (Kilmartin and Adams, 1984). Cdc14-3HA immunofluorescence was performed as described in (Marston *et al.*, 2003). To perform immunofluorescence of Nud1-13Myc, cells were subjected to a 15 minute fixation with 3.7% formaldehyde in 0.1M potassium phosphate (KPi pH 6.4) buffer and prepared for staining as described by (Marston *et al.*, 2003). Cells were incubated with an anti-Myc (9E10 epitope, Covance, Princeton, NJ) primary antibody at 1:2000 and anti-mouse-Cy3 (Jackson Laboratory, Bar Harbor, ME) secondary antibody at 1:500 for at least two hours each. For Bfa1-GFP imaging, cells were fixed for 15 minutes with 3.7% formaldehyde in KPi. Cells were resuspended in 0.1M KPi/1.2M sorbitol/1% Triton. Cells were resuspended in 0.05 μ g/ml DAPI in KPi/Sorbitol. Cells were imaged with a Zeiss Axioplan 2 microscope and a Hamamatsu ORCA-ER digital camera.

Immunoblot analysis

For immunoblot analysis of Dbf20-ProA, Dbf2-ProA, Tem1-9Myc, Cdc15-9Myc, 3Myc-Dbf2, 3Myc-Dbf20, Mob1-13Myc, Nud1-13Myc, Lte1-13Myc, 3HA-Mob1, and Pgk1, cells were incubated for at least 10 minutes in 5% trichloroacetic acid. Cell pellets were washed once in acetone and dried overnight. Cells were lysed in 100 μ l lysis buffer (50mM Tris-Cl at pH 7.5, 1mM EDTA, 2.75 mM DTT, complete protease inhibitor cocktail [Roche]) with a bead mill. After sample buffer was added, cell lysates were boiled. Myc-tagged proteins were detected with anti-Myc (9E10 epitope, Covance, Princeton, NJ) antibodies at a dilution of 1:500. HA-tagged proteins were detected with anti-HA (HA.11, Covance, Princeton, NJ) antibodies at a dilution of 1:1000. ProA-tagged proteins were detected by incubation with rabbit IgG (Sigma-Aldrich, St. Louis, MO) at a concentration of 1:5000. Pgk1 was detected with an anti-Pgk1 antibody

(Invitrogen, Carlsbad, CA) using a 1:5000 dilution. Quantification was performed using ImageQuant software.

Dbf2 and Dbf20 kinase assays

Dbf20 kinase assays were performed as previously described (Visintin and Amon, 2001) with several modifications. Cells were lysed in a cold block in a bead mill, and 1-2mg of lysate was used in immunoprecipitations (total volume of 150 μ l). Dbf20-ProA was immunoprecipitated using IgG-coupled Dynabeads and incubated for one hour at 4°C. To prepare IgG-coupled beads, Dynabeads (Invitrogen Dynal AS, Oslo, Norway) were incubated with 0.33 mg/ml rabbit IgG (Sigma-Aldrich, St. Louis, MO) and 1M ammonium sulfate in 0.1M sodium phosphate pH 7.4 buffer at 37°C overnight.

Phosphorylation of histone H1 was quantified with the PhosphorImaging system.

Co-immunoprecipitation assays

Cells were resuspended in 200 μ l of NP40 lysis buffer (150mM NaCl, 50mM Tris-Cl pH 7.5, 1% NP40, 60mM β -glycerophosphate, 0.1mM sodium orthovanadate, 15mM p-Nitrophenylphosphate, 1mM DTT, complete protease inhibitor cocktail [Roche, Mannheim, Germany]) and lysed in a bead mill. 1-2mg (meiosis) or 500 μ g (mitosis) of extract were used in immunoprecipitation experiments (volume of 150 μ l) with agarose beads directly conjugated to the V5 epitope (Sigma-Aldrich, St. Louis, MO). Lysates were incubated for 45 minutes at 4°C, washed, and beads were resuspended in sample buffer, boiled, and proteins were analyzed by Western blot analyses.

