Cell Cycle Regulation During Gametogenesis in Budding Yeast

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ABSTRACT

Sexual reproduction depends on meiosis, the specialized cell division that gives rise to gametes. During meiosis, two consecutive rounds of chromosome segregation follow one round of DNA replication to yield four haploid gametes from one diploid progenitor. In meiosis I, homologous chromosomes segregate and in meiosis II, sister chromatids split. Much of the same cell cycle machinery controls mitosis and meiosis. However, segregation of homologous chromosomes in meiosis I and progression into meiosis II directly after meiosis I necessitate several modifications to the basic cell cycle machinery. In this thesis, I have investigated how cell cycle regulators function during gametogenesis. First, I show that the mitotic exit network, which is a signaling pathway essential for mitotic exit, is dispensable for the meiotic divisions, and in fact signals via a mechanism distinct from mitosis. Second, I present data that the Polo kinase Cdc5, which activates mitotic exit in budding yeast, has gained additional roles during meiosis I. I show that *CDC5* is required for the removal of cohesin from chromosome arms in meiosis I, which is a prerequisite for meiosis I segregation. Despite the central role of *CDC5* in regulating meiosis I, *CDC5* is dispensable during meiosis II. In sum, understanding how cell cycle regulators control the specialized meiotic divisions has improved our understanding of how different cell division types are established.

Thesis supervisor: Angelika Amon
Title: Professor of Biology

This thesis is dedicated with love to my parents, Janet and David, and my sister, Rachel

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Chapter 1:

Introduction

A comparison between mitosis and meiosis

Accurate chromosome segregation lies at the heart of all cellular reproduction. Asexual reproduction and the development of a multicellular organism from a single cell are just two processes requiring faithful cell division. The cell division cycle is achieved by alternating DNA replication (S phase) with chromosome segregation (mitosis). During mitosis, chromosomes are segregated such that two cells genetically identical to the progenitor cell are formed. In sexually reproducing organisms, a specialized cell division called meiosis occurs in cells destined to become gametes. During meiosis, two rounds of chromosome segregation follow one round of DNA replication to produce four cells with half the genomic content of the progenitor cell. In diploid organisms, the fusion of two haploid gametes creates a zygote with the proper ploidy in the next generation.

Mitosis and meiosis have many similarities (Figure 1). Cyclin-dependent kinase (CDK) activity and regulated proteolysis drive progression through both cell division types. Both mitosis and meiosis require sister chromatids to be attached to a microtubule-based spindle and to be linked to each other by a protein complex called cohesin. Upon dissolution of cohesion, chromosomes segregate. Despite these general similarities, mitosis and meiosis have several very important distinctions. First, at exit from mitosis, CDKs are inactivated. The rapid resetting of the cell to a low CDK state creates conditions that allow cytokinesis and entry into the next cell cycle. In contrast, it is thought that CDKs are partially inactivated during meiosis I exit in order to ensure that cells disassemble the meiosis I spindle but immediately undergo a second division without exiting the meiotic program. Second, during mitosis sister chromatids segregate. However, during meiosis I, homologous chromosomes rather than sister chromatids

segregate. Understanding how signaling pathways and key mitotic events are modulated during meiosis has provided critical insights into how different cell division types are established. In this chapter, I will provide a broad overview of the mitotic cell cycle and review how one such cell cycle regulatory pathway, the mitotic exit network (MEN), functions in mitosis. I will then describe the meiotic cell divisions in more detail, focusing on the parallels between mitosis and meiosis and the specializations required for homologous chromosomes to segregate during meiosis I.

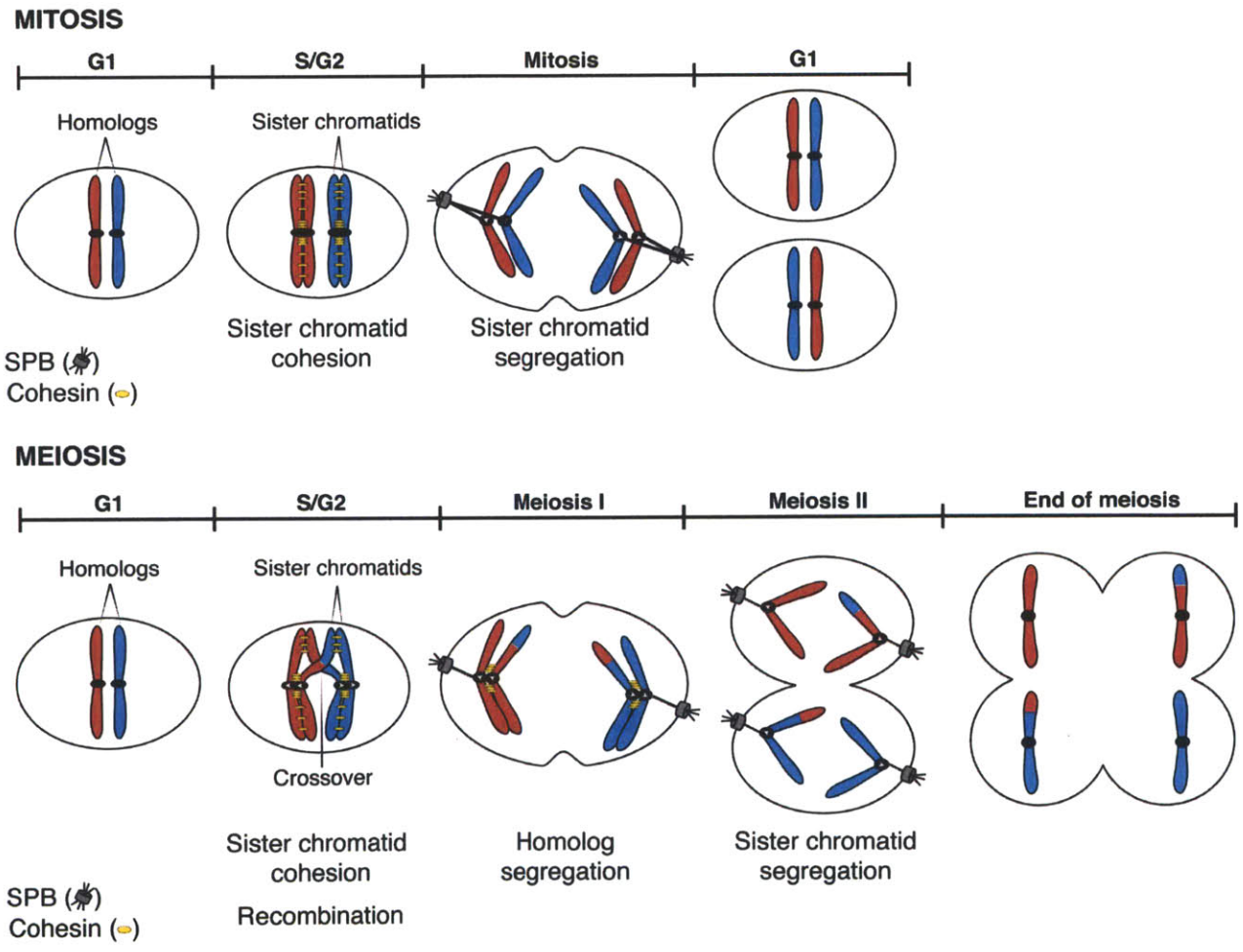

Figure 1. Comparison between mitosis and meiosis

(Top panel) During mitosis, two cells genetically identical to the original cell are formed. This is achieved through alternating rounds of DNA replication and chromosome segregation. During S phase, DNA replicates and sister chromatids are held together by cohesin complexes. In mitosis, chromosomes attach to the mitotic spindle, and sister chromatids are separated. (Bottom panel) During meiosis, the genomic complement of the original cell is halved. In most cases, a diploid progenitor produces four haploid gametes. In meiosis, much of the fundamental cell cycle machinery is the same as in mitosis. However, during meiosis, two rounds of chromosome segregation follow one round of DNA replication. Meiosis II is a mitosis-like division in which sister chromatids split. However, meiosis I is unique in that homologous chromosomes segregate. Figure used with permission from Matt Miller and Elçin Ünal.

Mitotic cell cycle

Mitosis is the cell cycle stage when chromosomes are segregated. Chromosome segregation must be accurate; chromosome missegregation yields aneuploid cells with an incorrect amount of DNA. Remarkably, countless mitoses occur in a developing human embryo from fertilization until birth, and the resulting cells in the newborn have the correct genomic complement. Furthermore, chromosome missegregation is a hallmark of cancer. Understanding the fundamental principles underlying correct and aberrant cell division is therefore of important medical relevance. The core components required for mitosis are conserved across species; therefore, the genetically tractable, simple eukaryote *Saccharomyces cerevisiae* (budding yeast) has provided important insights into how chromosome segregation occurs in all organisms.

