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 active only 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.**

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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 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 believed to activate Cdc15 (Asakawa *et al.*, 2001). Cdc15 in turn recruits Dbf2, the founding

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Abbreviations used: FEAR, Cdc14 early-anaphase release; MEN, mitotic exit network; SPB, spindle pole body.

member of the nuclear Dbf2-related (NDR) 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 believed 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 occurs only 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 activated only 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 and 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.

Although 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 after 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. Taken together, these observations raise the question of whether a pathway that 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 active only during meiosis II. Our analysis further reveals 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 occurs only 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 used 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 before 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 used 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 analogue, 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 later discussion of 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 1, A–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

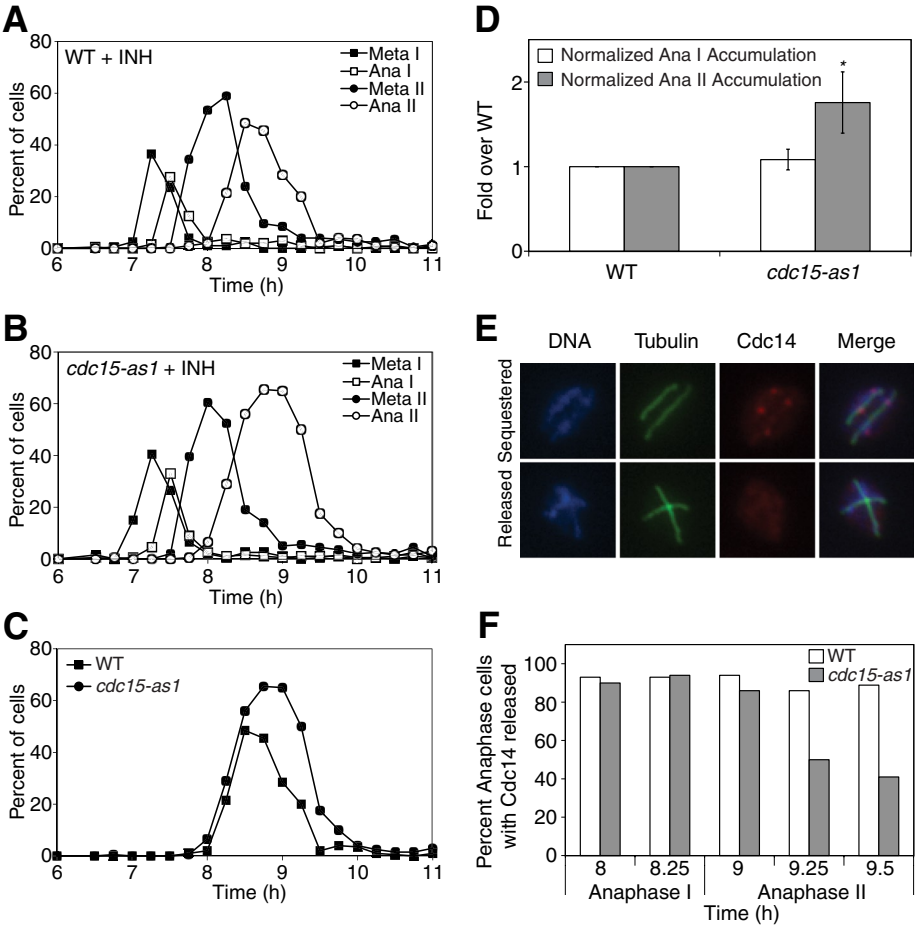

FIGURE 1: The MEN is dispensable for exit from meiosis I but is required for the timely exit from anaphase II. (A–D) Wild-type (A, A14201) or *cdc15-as1* (B, A19440) cells harboring a *GAL-NDT80* fusion as the sole source of *NDT80* were induced to sporulate. β-Estradiol, 1 μM, and 1-NA-PP1, 10 μM, were added to cultures 6 h 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. Two hundred 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; E) or released into the nucleus and cytoplasm (bottom; 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 h, $n = 35$, and wild type 9 h, $n = 36$).

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 (Supplemental 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 (Supplemental Figure S1, B–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 II. 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 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 1, E and F, and Supplemental 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 whereas 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 activated only 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 (Supplemental Figure S2, A–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 (Supplemental Figure S2, E–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 unclear.