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FIGURE LEGENDS

Fig 1

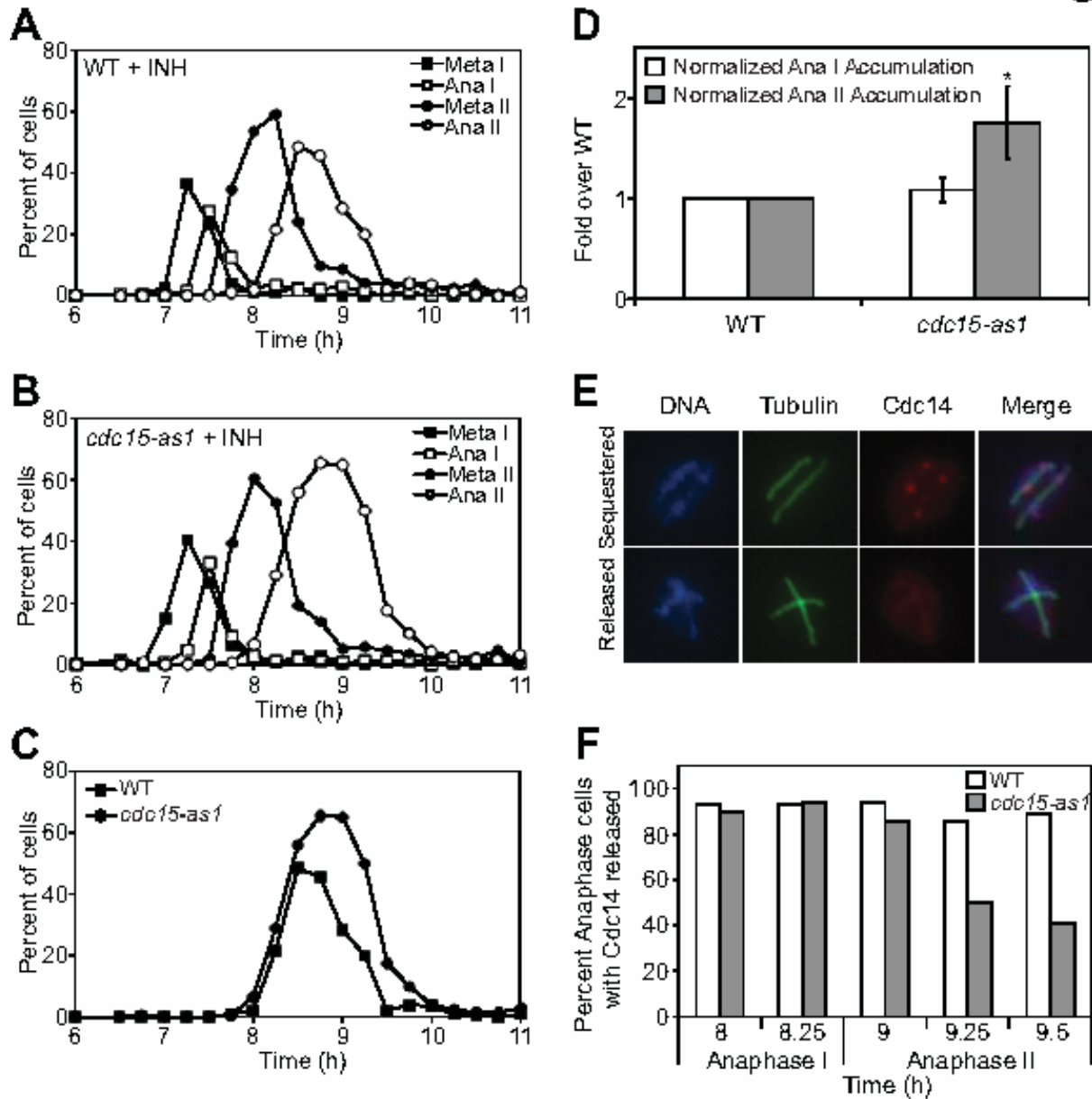


Figure 1. The MEN is dispensable for exit from meiosis I but is required for the timely exit from anaphase II. (A - D) Wild-type (A, A14201) or *cdc15-as1* (B, A19440) cells harboring a *GAL-NDT80* fusion as the sole source of *NDT80* were induced to sporulate. 1 μ M β -estradiol and 10 μ M 1-NA-PP1 was added to cultures six hours after transfer into sporulation medium. The percentage of cells with metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), anaphase II (open circles) was determined at the indicated times. 200 cells were analyzed at each time point. (C)