CDK activity drives progression through the cell cycle

Progression through the cell cycle is driven by cyclin-dependent kinase (CDK) activity, initially discovered through seminal genetic screens identifying mutants that arrest in specific cell cycle stages and biochemical experiments that identified the existence of a cell-cycle oscillator and purified CDK activity (Evans et al., 1983; Hara et al., 1980; Hartwell et al., 1970; Lohka et al., 1988; Masui and Markert, 1971; Nurse, 1975). CDKs are composed of two subunits: a catalytic kinase subunit and a regulatory cyclin subunit. In budding yeast, there is one CDK subunit, encoded by *CDC28*, which is expressed throughout the cell cycle. Budding yeast contain nine cyclins: three G1-cyclins *CLN1-3*, and six B-type cyclins, encoded by *CLB1-6* (Mendenhall and Hodge, 1998). *CLB5-6* promote S-phase, and *CLB1-4* predominantly control mitotic events (Fitch et al., 1992; Schwob and Nasmyth, 1993). CDK activity of the various cyclin-CDK complexes in budding yeast is illustrated in Figure 2. Changes in CDK activity lead to changes in substrate phosphorylation, and thus substrate function. CDKs phosphorylate a wide range of substrates to control cell cycle processes. I will discuss the regulation and role of CDKs as it becomes relevant.

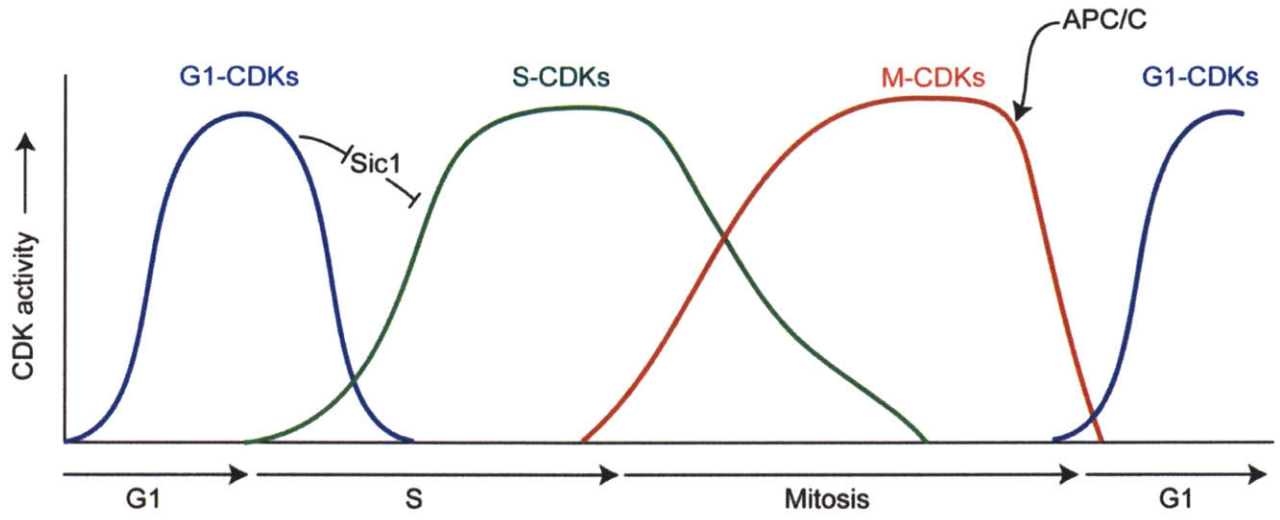

Figure 2. CDK activity oscillations throughout the budding yeast cell cycle. G1-CDK activity is high in G1, and serves the important role of inactivating the B-type-CDK inhibitor Sic1. Sic1 degradation triggers the rise of S-CDK activity. M-CDK activity peaks during mitosis. M-CDK activity declines precipitously at mitotic exit when B-type cyclin degradation is triggered by the E3 ubiquitin ligase APC/C. Exit from mitosis also establishes conditions necessary to begin accumulating the next round of G1 cyclins.

Overview of cell cycle events leading to mitosis

During G1, cells prepare to replicate DNA by assembling pre-replicative complexes (pre-RCs) onto origins of replication. Pre-RCs consist of an origin recognition complex (ORC) bound to origin DNA, Cdc6, and Cdt1. The major role of the pre-RC is to load the replicative helicase Mcm2-7 (Bell and Dutta, 2002). S-CDKs (Clb5/6-CDK) promote DNA replication and are activated in part by Cln-CDK-dependent degradation of the Clb-CDK inhibitor Sic1 (Schwob et al., 1994; Tyers, 1996). S-CDKs promote DNA replication through two major functions. First, S phase-CDKs inhibit pre-RC components, thus ensuring that pre-RCs are not reassembled during subsequent cell cycle stages. Second, S-CDKs in conjunction with the Dbf4-dependent kinase DDK

control the formation of the preinitiation complex and helicase activation (Bousset and Diffley, 1998; Donaldson et al., 1998). Two critical substrates of S-CDK in budding yeast are Sld2 and Sld3, two essential components of the replication pre-initiation complex. CDK-dependent phosphorylation of Sld2/3 promote their association with other factors in the pre-initiation complex, and is required for DNA replication to occur (Masumoto et al., 2002; Tanaka et al., 2007; Zegerman and Diffley, 2007).

During S phase, deposition of cohesin molecules, in addition to DNA replication, occurs (Hirano, 2000; Uhlmann and Nasmyth, 1998). Cohesin is the protein complex that provides linkages between sister chromatids, and cohesion between sister chromatids is critical for the execution of mitosis (Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997). Cohesin is a ring-shaped, evolutionarily conserved protein complex made up of four subunits: two SMC (structural maintenance of chromosomes) proteins Smc1 and Smc3, the cohesin subunit Scc3, and the α-kleisin Scc1/Mcd1 (Figure 3). Scc1/Mcd1 (henceforth referred to as Scc1) is the α-kleisin subunit of cohesin that connects the SMC dimer. How cohesin grasps sister chromatids is under debate. One model posits that the cohesin ring encircles the two sister chromatids, and a second model posits that cohesin rings oligomerize to tether sister chromatids (Nasmyth and Haering, 2009; Onn et al., 2008; Figure 3).

Cohesin binds chromatin during the G1/S transition in budding yeast at pericentromeric regions and on chromosome arms. Cohesin binds at a lower density around chromosome arms and is concentrated at cohesin-associated regions (CARs) which occur at approximately 15kb intervals (Blat and Kleckner, 1999; Glynn et al., 2004; Laloraya et al., 2000). Through a poorly defined mechanism, cohesin associates

with chromatin in a process dependent on *SCC2* and *SCC4* (Ciosk et al., 2000). Cohesin loading onto chromosomes does not automatically create cohesion between sister chromatids. Cohesion is established by the evolutionarily conserved protein Eco1/Ctf17 (Skibbens et al., 1999; Toth et al., 1999). Eco1 acetylates the Smc3 subunit of cohesin to promote the cohesive state of cohesin (Unal et al., 2008). Eco1 is not needed for the maintanence of cohesion; once cohesion is generated, Eco1 is dispensable (Skibbens et al., 1999; Toth et al., 1999). After S phase is complete, mammalian cells enter a second gap phase, G2. In contrast, G2 in budding yeast is not clearly defined.

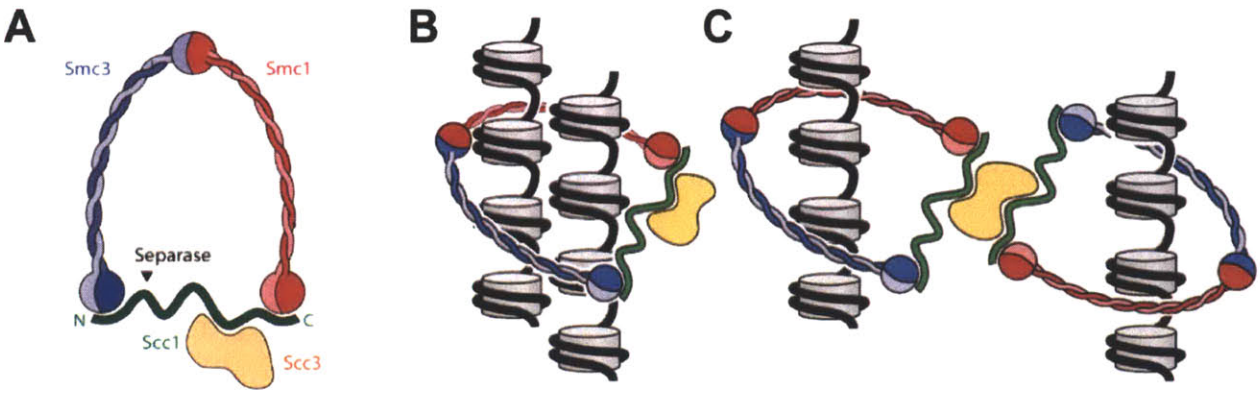

Figure 3. Structure of the cohesin complex.
(A) Structure of the cohesin complex. Cohesin is a large ring-shaped structure. Smc1 and Smc3 contain long coiled-coil domains. Scc1 (also called Mcd1 and Rad21) links the two head regions of the Smc proteins. Scc1 is the subunit of cohesin that is cleaved by separase. During meiosis, Scc1 is replaced by the meiosis-specific α-kleisin subunit Rec8. (B-C) Models for how cohesin links sister chromatids. Cohesin might encircle both chromatids (B) or cohesin molecules might associate (C). It is also possible that cohesin molecules catenate (not shown). Adapted from Haering and Nasmyth 2009.