Of the two Dbf2-like kinases present in *S. cerevisiae*, only Dbf20 was expressed at appreciable levels during meiosis (Supplemental Figure S2, I–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 2, A and B, and Supplemental Figure S3A; note that slower-migrating forms of Dbf20 can be detected only with the Dbf20-Myc fusion and not with the Dbf20-ProA fusion).

As during mitosis, in which 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 and Supplemental Figure S3, B and C). We conclude that the MEN is active only 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 not been able to determine the mechanisms that restrict MEN activity to exit from meiosis II, but, as described later, our analyses of MEN regulation during meiosis II revealed that the pathway is regulated in fundamentally different ways during meiosis II than during mitosis.

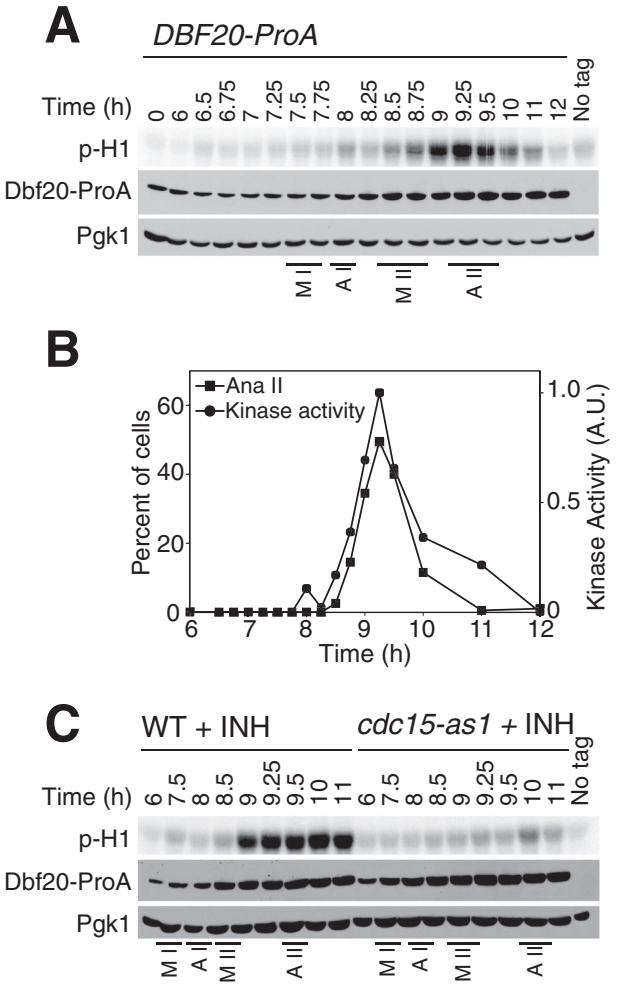

FIGURE 2: Dbf20 kinase activity peaks in anaphase II and depends on Cdc15 kinase activity. (A) Cells containing a *DBF20-ProA* fusion (A23162) were sporulated as described in Figure 1A, except that 1-NA-PP1 was not added. Samples were taken at the indicated times to determine Dbf20-associated kinase 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 (MI, metaphase I; AI, anaphase I; MII, metaphase II; AII, anaphase II). (B) Quantification of Dbf20-associated kinase activity (closed circles) and anaphase II spindles (closed squares; $n = 200$ 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.

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 green fluorescent protein (GFP), Myc, or hemagglutinin (HA), were never detected at SPBs in any stage of meiosis (Supplemental Figure S4, A–D). In addition, despite the role of Cdc15 in spore wall formation (Kamieniecki *et al.*, 2005; Pablo-Hernando *et al.*, 2007), Cdc15–enhanced GFP (eGFP) was not detected on SPBs during spore formation (Supplemental Figure S4B, bottom). However, the protein required for tethering these proteins to SPBs during mitosis, Nud1, was present at all SPBs throughout meiosis (Supplemental Figure S5, A and B).