Comparison of the percentage of anaphase II cells in wild-type (closed squares) and *cdc15-as1* mutants (closed circles). (D) Quantification of the accumulation of anaphase I and anaphase II spindles in wild-type and *cdc15-as1* mutants (n=6 experiments). The area under the lines for anaphase I and anaphase II spindles was determined and expressed as a fold change of wild-type (anaphase I: mean=1.083, SD=0.122, p=0.598 (two-tailed, paired t-test); anaphase II: mean=1.757, SD=0.362, p=0.005 (two-tailed, paired t-test)). (E - F) Examples of anaphase II cells with Cdc14 sequestered in the nucleolus (top panels; E) or released into the nucleus and cytoplasm (bottom panels; E). Cdc14-HA is shown in red, DNA in blue and microtubules in green. (F) Quantification of Cdc14 release in anaphase II cells. WT (A22130; white bars) or *cdc15-as1* (A22129; gray bars) cells carrying *CDC14-HA* fusions were induced to sporulate as in (A). At the time points indicated, the percentage of anaphase I or anaphase II cells with Cdc14 released was determined (n=100 for each time point except for wild-type 8.25 hr, n=35 and wild-type 9 hr, n=36).

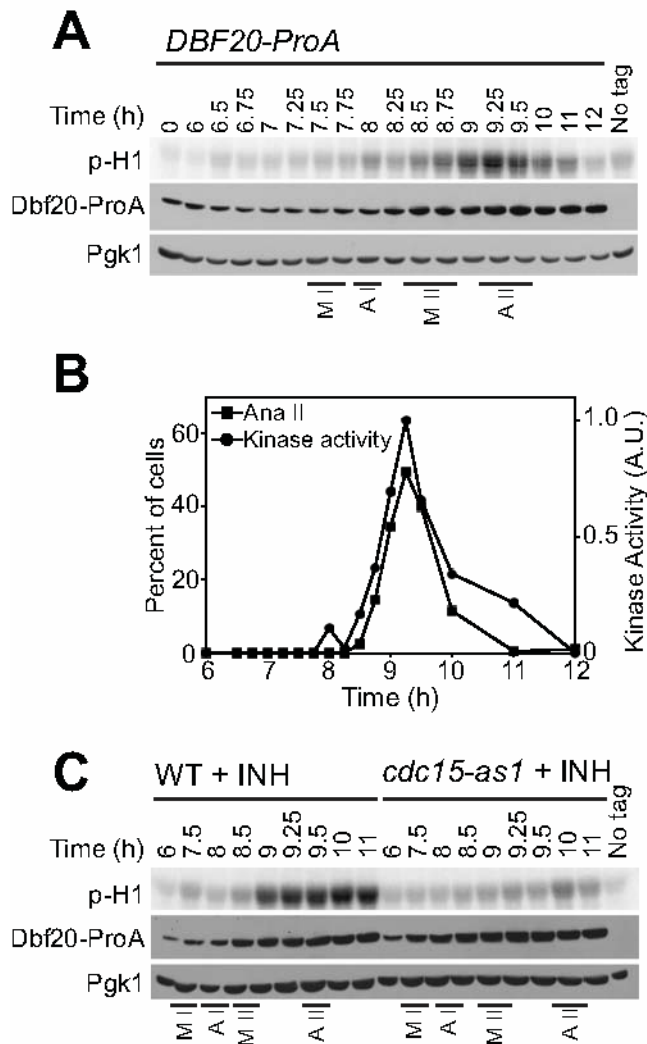
Fig 2

Figure 2. Dbf20 kinase activity peaks in anaphase II and depends on Cdc15 kinase activity.

(A) Cells containing a *DBF20-ProA* fusion (A23162) were sporulated as described in Figure 1A except 1-NA-PP1 was not added. Samples were taken at the indicated times to determine Dbf20-associated kinase activity and Dbf20-ProA protein levels. Dbf20-associated kinase activity was assessed by phosphorylation of the substrate Histone H1 (p-H1). Pgk1 was used as a loading control. The peak of each stage of meiosis is indicated below the blot (M I - metaphase I, A I - anaphase I, M II - metaphase II, A II - Anaphase II.)

(B) Quantification of Dbf20-associated kinase activity (closed circles) and anaphase II spindles (closed squares) are shown (n=200 cells counted).

(C) Wild-type (A23162) or *cdc15-as1* (A23733) cells containing a *DBF20-ProA* fusion were induced to sporulate as described in Figure 1A. Dbf20-associated kinase activity and Dbf20-ProA protein levels were determined at the indicated times.