Overview of prophase and metaphase events

Ultimately, in all eukaryotes analyzed, mitotic cyclin dependent kinase (CDK) activity promotes entry into mitosis (Morgan, 1997). Activation of M-CDKs in most organisms (other than budding yeast) depends on the dephosphorylation of an inhibitory tyrosine on the catalytic CDK subunit by Cdc25-family phosphatases and an ensuing positive feedback loop (Nurse, 1990). M-CDKs trigger the events of mitosis: chromosome condensation, centrosome splitting, nuclear envelope breakdown, spindle formation, metaphase I-anaphase I transition and spindle elongation (Miele, 2004; Nigg, 2001; Rahal and Amon, 2008). M-CDKs govern mitosis in all eukaryotes, but the mechanics of mitosis vary slightly between organisms. Notably, budding yeast undergoes mitosis with an intact nucleus and with the centrosomes, called spindle pole bodies (SPBs), embedded in the nuclear envelope. Differences in cyclins also exist. Interestingly, in yeast, different B-type cyclins (Clbs) have different abilities to promote mitotic events. Only Clb2 is able to sustain mitotic progression on its own (Fitch et al., 1992).

In addition to CDK activity, an additional conserved kinase coordinates multiple aspects of mitosis. This family of kinases, polo kinases, is defined by a kinase domain at its N-terminus and a polo-box domain at its C-terminus. The polo-box domain can recognize phosphorylated serines and threonines in certain contexts, thus targeting the polo kinase to proteins that have already been phosphorylated (Elia et al., 2003). In most eukaryotes, Polo kinases play a critical role in the full activation of M-CDKs, cohesin removal, centrosome separation, spindle assembly, exit from mitosis, and

cytokinesis (Archambault and Glover, 2009). In most organisms examined, polo kinase mutants build abnormal mitotic spindles (Lane and Nigg, 1996; Sunkel and Glover, 1988). In contrast, temperature-sensitive mutants in the sole budding yeast polo kinase *CDC5* arrest in anaphase (Kitada et al., 1993). Therefore, the essential role for polo kinase in budding yeast is in exit from mitosis.

In prophase, chromosomes condense, a process mediated by condensin complexes, in order to transform chromosomes into discrete, movable units (Hirano, 2005). In addition, the majority of arm cohesion is lost during prophase in vertebrates. The mammalian polo-like kinase, Plk1, mediates cohesion loss via the prophase pathway (Losada et al., 2002; Sumara et al., 2002). The vertebrate homolog of Scc3, SA2, is the critical target of Plk1. An allele of SA2 containing the Plk1 phosphorylation sites mutated to alanine blocks prophase removal. Furthermore, the cohesin removed from chromosomes during prophase contains phosphorylated SA2 (Hauf et al., 2005). In addition, prophase removal of cohesion depends on another factor called Wapl, as cells depleted for Wapl contain phosphorylated SA2 on chromosome arms in metaphase (Gandhi et al., 2006; Kueng et al., 2006). Cohesion is protected around centromeric regions by a shugoshin/MEI-S332 family member (Kerrebrock et al., 1995; Watanabe and Kitajima, 2005). Indeed, in human cells depleted for Sgo1, cohesin and cohesion are lost from the entire chromosome prematurely (McGuinness et al., 2005; Salic et al., 2004). In contrast to mammalian cells, budding yeast lose all cohesion at the metaphase - anaphase transition. Although the budding yeast polo kinase Cdc5 does not promote prophase removal of cohesin, Cdc5 does phosphorylate Scc1 to facilitate its cleavage (Alexandru et al., 2001; Hornig and Uhlmann, 2004).

During metaphase, chromosomes attach to the mitotic spindle. Attachment occurs via the kinetochore, a large protein complex that links the chromosomes to spindle microtubules. The inner kinetochore binds centromeric regions, and the outer kinetochore captures microtubules (Cheeseman and Desai, 2008). In yeast, where only one microtubule binds to a kinetochore, it is thought that the outer kinetochore component Dam1 forms a collar around the microtubule and links to the outer kinetochore by binding to the Ndc80 complex (Cheeseman et al., 2001; Westermann et al., 2006). The conserved Ndc80 complex contains microtubule binding activity as well, thus linking the outer kinetochore to spindle microtubules (Cheeseman et al., 2006; DeLuca et al., 2006; Wei et al., 2007). The metaphase – anaphase transition is not triggered until sister chromatids are properly attached to microtubules emanating from opposite spindles poles. Tension on the mitotic spindle can be achieved because cohesion between the sister chromatids resists the pulling force of the microtubules. The spindle assembly checkpoint (SAC) senses the proper biorientation of sister chromatids on the mitotic spindle, and inhibits the metaphase - anaphase transition until all chromosomes are bioriented. Unattached kinetochores send a "wait anaphase signal" that inhibits the E3 ubiquitin ligase called the anaphase promoting complex/cyclosome (APC/C), which is required for the metaphase-anaphase transition, until all chromosomes are properly bioriented on the spindle (Musacchio and Hardwick, 2002; Musacchio and Salmon, 2007). If chromosomes are not bioriented, and thus not under tension, the Aurora B kinase phosphorylates kinetochore components including Dam1, causing destabilization of the microtubule-kinetochore interaction (Cheeseman et al., 2002).

The dissolution of sister chromatid cohesion triggers the metaphase - anaphase transition, and is achieved by the proteolytic cleavage of the Scc1 subunit of the cohesin complex by the protease separase (Uhlmann et al., 1999). When the SAC is satisfied, the APC/C in association with its activating subunit Cdc20 targets the separase inhibitor securin for destruction. Securin degradation frees separase to cleave Scc1, thus triggering anaphase, the specific stage of mitosis when chromosomes segregate (Ciosk et al., 1998; Cohen-Fix et al., 1996). After chromosomes segregate, cells exit from mitosis. During this stage of mitosis, the mitotic spindle is disassembled, chromosomes decondense, and mitotic CDKs are inactivated. Exit from mitosis establishes conditions necessary for cells to undergo cytokinesis, enter G1 of the next cell cycle, and license origins for the next round of DNA replication (Stegmeier and Amon, 2004).

Function of Cdc14 in mitotic exit

In higher eukaryotes, most mitotic CDKs are inactivated at the metaphase-anaphase transition (Peters, 2002). However, in budding yeast a significant pool of mitotic CDKs remains after chromosome segregation (Wasch and Cross, 2002). CDK inactivation occurs through the activity of the essential phosphatase Cdc14. Cdc14 reverses CDK-dependent phosphorylation, and has several key targets that promote the inactivation of CDKs (Visintin et al., 1998). First, Cdc14 de-phosphorylates the APC/C activating subunit Cdh1 (Jaspersen et al., 1999; Visintin et al., 1998). De-phosphorylation of Cdh1 promotes its association with the APC/C, and thus the destruction of mitotic cyclins and polo kinase. Cdc14 also de-phosphorylates (and thus activates) the Clb inhibitor Sic1 and the *SIC1* transcription factor, Swi5, to further ensure

that Clbs are inhibited (Toyn et al., 1997; Visintin et al., 1998). Cdc14 activity is tightly controlled; it is bound by its inhibitor Cfi1/Net1 in the nucleolus for most of the cell cycle, and is released into the nucleus and cytoplasm during anaphase (Shou et al., 1999; Visintin et al., 1999). It is thought that dissociation of the Cdc14-Cfi1/Net1 complex is promoted by the phosphorylation of both Cdc14 and Cfi1/Net1 (Shou et al., 2002; Shou et al., 1999; Visintin et al., 2003; Yoshida and Toh-e, 2002). Two signaling pathways control the dissociation of the Cdc14-Cfi/Net1 complex, thus releasing Cdc14 from the nucleolus: the non-essential FEAR (Cdc-fourteen early anaphase release) network, and the essential mitotic exit network (MEN).