Bfa1 localized to both SPBs during anaphase I as judged by colocalization with the SPB component Spc42. During anaphase II, Bfa1 localized to only two of the four SPBs (Supplemental Figure S5, C and 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

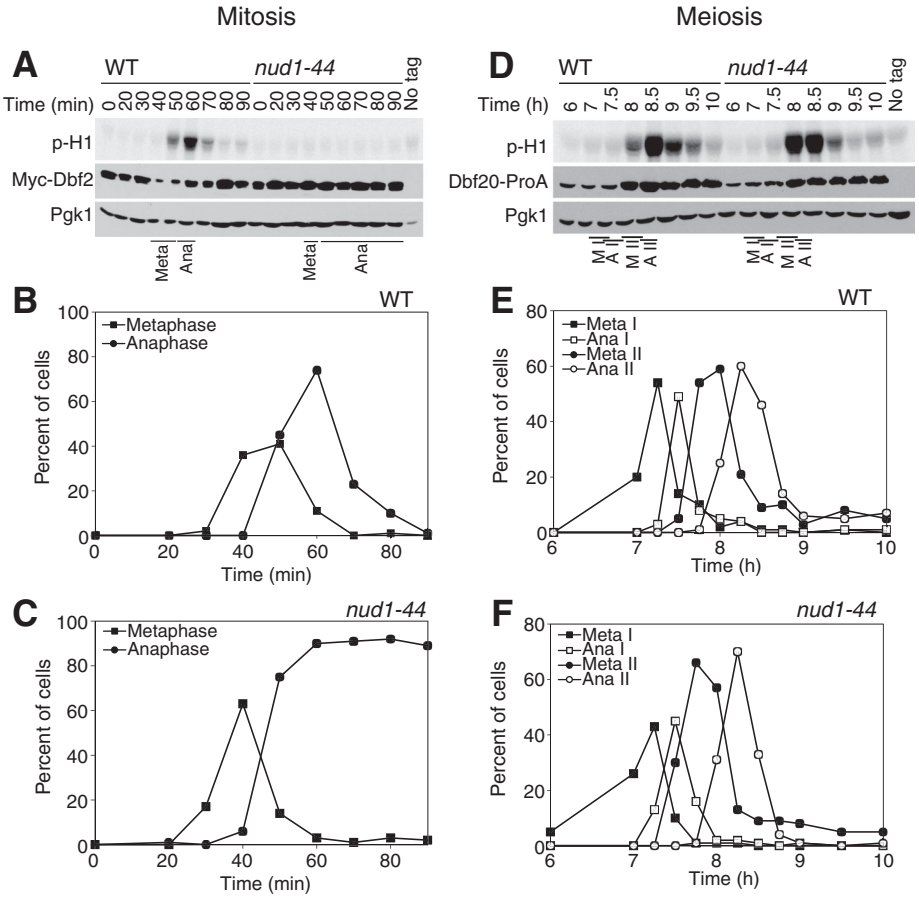

FIGURE 3: MEN signaling occurs in a *NUD1*-independent manner during meiosis. (A–C) The temperature 34°C is restrictive for *nud1-44*. Wild-type (A8499) and *nud1-44* (A28757) cells containing a *3MYC-DBF2* fusion were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium for 2 h at room temperature and then shifted to 34°C for 30 min. 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 time point). (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 5 h. At 6 h, cultures were induced with 1 μM 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).

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, we arrested *nud1-44* cells in G1 with pheromone. On release from the G1 arrest, *nud1-44* cells arrested in anaphase and failed to activate Dbf2 kinase at this temperature (Figure 3, A–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 3, D–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 (Supplemental Figure S6, A–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 does 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 division 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 4, A and 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 4, A–D). Expression of Dbf2 during the meiotic divisions led to Dbf2 kinase activity that is regulated similarly to that of Dbf20 (Figure 4, E–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 4, A–D; Visintin and Amon, 2001), but Dbf20 is largely inactive throughout the mitotic cell cycle (Figure 4, A–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.