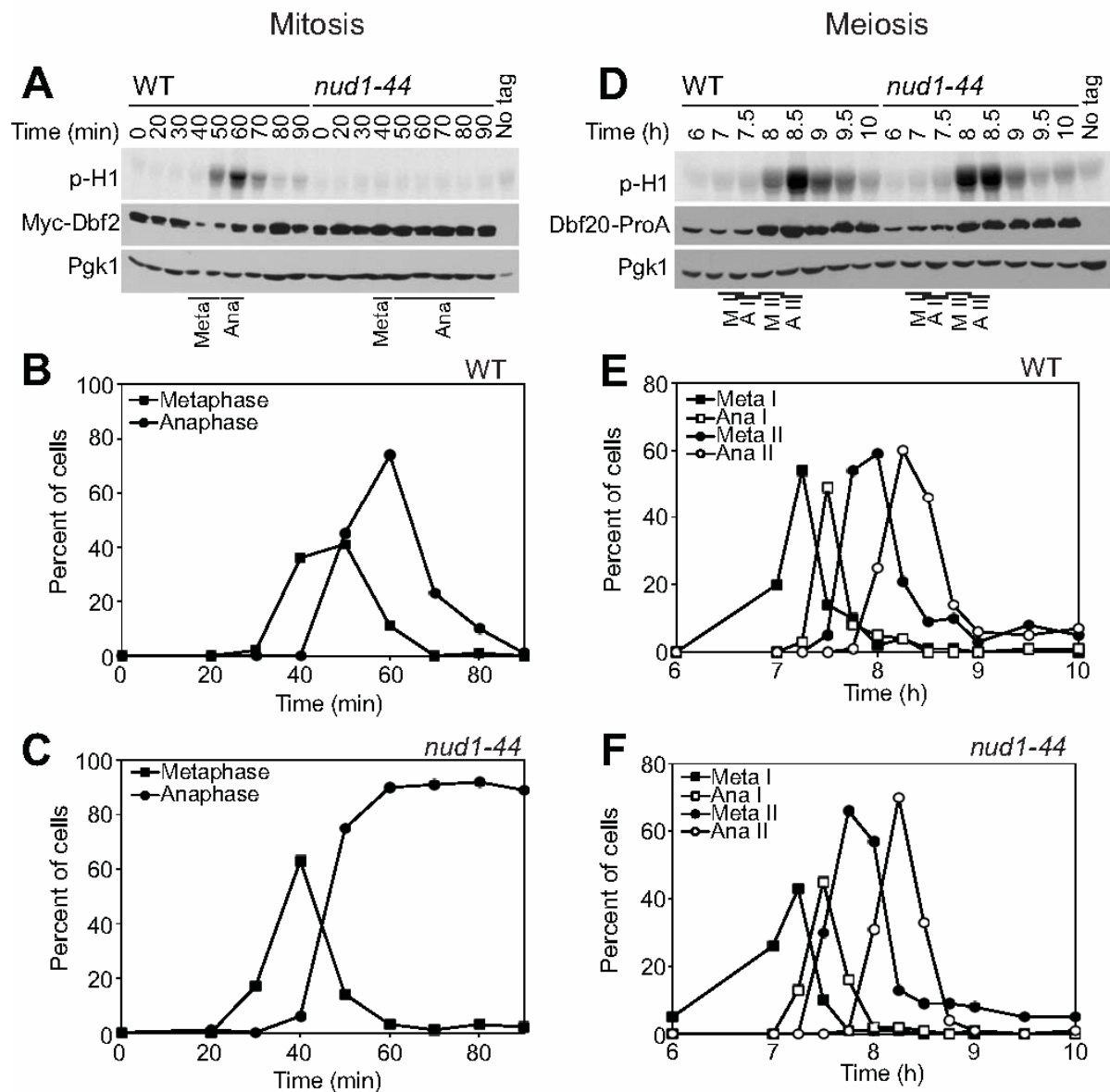
Fig 3

Figure 3. MEN signaling occurs in a *NUD1*-independent manner during meiosis. (A - C) 34°C is a restrictive temperature for *nud1-44*. Wild-type (A8499) and *nud1-44* (A28757) cells containing a 3MYC-DBF2 fusion were arrested in G1 with α -factor pheromone (5 μ g/ml) in YEPD medium for 2 hours at room temperature and then shifted to 34°C for 30 minutes. Cells were released into pheromone-free YEPD medium at 34°C thereafter. Dbf2 protein and kinase activity (A) and the percentage of cells in metaphase and anaphase (B, C) were analyzed at the indicated times (n=100 cells per timepoint). (D - F) Wild-type (A23162) or *nud1-44* (A27697) cells containing a DBF20-