The FEAR network

The FEAR network is a non-essential pathway that promotes a transient burst of Cdc14 release during early anaphase (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). FEAR network components include separase, the separase-binding protein Slk19, the replication fork block protein Fob1, a protein of unknown function Spo12, the polo kinase Cdc5, and the phosphatase $PP2A^{Cdc55}$. The FEAR network controls Cdc14-Cfi1/Net1 dissociation through a poorly defined signaling mechanism (Figure 4). First, separase links the metaphase-anaphase transition to exit from mitosis. Indeed, mitotic exit is inhibited when securin is stabilized, as Clb2 is not degraded in these cells (Cohen-Fix and Koshland, 1999). Separase not only causes dissolution of sister chromatid cohesion, but also acts in conjunction with Slk19 to mediate the early release of Cdc14, possibly by downregulating PP2A and allowing CDKs to phosphorylate Cfi1/Net1 (Azzam et al., 2004; Queralt et al., 2006). Activation

of the proteins Zds1 and Zds2 may also contribute to PP2A downregulation (Queralt and Uhlmann, 2008). Second, the *ESP1-SLK19* branch of the FEAR network acts upstream and parallel to *SPO12*. Spo12, a protein activated by CDK-dependent phosphorylation in anaphase and dephosphorylated by Cdc14, acts positively in the FEAR network, likely by inhibiting Fob1, a nucleolar protein that stabilizes the Cdc14-Cfi/Net1 interaction (Stegmeier et al., 2004; Tomson et al., 2009; Visintin et al., 2003). Third, the polo kinase Cdc5 promotes phosphorylation of both Cdc14 and Cfi1/Net1 (Shou et al., 2002; Visintin et al., 2003; Yoshida and Toh-e, 2002).

Although non-essential, the FEAR network serves important roles in coordinating anaphase. Proper segregation of the rDNA repeats, which encode the rRNA and define the nucleolus, is dependent upon the FEAR network (Rock and Amon, 2009). Cdc14 dephosphorylates several microtubule-binding proteins, resulting in the stabilization of the mitotic spindle (Higuchi and Uhlmann, 2005; Woodbury and Morgan, 2007). Finally, FEAR-released Cdc14 plays an important role in activating the MEN (Jaspersen and Morgan, 2000; Stegmeier et al., 2002). Importantly, in contrast to the 10-20 minute mitotic exit delay that FEAR mutants display, the FEAR network plays an essential role in regulating the meiosis I-meiosis II transition (Buonomo et al., 2003; Marston et al., 2003). I will discuss the meiotic role for the FEAR network later in this chapter.

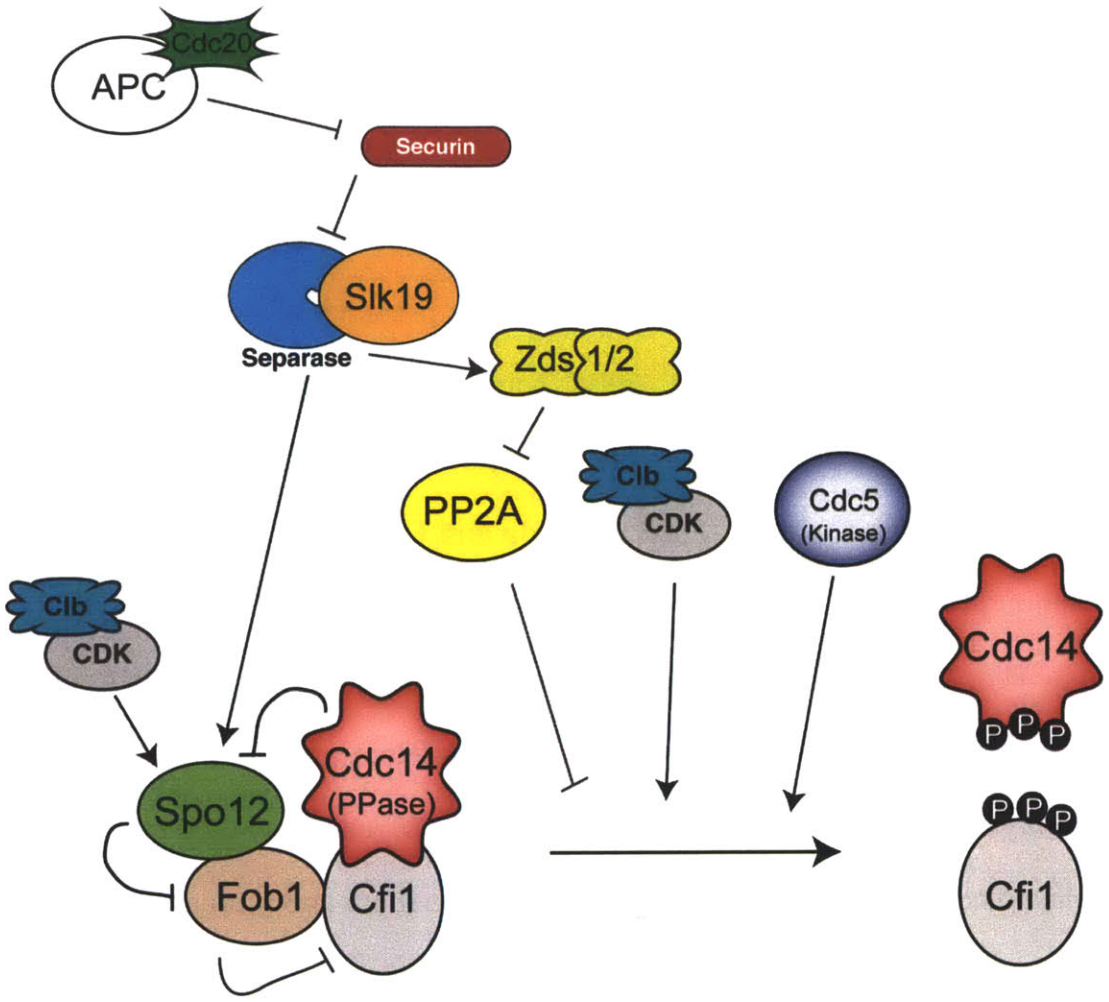

Figure 4. Schematic of the FEAR network

The FEAR network links the metaphase – anaphase transition to mitotic exit. Upon APC/C^{Cdc20}- dependent degradation of securin, separase promotes FEAR network activity. Separase-Slk19 inhibits the PP2A phosphatase and acts upstream of Spo12. Spo12 inhibits the Fob1 inhibitor during anaphase. Together with CDK activity and Cdc5 activity, Cdc14 is released from the nucleolus early in anaphase.

The Mitotic Exit Network (MEN)

The MEN is essential for mitotic exit in budding yeast. MEN mutants arrest in late anaphase with high CDK activity and Cdc14 sequestered in the nucleolus (Jaspersen et

al., 1998; Shou et al., 1999; Visintin et al., 1999). The MEN is a conserved GTPase signaling cascade, consisting of a small GTPase, a two-component GAP, a sterile 20-like kinase, an NDR kinase, a Mob1-family protein (activating subunit for NDR kinase), and a scaffold (Stegmeier and Amon, 2004). The small GTPase central to MEN signaling is encoded by *TEM1* (Shirayama et al., 1994) and is negatively regulated by Bub2-Bfa1, a two-component GAP (Bardin et al., 2000; Fesquet et al., 1999; Geymonat et al., 2002; Pereira et al., 2000; Wang et al., 2000). During anaphase, Tem1 is activated and propagates a signal to the sterile 20-like kinase Cdc15, which then activates the NDR-kinase complex Dbf2-Mob1 (Asakawa et al., 2001; Mah et al., 2001; Visintin and Amon, 2001). Nud1, a component of the spindle pole body (SPB), acts as a scaffold for MEN signaling at the SPB (Adams and Kilmartin, 1999; Bardin et al., 2000; Geymonat et al., 2002; Gruneberg et al., 2000; Visintin and Amon, 2001). Finally, although not a core component of the MEN, the polo kinase Cdc5 activates the MEN at multiple points (Hu et al., 2001; Rock and Amon, 2011). A schematic diagram of the MEN is shown in Figure 5.