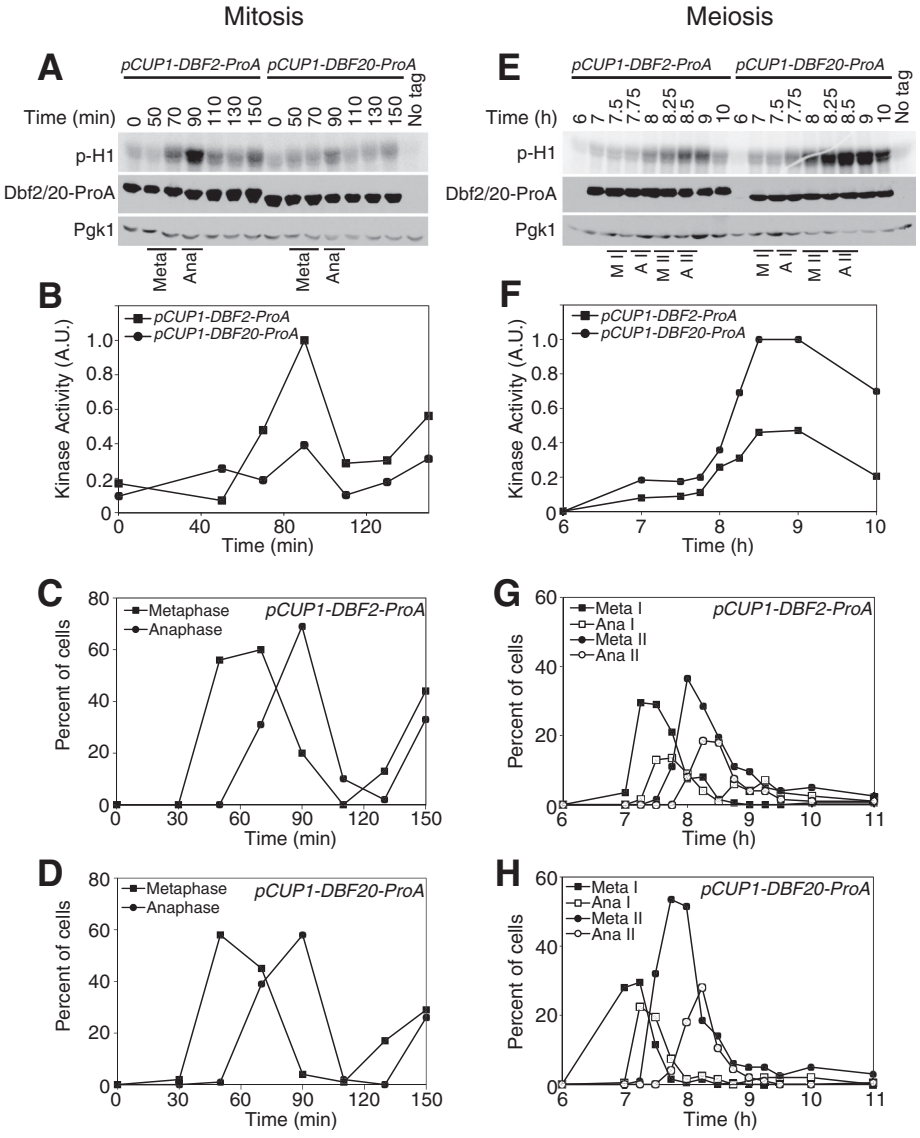

FIGURE 4: Differential regulation of Dbf2 and Dbf20 activity. (A–D) *pCUP1-DBF2-ProA* (A25020) or *pCUP1-DBF20-ProA* (A25193) cells were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium. $CuSO_4$, 50 μM, was added to the medium 2 h into the arrest. When the arrest was complete (2 h, 30 min), cells were released into pheromone-free YEPD medium containing 50 μM $CuSO_4$. 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 by the graph in B. (E–H) *pCUP1-DBF2-ProA* (A25028) or *pCUP1-DBF20-ProA* (A25195) cells were sporulated via the Ndt80 block-release protocol. β-Estradiol, 1 μM, and $CuSO_4$, 50 μM, were added to the medium 6 h 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 by the graph in F.