ProA fusion were induced to sporulate via the Ndt80-block release protocol. Cells were incubated in sporulation medium at room temperature and shifted to 34°C after five hours. At six hours, cultures were induced with 1µM β-estradiol. Cells were maintained at 34°C throughout the rest of the experiment. Samples were taken at the indicated times to determine Dbf20 protein levels and Dbf20 kinase activity (D) and meiotic progression (E, F; n=100 cells per time point).

Fig 4

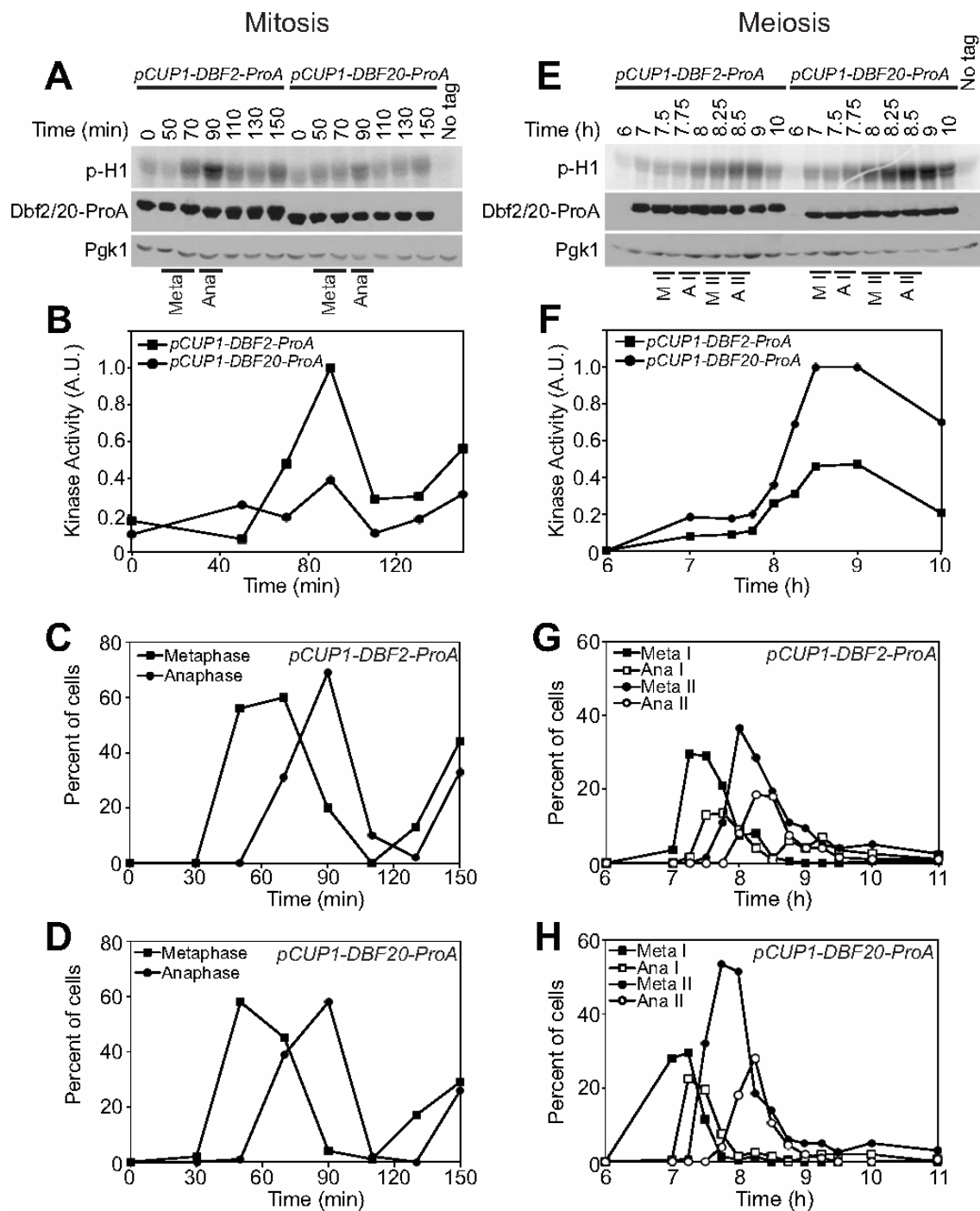


Figure 4. Differential regulation of Dbf2 and Dbf20 activity.