MEN components are organized into signaling modules at the SPB. *NUD1*, which encodes the scaffold at the SPB, is required for the localization of MEN components to the SPB, and *NUD1*-temperature sensitive mutants arrest in anaphase (Adams and Kilmartin, 1999; Gruneberg et al., 2000; Visintin and Amon, 2001). Tem1 localizes to SPBs from metaphase until late anaphase, and SPB localization is essential for Tem1 function (Bardin et al., 2000; Pereira et al., 2000; Valerio-Santiago and Monje-Casas, 2011). Upon Tem1 activation in anaphase, Tem1-GTP is thought to recruit Cdc15 to SPBs (Asakawa et al., 2001). Although Tem1 is enriched on the daughter-bound

spindle pole, Cdc15 is recruited to both SPBs, which in turn recruits Dbf2-Mob1 to SPBs (Cenamor et al., 1999; Luca et al., 2001; Visintin and Amon, 2001; Xu et al., 2000; Yoshida and Toh-e, 2001). Cdc15 then promotes Dbf2-Mob1 activation through two mechanisms. First, Cdc15 has been shown to phosphorylate Dbf2 directly *in vitro*, and this phosphorylation likely contributes to Dbf2-Mob1 activation *in vivo* (Mah et al., 2001). Second, Cdc15 recruits Dbf2-Mob1 to SPBs by phosphorylating the scaffold Nud1, which creates a binding site for Mob1, the activating subunit for the Dbf2 kinase (Rock et al., personal communication). How the MEN causes sustained Cdc14 release is not completely clear, but occurs at least partially through Dbf2-dependent phosphorylation near the nuclear localization signal of Cdc14 (Mohl et al., 2009).

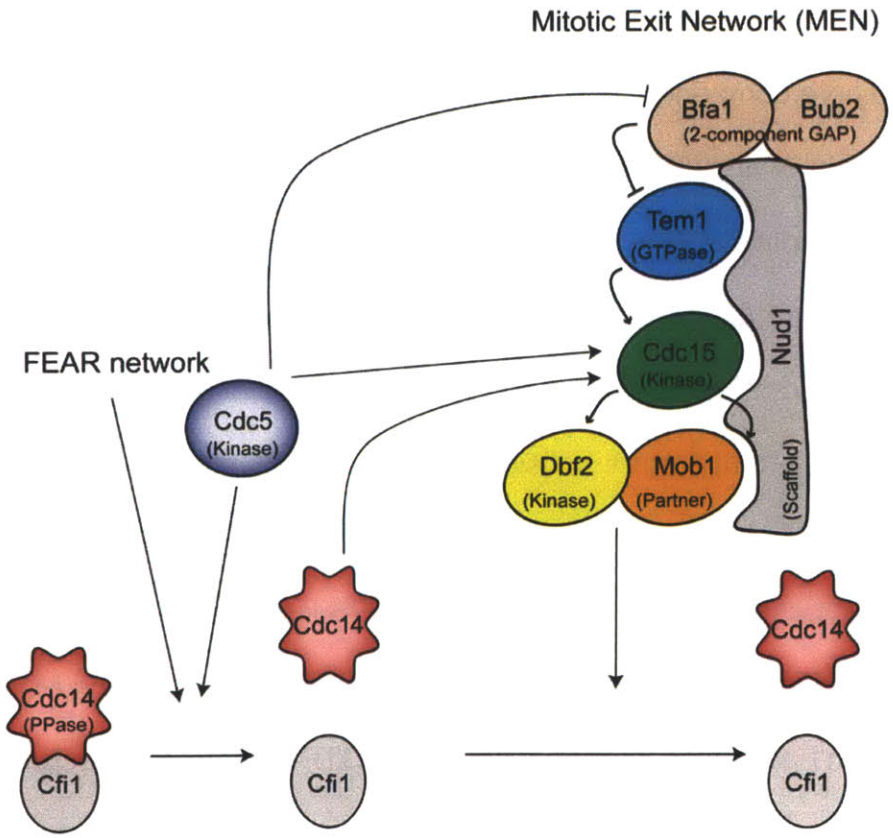

Figure 5. Diagram of the Mitotic Exit Network

The MEN is a GTPase signaling cascade anchored at the SPB. The FEAR network and Cdc5 promote an early burst of Cdc14 activity that contributes to the activation of the MEN. The MEN is required for sustained release of Cdc14 from its inhibitor in the nucleolus and mitotic exit.

Control of the MEN by spatial and temporal signals

The MEN integrates multiple spatial and temporal cellular signals, thus ensuring that MEN activation only occurs during anaphase. Much progress has been made in understanding the spatial control of MEN activation by spindle position. Because budding yeast divide asymmetrically, an additional challenge to proper chromosome segregation is present: one complement of the genome must be segregated into the bud. The bud site is selected in G1, so the axis of cell division is determined prior to mitosis. Therefore, budding yeast must only activate the MEN once the spindle is properly positioned along the mother-bud axis. Tem1, the GTPase central to MEN signaling which is enriched on the daughter-bound SPB, acts as a sensor moving between two zones: an inhibitory zone in the mother cell and an activating zone in the daughter cell (Bardin et al., 2000; Pereira et al., 2000). A diagram of the zone model is shown in Figure 6.

The inhibitory zone is created by the protein kinase Kin4, which localizes to the mother cell cortex throughout the cell cycle and to the SPB of the mother cell during anaphase (Maekawa et al., 2007). Kin4 was initially identified as a central component of the spindle position checkpoint (SPOC). In response to a mispositioned spindle, Kin4

inhibits MEN activation until the spindle is properly positioned. Indeed in *kin4Δ* mutants, cells with mispositioned spindles will exit from mitosis with both nuclei contained in the mother cell, whereas wild-type cells will delay mitotic exit until the spindle has been properly positioned (D'Aquino et al., 2005; Pereira and Schiebel, 2005). In cells in which an anaphase spindle has elongated within the mother cell, Kin4 localizes to both SPBs, and Tem1 is no longer localized to SPBs (D'Aquino et al., 2005). Interestingly, tethering of Tem1 to SPBs suppresses the anaphase arrest caused by overexpression of *KIN4*, suggesting that a critical function for the SPOC is keeping Tem1 off SPBs (Valerio-Santiago and Monje-Casas, 2011). Kin4 also promotes phosphorylation of Bfa1 in a manner that protects it from inhibitory phosphorylation by Cdc5. (Maekawa et al., 2007). It is tempting to speculate that through activating Bfa1, Kin4 promotes Tem1 in a GDP-bound state, which in turn promotes Tem1 loss from SPBs. Finally, Kin4 itself is regulated; it functions in its dephosphorylated form in anaphase and is regulated by the kinase Elm1 and the phosphatase PP2A bound to its specificity subunit Rts1 (Caydasi et al., 2010; Chan and Amon, 2009; Moore et al., 2010).

The activating zone is created in the bud by the protein Lte1, which primarily localizes to the bud cortex (Seshan et al., 2002). Lte1 contains putative GEF domains, and was long thought to positively regulate MEN signaling by acting as a GEF for Tem1, despite the observations that Lte1 was not seen on SPBs and does not exhibit GEF activity *in vitro* (Geymonat et al., 2009). Recently, it has become clear that Lte1 acts positively within the MEN by inhibiting Kin4. Two parallel mechanisms ensure that Kin4 does not inhibit the MEN in the daughter cell: restriction of Kin4 to the mother cell cortex and inhibition of Kin4 loading onto the dSPB by *LTE1*. In the absence of *LTE1*, Kin4

loads onto the dSPB in a fraction of cells (Falk et al., 2011). Loading of Kin4 onto the dSPB is enhanced in the absence of *LTE1* when a mutant version of Kin4 that localizes to the mother and bud cortex is expressed (Chan and Amon, 2010; Falk et al., 2011). Furthermore, expression of a symmetric mutant of *LTE1* that localizes to both the mother and daughter cell inhibits Kin4 localization to the mother SPB (Bertazzi et al., 2011; Falk et al., 2011). An alternative to the zone model posits that interactions between cytoplasmic microtubules in the bud neck control the SPOC. In support of this model, laser ablation of cytoplasmic microtubules leads to mitotic exit in cells with mispositioned spindles. Further, the Elm1 kinase that regulates Kin4 localizes to the bud neck (Caydasi et al., 2010; Moore et al., 2010). However, the observation that mutants lacking cytoplasmic microtubules only exhibit a minor defect in SPOC function in cells that misposition spindles suggests that this is not the major mechanism cells use to coordinate spindle position with mitotic exit (Adames et al., 2001; Moore et al., 2009). The data therefore support the zone model: in every cell cycle only when a Tem1-containing SPB escapes the inhibitory zone created by Kin4 in the mother and enters the activating zone created by Lte1 in the bud can mitotic exit be triggered.