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 coimmunoprecipitation 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 5, A and 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 coimmunoprecipitation (Figure 5, C and 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 5, E and 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 6, A–C). In contrast, the Dbf20-Mob1 interaction occurred independently of *CDC15* during mitosis (Figure 6, D–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

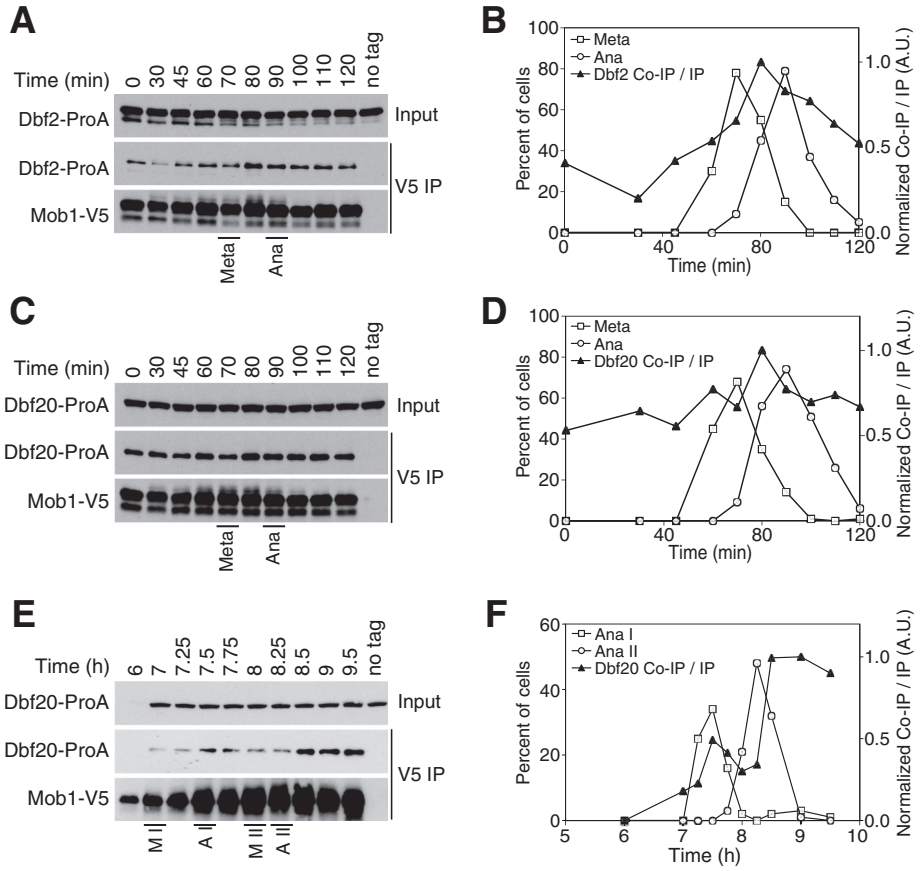

FIGURE 5: The Dbf20–Mob1 interaction is under cell cycle control in meiosis but not mitosis. (A, B) Cells containing *pCUP1-DBF2-ProA* and a *MOB1-V5* fusion (A27687) or *MOB1* (A25020, no tag control) were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium. $CuSO_4$, 50 μM, was added to the medium 2 h into the arrest. When the arrest was complete (2 h, 30 min), cells were released into pheromone-free YEPD medium containing 50 μM $CuSO_4$. Western blots in A show total Dbf2-ProA protein (input), immunoprecipitated Mob1-V5, and coimmunoprecipitated Dbf2-ProA at the indicated time points. (B) Quantification of coimmunoprecipitation expressed as the amount of Dbf2 coimmunoprecipitated over the amount of Mob1-V5 immunoprecipitated . 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 and 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 μM β-estradiol and 50 μM $CuSO_4$ at 6 h after transfer to sporulation medium. Western blots show immunoprecipitated Mob1-V5, coimmunoprecipitated Dbf20-ProA, and total Dbf20 protein (input) at the indicated time points (E). Quantification of the amount of Dbf20 coimmunoprecipitated 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.