(A - D) *pCUP1-DBF2-ProA* (A25020) or *pCUP1-DBF20-ProA* (A25193) cells were arrested in G1 with α -factor pheromone (5 μ g/ml) in YEPD medium. 50 μ M CuSO₄ was added to the medium 2 hours into the arrest. When the arrest was complete (2 hours

30 minutes), cells were released into pheromone-free YEPD medium containing 50 μ M CuSO₄. Dbf2 and Dbf20 kinase activity, Dbf2 and Dbf20 protein (A, B) and mitotic progression (C, D; n=100 cells per time point) were examined at the indicated times. Quantifications of Dbf2 and Dbf20 kinase activity are shown in the graph in (B). (E - H) *pCUP1-DBF2-ProA* (A25028) or *pCUP1-DBF20-ProA* (A25195) cells were sporulated via the Ndt80 block-release protocol. 1 μ M β -estradiol and 50 μ M CuSO₄ were added to the medium six hours after transfer into sporulation medium. Dbf2 and Dbf20 kinase activity, Dbf2 and Dbf20 protein (E, F) and meiotic progression (G, H; n=100 cells per time point) were examined at the indicated times. Quantifications of Dbf2 and Dbf20 kinase activity are shown in the graph in (F).

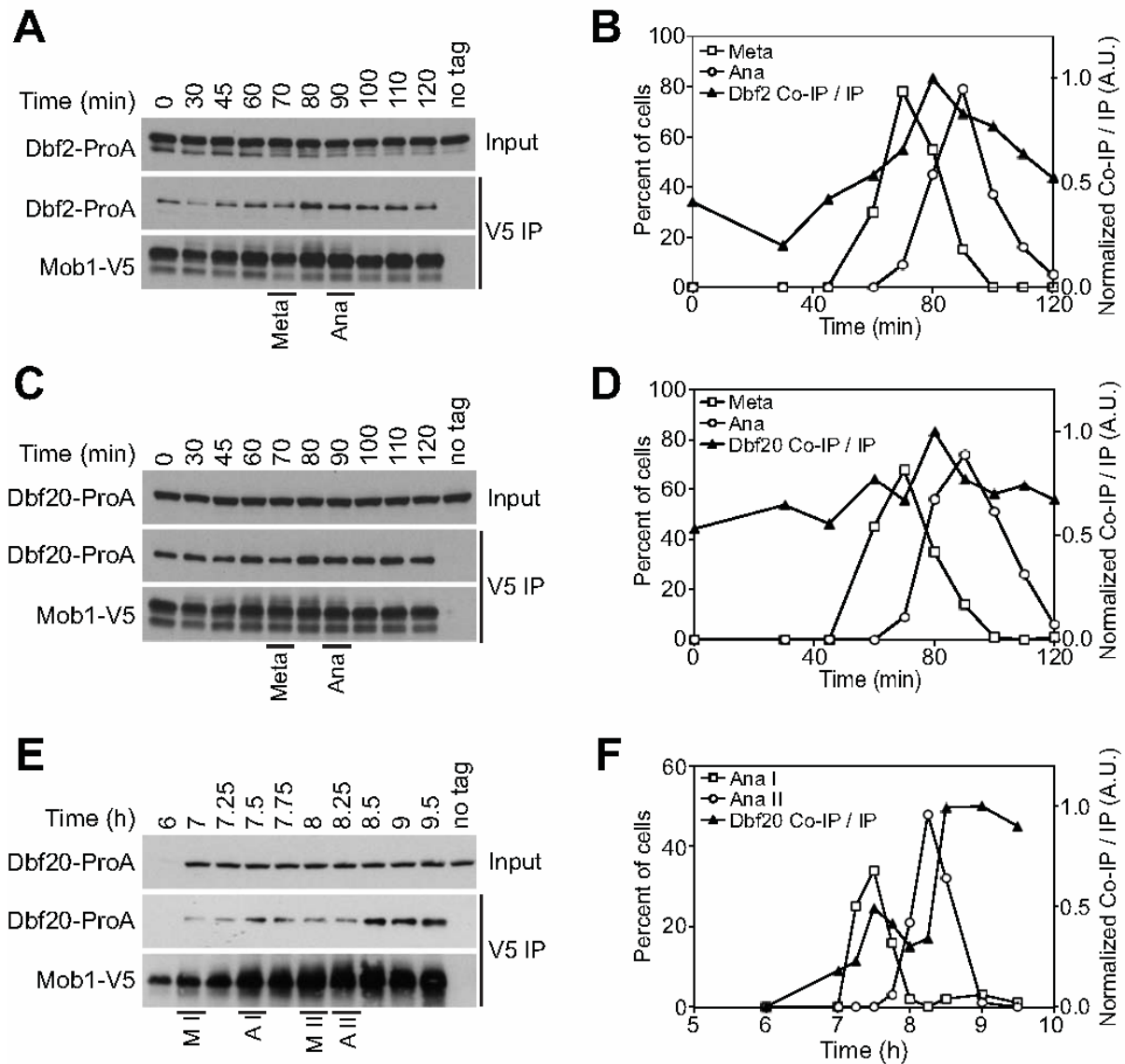
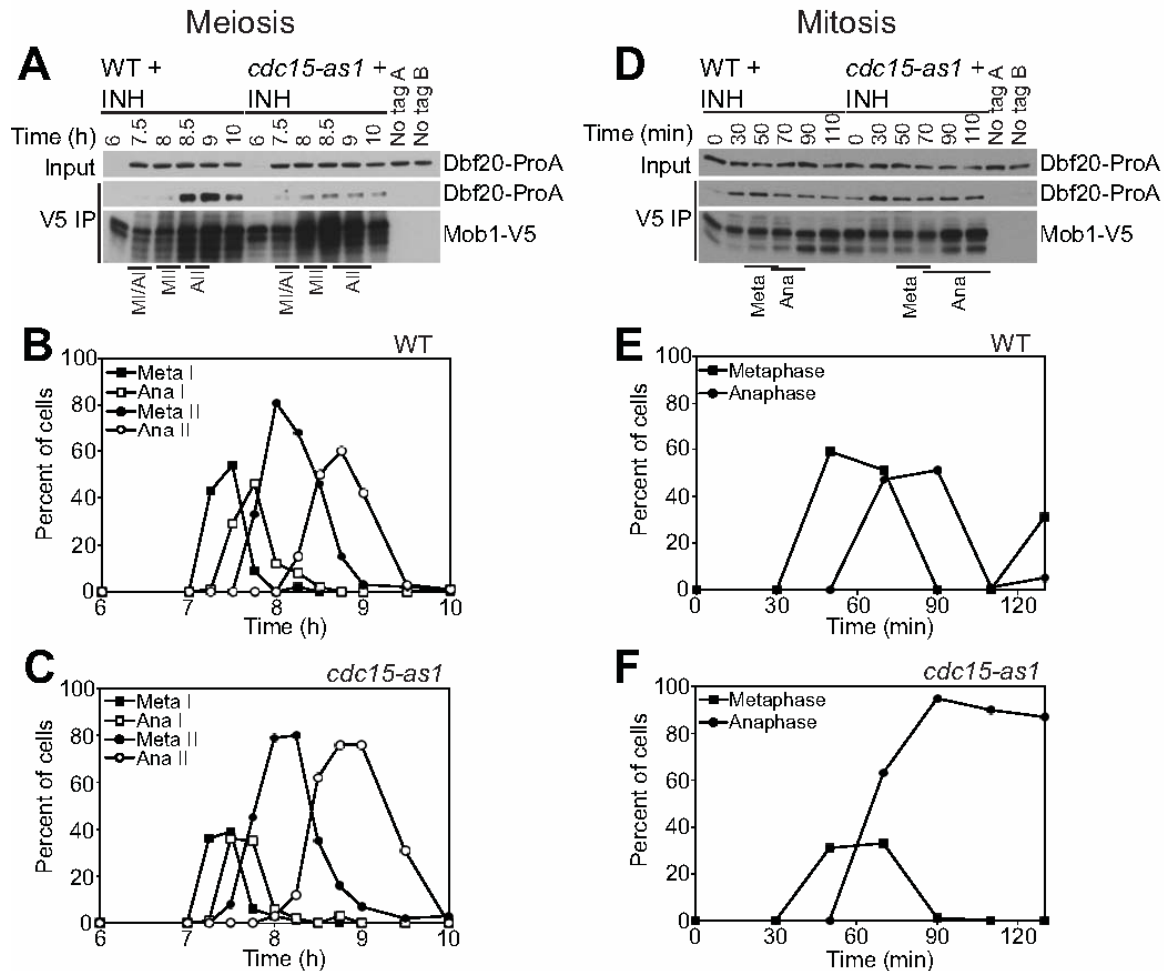
Fig 5