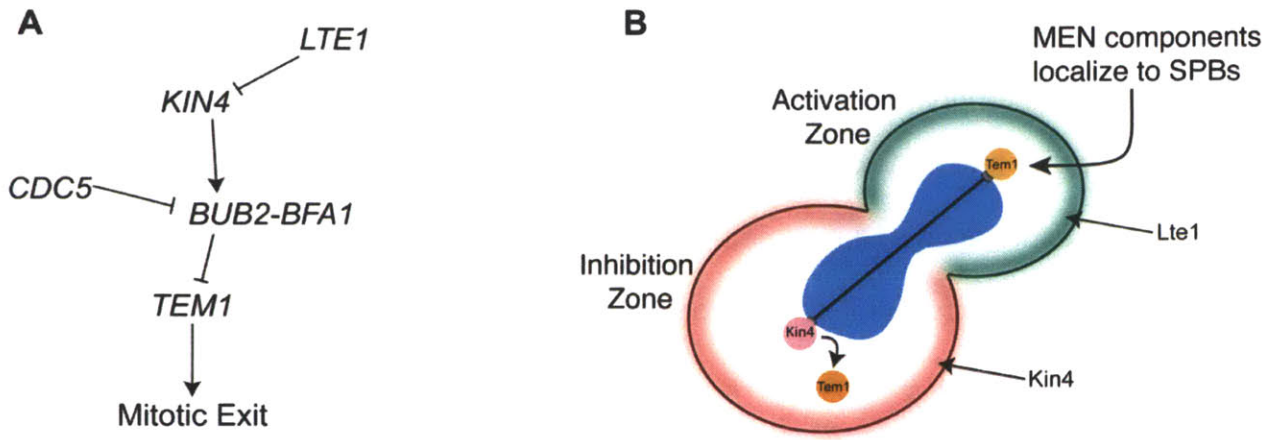

Figure 6. Control of MEN activation by spindle position

(A) Diagram of the genetic interactions between components controlling the activation of Tem1, and thus MEN signaling. (B) Diagram of the zone model. Kin4 (pink) resides in the mother cell and establishes an inhibitory zone. Lte1 (green) resides in the daughter cell and establishes an activating zone. Tem1 (orange) acts as a sensor moving between the zones, and only when Tem1 contacts the daughter cell can mitotic exit be triggered. The nucleus is shown in blue, and the spindle microtubules are indicated by the black line.

Spatial control is not necessary for mitotic exit in cells with properly positioned spindles, as *lte1Δ kin4Δ* double mutants still activate MEN signaling in anaphase (Rock and Amon, 2011). Therefore, temporal control of MEN activation exists, and the Tem1 GTPase as well as the polo-like kinase Cdc5 have been implicated in this temporal control. Evidence that *TEM1* mediates temporal control is seen in the observation that cells lacking *TEM1*, but that are kept alive by overexpression of *CDC15*, prematurely activate Dbf2-Mob1 in metaphase (Rock and Amon, 2011). Moreover, tethering Tem1 to SPBs results in premature localization of Cdc15 to SPBs, further suggesting that Tem1 mediates temporal control of MEN activation (Valerio-Santiago and Monje-Casas,

2011). Furthermore, *CDC5* is required for the recruitment of Cdc15 to SPBs in anaphase (Rock and Amon, 2011). In addition, *CDC5* promotes MEN activation in several other ways. Cdc5 phosphorylates Bfa1 to reduce its GAP activity, and thus promote MEN activation (Hu et al., 2001; Lee et al., 2001). Also, as part of the FEAR network, *CDC5* controls the early release of Cdc14, which leads to the dephosphorylation of Cdc15 and further promotes MEN activation (Jaspersen and Morgan, 2000; Stegmeier et al., 2002). Taken together, the polo kinase Cdc5 acts at multiple points to activate MEN signaling.

After mitotic exit has been achieved, MEN and FEAR signaling must be reversed. Spo12 and Cdc5 are both degraded by the APC/C^{Cdh1} at exit from mitosis. However, FEAR signaling is inactivated prior to exit from mitosis. Spo12 phosphorylation is an indication of FEAR activity, and is antagonized by Cdc14 (Tomson et al., 2009). Therefore FEAR-released Cdc14 may contribute to the inactivation of FEAR signaling. MEN signaling is inactivated at least in part by APC/C^{Cdh1} dependent degradation of Cdc5 as well as Cdc14 activity, which both promote the return of Cdc14 to the nucleolus (Manzoni et al., 2010; Visintin et al., 2008).

NDR-kinase signaling in higher eukaryotes

The MEN has served as an important paradigm for many problems in cell biology. First, studying the MEN has elucidated important concepts in how the cell cycle is reset after chromosome segregation. Second, studying the MEN has shed light on how asymmetric cell division is achieved. Asymmetric cell division is not unique to budding yeast. For example, a major way that cells in a developing embryo become

different from each other is through asymmetric cell division. Third, the MEN is an important paradigm for understanding how homologous signaling pathways function. How NDR-kinase signaling pathways function in different cellular contexts will be addressed in Chapter 2, and insights from other organisms will be described briefly below.

The core signaling components of the MEN are conserved (Figure 7). This pathway is known as the septation initiation network (SIN) in fission yeast, where it controls cytokinesis. Like the MEN, the SIN pathway is a GTPase signaling cascade that utilizes spindle pole bodies to assemble signaling modules (Krapp et al., 2004b). Similarly, polo kinase activity positively regulates the SIN (Krapp et al., 2004a; Ohkura et al., 1995). In contrast, the fission yeast Cdc14 homolog Clp1/Flp1 is not required for cyclin degradation, and its release from the nucleolus is not dependent on the SIN (Cueille et al., 2001; Trautmann et al., 2001).

Homologous signaling pathways also exist in higher eurkaryotes, although the pathway has evolved to control organ growth. Hippo signaling in *Drosophila* and NDR-kinase signaling in mammals use the same core signaling module as the MEN. The Cdc15-Dbf2-Mob1 module of the MEN is conserved in the hippo/NDR kinase signaling pathway as Hippo-Warts-Mats in *Drosophila* and Mst1/2-Lats1/2-Mob1A/B in mouse. The Hippo pathway effector is not Cdc14, but a transcriptional co-activator Yorkie (YAP/TAZ in mammals). Yorkie promotes transcription of pro-growth genes, and Hippo pathway activity leads to phosphorylation and nuclear exclusion of Yorkie (Halder and Johnson, 2011; Hergovich et al., 2006). Therefore, Hippo/NDR kinase signaling pathways restrain cellular proliferation. Phosphorylation of Yorkie by the Warts kinase

results in the binding of Yorkie to 14-3-3 proteins and cytoplasmic retention (Basu et al., 2003; Zhao et al., 2007). The Hippo pathway also contains a scaffold, Salvador/WW45, which appears to be regulated by phosphorylation, similarly to Nud1. It is becoming clear that Sav/WW45 can interact with both Hpo/Mst and with Warts/Lats, thus enabling Hpo/Mst to phosphorylate and activate the Warts/Lats kinase (Callus et al., 2006; Guo et al., 2007).

The upstream signals regulating the Hippo kinase are beginning to emerge, and, analogous to yeast, involve the integration of information from other signaling pathways and positional cues (Yu and Guan, 2013). Upstream signals restrain Hippo signaling when division is needed, and activate Hippo signaling when cells are contact-inhibited. Upstream signals regulating the Hippo pathway are most clear in *Drosophila*, and many signals exist. Signals transmitted from the atypical cadherin Fat and the transmembrane apical-basal polarity factor Crumbs, activate Hippo signaling through a poorly defined mechanism mediated in part by the Expanded/Merlin/Kibra complex. (Baumgartner et al., 2010; Hamaratoglu et al., 2006; Ling et al., 2010; Reddy and Irvine, 2008). Ajuba proteins, proteins that localize to adherens junctions, inhibit Hippo signaling (Das Thakur et al., 2010; Marie et al., 2003) when a cell is not contact-inhibited. Finally, crosstalk between signaling pathways influences Hippo signaling. Mst proteins have been shown to interact with RASSF proteins, which may allow crosstalk between the Ras and Hippo pathways (Guo et al., 2007; Matallanas et al., 2007). Taken together, although many inputs for Hippo/NDR kinase signaling have been identified, much remains to be learned about the mechanisms controlling activation or inhibition of the Hippo pathway. Moreover, the NDR kinase pathway appears to be regulated differently

in different cell types and contexts, with much remaining to discover (Halder and Johnson, 2011).