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 play only 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, 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 coopted to fulfill a novel meiosis-specific function—spore-wall morphogenesis (Kamieniecki *et al.*, 2005; Pablo-Hernando *et al.*, 2007; Attner, unpublished observations). Because spore wall formation must occur only after the completion of the two meiotic divisions, it is perhaps not surprising that the MEN is active only 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 hyperactivate the MEN during mitosis failed to do so in

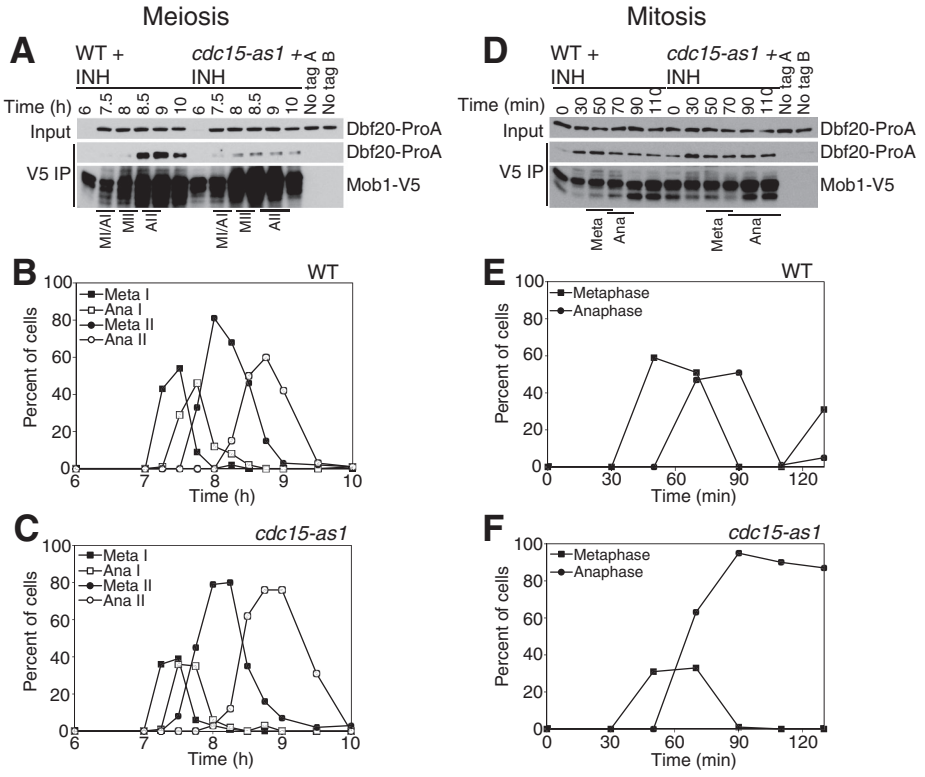

FIGURE 6: The Dbf20-Mob1 interaction depends on *CDC15* in meiosis. (A–C) Cells containing *pCUP1-DBF20-ProA*, a *MOB1-V5* fusion, and *CDC15* (A27371) or *cdc15-as1* (A29149) were induced to sporulate via the Ndt80 block-release protocol as in Figure 1A with the addition of 10 μM 1-NA-PP1 and 50 μM $CuSO_4$ at 6 h after transfer to sporulation medium. Western blots in A show total Dbf20-ProA protein (input), immunoprecipitated Mob1-V5, and coimmunoprecipitated Dbf20-ProA at the indicated time points. 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). (B, C) Meiotic progression in the two strains. (D–F) Cells containing *pCUP1-DBF20-ProA*, a *MOB1-V5* fusion, and *CDC15* (A27367) or *cdc15-as1* (A29125) were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium as in Figure 4A. $CuSO_4$, 50 μM, was added to the medium 2 h into the arrest. When the arrest was complete (2 h, 30 min), cells were released into YEPD medium containing 10 μM 1-NA-PP1 and 50 μM $CuSO_4$. (D) Total Dbf20-ProA protein (input), coimmunoprecipitated Dbf20-ProA, and immunoprecipitated Mob1-V5 for the indicated time points. (E, F) Mitotic progression of the two strains . 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).