Figure 5. The Dbf20-Mob1 interaction is under cell cycle control in meiosis but not mitosis.

(A, B) Cells containing *pCUP1-DBF2-ProA* and a *MOB1-V5* fusion (A27687) or *MOB1* (A25020, no tag control) were arrested in G1 with α -factor pheromone (5 μ g/ml) in YEPD medium. 50 μ M CuSO_4 was added to the medium two hours into the arrest. When the arrest was complete (2 hours 30 minutes), cells were released into pheromone-free YEPD medium containing 50 μ M CuSO_4 . Western blots in (A) show total Dbf2-ProA protein (input), immunoprecipitated Mob1-V5 and co-immunoprecipitated Dbf2-ProA at

the indicated timepoints. Quantification of co-immunoprecipitation expressed as the amount of Dbf2 co-immunoprecipitated over the amount of Mob1-V5 immunoprecipitated are shown in (B). Values were normalized so that the maximum value was set to 1.0 (closed triangles). For comparison, the percentage of cells in metaphase (open squares) and anaphase (open circles) is shown. (C, D) Cells containing *pCUP1-DBF20-ProA* and a *MOB1-V5* fusion (A27367) or *MOB1* (A25191, no tag control) were grown and analyzed as described in (A, B). (E, F) Cells containing *pCUP1-DBF20-ProA* and a *MOB1-V5* fusion (A27370) or *MOB1* (A25195, no tag control) were sporulated via the Ndt80 block-release protocol. Cells were induced with 1 μ M β -estradiol and 50 μ M CuSO₄ six hours after transfer to sporulation medium. Western blots show immunoprecipitated Mob1-V5, co-immunoprecipitated Dbf20-ProA, and total Dbf20 protein (input) at the indicated timepoints (E). Quantification of the amount of Dbf20 co-immunoprecipitated with Mob1-V5 is shown as a ratio of the two values. Values were normalized so that the maximum value was set to 1.0. For comparison, meiotic progression was also analyzed.

Fig 6**Figure 6.** The Dbf20-Mob1 interaction depends on *CDC15* in meiosis.

(A - C) Cells containing *pCUP1-DBF20-ProA*, a *MOB1-V5* fusion, and *CDC15* (A27371) or *cdc15-as1* (A29149) were induced to sporulate via the Ndt80 block-release protocol as in Figure 1A with the addition of 10 μ M NA-PP1 and 50 μ M CuSO₄ six hours after transfer to sporulation medium. Western blots in (A) show total Dbf20-ProA protein (input), immunoprecipitated Mob1-V5 and co-immunoprecipitated Dbf20-ProA at the indicated timepoints. The genotype of no tag A is *pCUP1-DBF20-ProA* (A25195) and the genotype of no tag B is *pCUP1-DBF20-ProA cdc15-as1* (A29150). Meiotic progression in the two strains is shown in (B, C). (D - F) Cells containing *pCUP1-DBF20-ProA* and a *MOB1-V5* fusion, and *CDC15* (A27367) or *cdc15-as1* (A29125) were arrested in G1 with α -factor pheromone (5 μ g/ml) in YEPD medium as in Figure

4A. 50 μ M CuSO₄ was added to the medium 2 hours into the arrest. When the arrest was complete (2 hours 30 minutes), cells were released into YEPD medium containing 10 μ M NA-PP1 and 50 μ M CuSO₄. Total Dbf20-ProA protein (input), Co-immunoprecipitated Dbf20-ProA, and immunoprecipitated Mob1-V5 are shown for the indicated time points in (D). Mitotic progression of the two strains is shown in (E, F). The genotype of no tag A is *pCUP1-DBF20-ProA* (A25193) and the genotype of no tag B is *pCUP1-DBF20-ProA cdc15-as1* (A29124).