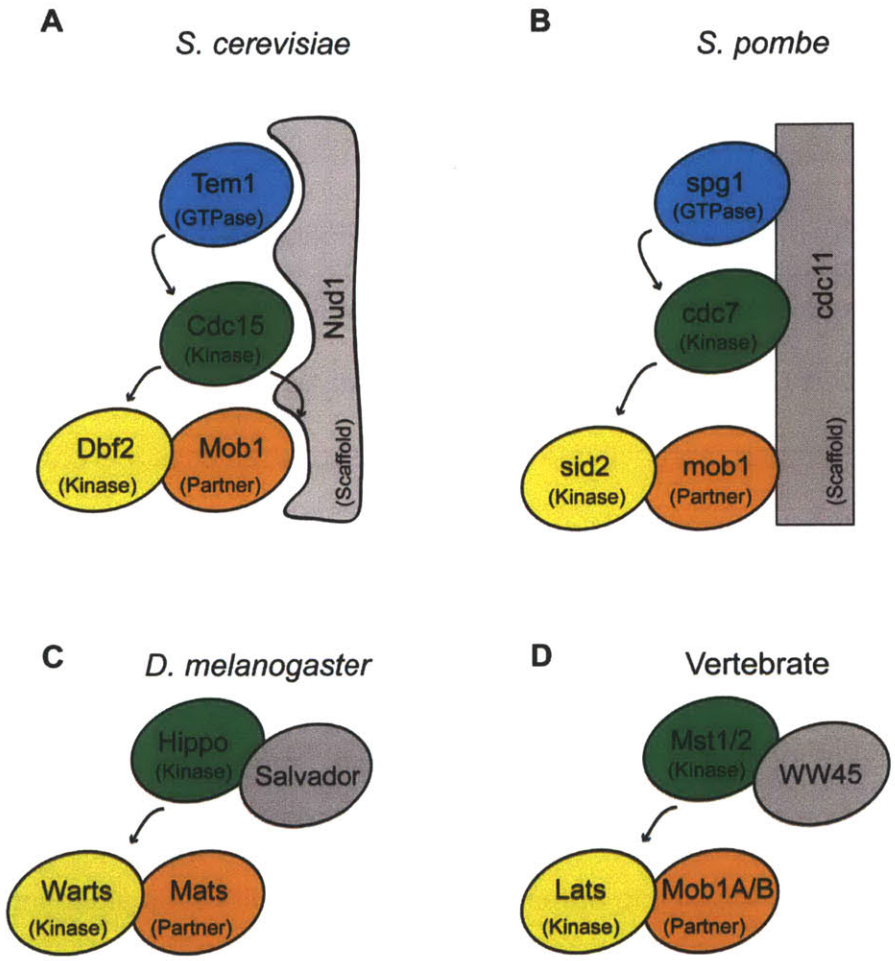

Figure 7. Conservation of the core signaling components in NDR-kinase signaling.

The mitotic exit network is conserved in higher eukaryotes. Although the upstream signals and downstream effectors vary between species, the core components of the pathway are conserved, shown for budding yeast (A), fission yeast (B), fly (C), and vertebrates (D). The GTPase is shown in blue, the sterile-20-like kinase in green, scaffold in gray, NDR kinase in yellow, and NDR-kinase activating subunit in orange.

Meiosis: A specialized cell division required for sexual reproduction

All sexual reproduction depends on meiosis, the specialized cell division that yields four meiotic products with half of the amount of genetic material. In diploid organisms, meiosis produces four haploid cells from one diploid progenitor. In yeast the products of meiosis, or gametes, are spores. It is essential that gametes with the correct number of chromosomes are produced; chromosome mis-segregation during meiosis causes miscarriages and Down's syndrome in humans (Hassold and Hunt, 2001).

During meiosis, two rounds of chromosome segregation follow one round of DNA replication. In meiosis I, homologous chromosomes separate and in meiosis II, sister chromatids split. While meiosis II segregation resembles mitosis, meiosis I segregation is unique in that homologous chromosomes segregate. Several modifications are required for the meiosis I chromosome segregation pattern. These specializations - creation of physical linkages between homologous chromosomes, stepwise loss of cohesion between sister chromatids, and attachment of sister kinetochores to microtubules from the same pole of the meiotic spindle - establish the meiosis I chromosome segregation pattern (Figure 8). Additionally, cells must exit from meiosis I without establishing conditions permissive for DNA re-replication (Marston and Amon, 2004). In the following section of this chapter I will describe the stages of meiosis in greater detail. I will highlight similarities between the cell cycle machinery in mitosis and meiosis and focus on the specializations required for homologous chromosomes to segregate in meiosis I.

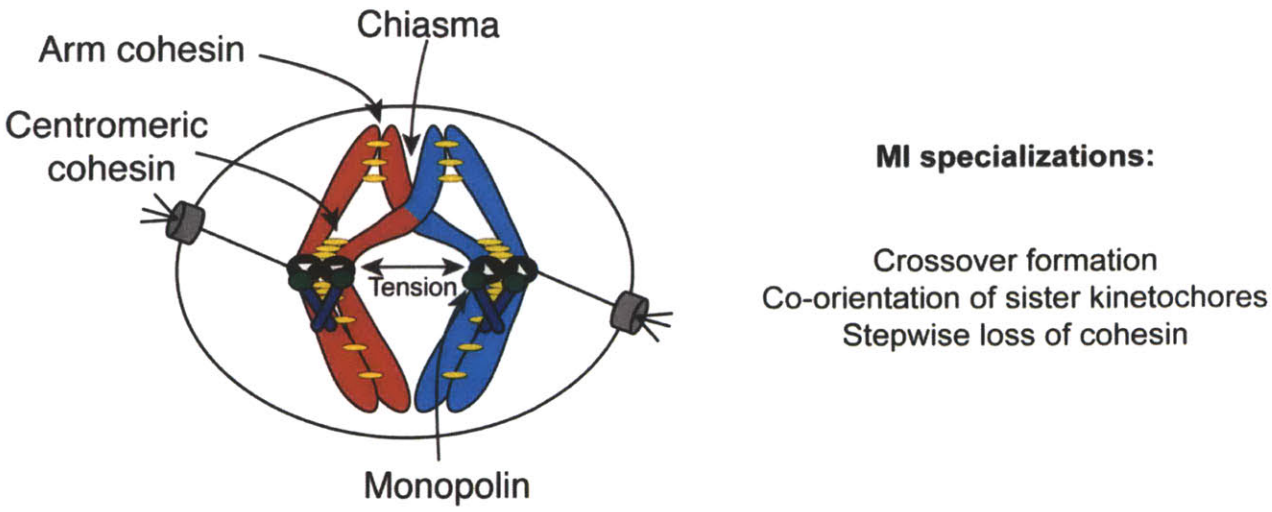

Figure 8. Meiosis I Specializations

Several modifications occur to establish conditions that allow homologous chromosomes, rather than sister chromatids to split in meiosis I. (1) Recombination links homologous chromosomes. Chiasmata are the physical manifestations of crossover events. (2) Cohesion links sister chromatids. Arm cohesion is removed in meiosis I, but centromeric cohesion is retained, thus allowing sister chromatids to be held together until meiosis II. (3) The monopolin complex links sister kinetochores so that they can be attached to microtubules from the same spindle pole. Together, these modifications allow tension to be achieved on the meiosis I spindle.

Entry into gametogenesis

In budding yeast, sporulation is a starvation response. If diploid cells are starved, they undergo meiosis coupled with the developmental program of spore formation to produce stress-resistant, haploid gametes (spores) that can return to growth when nutrients are restored. Therefore, entry into sporulation requires the integration of nutritional signals with mating-type information: only nutrient-starved (lack of a nitrogen source and a fermentable carbon source) cells of an a/α mating type will enter the

sporulation program. *IME1*, a transcription factor, is the master regulator of entry into gametogenesis in budding yeast. Cells lacking *IME1* do not sporulate, and cells overexpressing *IME1* will undergo meiosis even in nutrient rich conditions or in the a or α mating type (Kassir et al., 1988; Smith and Mitchell, 1989). The promoter of IME1 integrates information about mating type (which is a read-out for ploidy) and nutritional state. The *IME1* promoter is complex; it is approximately 2kb long and contains binding sites for proteins whose presence or absence at the promoter is reflective of mating type or nutritional state (van Werven and Amon, 2011). In addition, two non-coding RNAs present in the *IME1* promoter mediate mating-type control of *IME1* expression (van Werven et al., 2012).

Once expressed, Ime1 initiates a wave of transcription of early meiotic genes. These early meiotic genes encode proteins required for pre-meiotic DNA replication, chromosome condensation, and homologous recombination. One critical target of Ime1 is *IME2*, which encodes a protein kinase (Dirick et al., 1998; Mitchell et al., 1990; Yoshida et al., 1990). Indirectly, Ime1 promotes expression of *NDT80*, which encodes another transcription factor. Ndt80 promotes the expression of middle meiotic genes including polo kinase, cyclins, and other factors necessary for the meiotic divisions. Finally, in a late wave of transcription, genes that encode factors essential for building spore walls, are expressed (Chu and Herskowitz, 1998; Pak and Segall, 2002; Primig et al., 2000).