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 homologues is in part due to their expression levels, with Dbf2 levels being low during the meiotic divisions (Supplemental 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 and 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 is 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, either this mechanism is 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 in which Cdc15 is activated during meiosis II, which then promotes the interaction and activity of the Dbf20–Mob1 kinase complex, maintaining Cdc14 in its released form. These events may require posttranslational modifications on Dbf20 that are restricted to meiosis II and independent of *CDC15* (Supplemental Figure S2K; Attner, 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 homologue encoded in the *S. cerevisiae* genome, but functional homologues 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 so 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 coopting 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 homologues, Sid2 and Slk1. During mitosis, Sid2 functions in SIN signaling (Sparks *et al.*, 1999), but, as in budding yeast, the other Dbf2 homologue, 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 use 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 mammalian sterile 20–like kinase 1/2, NDR kinase, and hMob1, respectively, and they 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 are listed in Supplemental 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 5× to SK1.

Sporulation

Strains were grown overnight on yeast extract/peptone/glycerol plates (3% glycerol) and then transferred to 4% yeast extract/peptone/dextrose (YEPD; 4% glucose) plates in the morning. Cells were cultured in YEPD medium to saturation (~24 h) and then diluted into buffered YTA medium (1% yeast extract, 2% tryptone, 1% potassium acetate, 50 mM potassium phthalate) to OD_{600} = 0.35. Cells were grown overnight and then resuspended in sporulation medium (1% potassium acetate, pH 7) at 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 h. Due to the lack of *NDT80, GAL-NDT80* cells will arrest in pachytene. At t = 6 h, when most cells had reached pachytene, 1μM β-estradiol was added to the medium, allowing cells to progress through the meiotic divisions in a synchronous manner. For *cdc15-as1* experiments, 10 μM 4-amino-1-tert-butyl-3-(1′-naphthyl) pyrazolo[3,4-d]pyrimidine (1-NA-PP1; Toronto Research Chemicals, North York, Canada) 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-min fixation with 3.7% formaldehyde in 0.1 M 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 2 h each. For Bfa1-GFP imaging, cells were fixed for 15 min with 3.7% formaldehyde in KPi. Cells were resuspended in 0.1 M KPi/1.2 M sorbitol/1% Triton. Cells were resuspended in 0.05 μg/ml 4′,6-diamidino-2-phenylindole in KPi/sorbitol. Cells were imaged with a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) and a Hamamatsu ORCA-ER digital camera (Hamamatsu, Hamamatsu, Japan).

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 min in 5% trichloroacetic acid. Cell pellets were washed once in acetone and dried overnight. Cells were lysed in 100 μl of lysis buffer (50 mM Tris-Cl at pH 7.5, 1 mM EDTA, 2.75 mM dithiothreitol [DTT], complete protease inhibitor cocktail [Roche, Mannheim, Germany]) with a bead mill. After sample buffer was added, cell lysates were boiled. Myc-tagged proteins were detected with anti-Myc (9E10 epitope; Covance) antibodies at a dilution of 1:500. HA-tagged proteins were detected with anti-HA (HA.11; Covance) antibodies at a dilution of 1:1000. ProA-tagged proteins were detected by incubation with rabbit immunoglobulin G (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 1–2 mg of lysate was used in immunoprecipitations (total volume of 150 µl). Dbf20-ProA was immunoprecipitated using IgG-coupled Dynabeads (Invitrogen Dynal AS, Oslo, Norway) and incubated for 1 h at 4°C. To prepare IgG-coupled beads, Dynabeads were incubated with 0.33 mg/ml rabbit IgG (Sigma-Aldrich) and 1 M ammonium sulfate in 0.1 M sodium phosphate, pH 7.4, buffer at 37°C overnight. Phosphorylation of histone H1 was quantified with the PhosphorImaging system (GE Healthcare Biosciences).

Coimmunoprecipitation assays

Cells were resuspended in 200 µl of NP40 lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% NP40, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 15 mM *p*-nitrophenylphosphate, 1 mM DTT, complete protease inhibitor cocktail [Roche]) and lysed in a bead mill. From 1 to 2 mg (meiosis) or 500 µg (mitosis) of extract was used in immunoprecipitation experiments (volume of 150 µl) with agarose beads directly conjugated to the V5 epitope (Sigma-Aldrich). Lysates were incubated for 45 min at 4°C and washed, and beads were resuspended in sample buffer and boiled. Proteins were analyzed by Western blot analysis.

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