Entry into gametogenesis is quite different in mammals and other metazoans, but may require a master regulator. In multicellular organisms, germ cells are specified early in development. After specification, primordial germ cells (PGCs) migrate to the

gonad where they become meiosis-competent. It is likely that a master regulator of meiosis exists in mammals; the putative transcription factor *Stra8* is required for meiosis in both males and females (Baltus et al., 2006; Tedesco et al., 2009). *Stra8* also integrates multiple signals. Stra8 requires the RNA-binding protein *Dazl* for its expression, and *Dazl* is expressed only in PGCs in the gonad (Lin et al., 2008). *Stra8* also responds to an extracellular signal, the presence of retinoic acid (Bowles et al., 2006). Upon integration of intracellular and extracellular signals, *Stra8* then promotes entry into the gametogenesis program.

Pre-meiotic DNA replication

After cells have entered the gametogenesis program, DNA is replicated. In both pre-meiotic and pre-mitotic S phase, Clb5/6-CDK and Dbf4-dependent kinase (DDK) are required. Additionally, an important target of Ime1, the kinase Ime2, is required for entry into pre-meiotic S; degradation of the Clb-CDK inhibitor Sic1 depends on Ime2 (Benjamin et al., 2003; Dirick et al., 1998). Although similar origins of replication are used, meiotic S phase proceeds more slowly than pre-mitotic S phase, as a consequence of delayed initiation (Blitzblau et al., 2012). As in the vegetative cell cycle, during pre-meiotic S phase, cohesin molecules are deposited. Cohesin complexes are similar between mitosis and meiosis with one important difference: the α-kleisin subunit of cohesin (the Scc1 subunit that is cleaved by separase) is replaced by Rec8 in meiosis. Rec8 is utilized across species including mammals in meiotic cohesin complexes. The replacement of Scc1 by Rec8 has important consequences in prophase I. Rec8 plays multiple roles in pairing of homologous chromosomes, homologous

recombination, and synaptonemal complex assembly (Brar et al., 2009; Klein et al., 1999) that cannot be supported by Scc1.

Meiotic Prophase and homologous recombination

Once pre-meiotic S phase is completed, cells undergo an elongated prophase I (sometimes referred to as meiotic G2), in which homologous chromosomes pair and recombine. I will briefly discuss the relevance of homologous recombination to chromosome segregation. Recombination is an amazing and risky process in which a large amount of DNA damage is introduced into chromosomes and correctly repaired (Figure 9). The conserved endonuclease Spo11 is responsible for introducing these programmed double strand breaks (DSBs; (Keeney et al., 1997)). After DSBs are produced, the 5' ends of the DNA are resected, and in a process dependent on *DMC1*, a homology search biased toward the homolog begins (Bishop et al., 1992; Schwacha and Kleckner, 1997; Shinohara and Shinohara, 2004). DSBs can be processed as either crossovers (COs) or noncrossovers (NCOs), and this decision is made early, soon after strand invasion (Allers and Lichten, 2001). Therefore, double Holliday junctions are repaired into crossovers. A surveillance mechanism exists to ensure that cells do not progress into meiosis before all DNA damage is repaired. This checkpoint is conserved since mouse spermatocytes and oocytes failing to repair DNA damage do not undergo meiosis. In budding yeast, unrepaired damage leads to the inhibition of Clb-CDKs and Ndt80, the transcription factor required for expression of B-type cyclins, polo kinase, and progression into the meiotic divisions (Hochwagen and Amon, 2006).

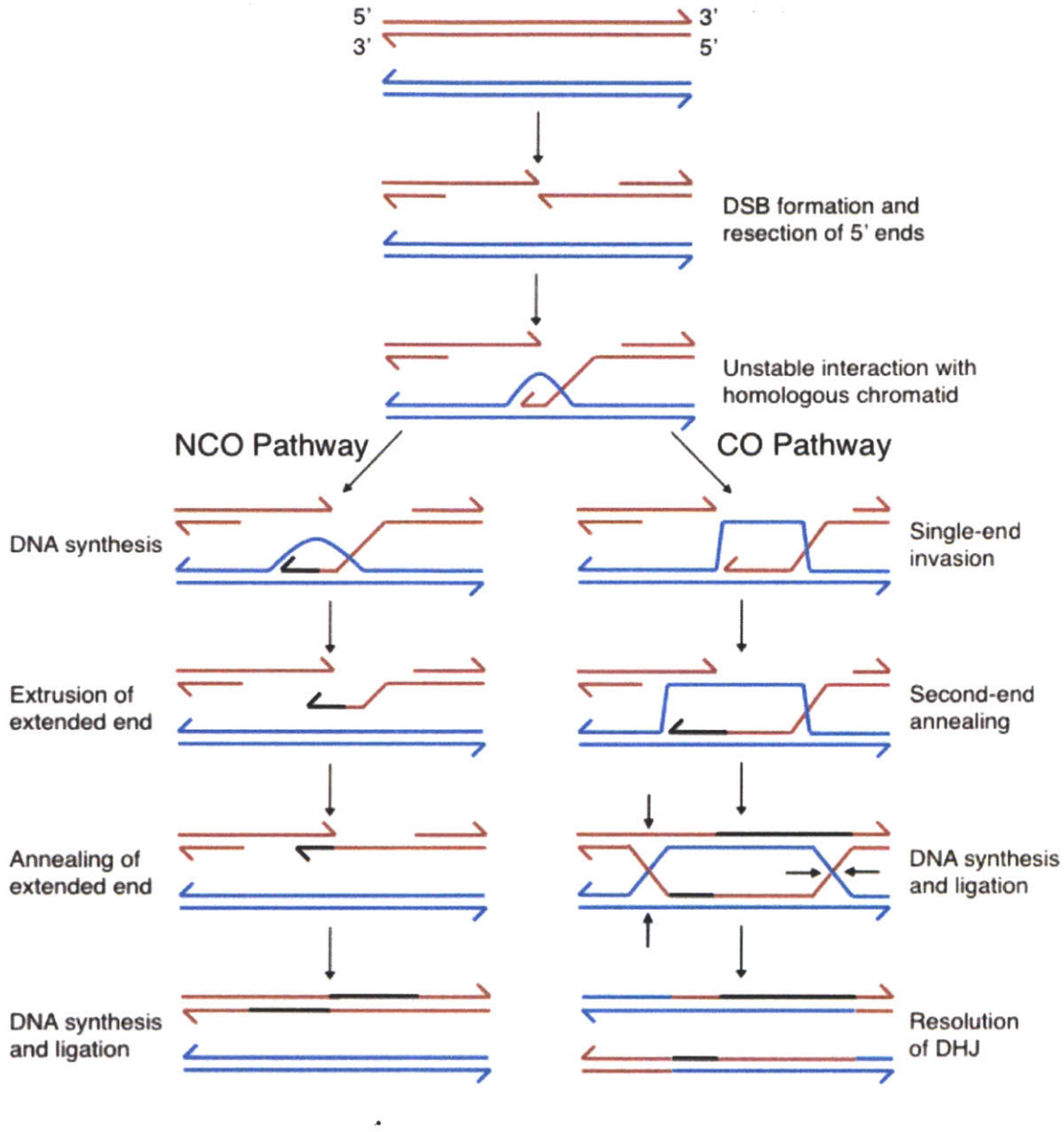

Figure 9. Schematic of homologous recombination.

During prophase, programmed double-strand breaks are created throughout the genome. DNA is resected, and single strands undergo a homology search. If the extended end is ejected, the break will be repaired as a non-crossover (NCO). If the extended end anneals, double Holliday junctions (DHJs) will form and ultimately resolve into a crossover. Adapted from Bishop and Zickler, 2004.

In addition to homologous recombination, the synaptonemal complex (SC), a proteinaceous scaffold, forms between the homologs during meiotic prophase to enable efficient homologous chromosome pairing and recombination (Page and Hawley, 2004). SC formation depends on cohesin, condensin, and DSBs (in budding yeast). The SC forms a zipper-like structure that synapses homologous chromosomes together during prophase. The lateral elements are composed of Red and Hop1 and are thought to promote inter-homolog repair (Hollingsworth et al., 1990; Smith and Roeder, 1997). In late prophase, transverse filaments containing Zip1 form and are required for crossover maturation (Borner et al., 2004). SC function varies in different organisms. In budding yeast, SC formation requires the initiation of recombination, whereas in *C. elegans*, the SC can form in the absence of recombination initiation, but is important for the completion of recombination (Colaiacovo et al., 2003; Dernburg et al., 1998; Roeder, 1997). In contrast, *Drosophila* males and fission yeast do not form SC at all (Page and Hawley, 2004).

Crucially for chromosome segregation, at least one crossover must be present on each pair of homologous chromosomes. The chiasmata produced by repair via crossovers provide the linkages between homologous chromosomes. Together with the cohesin molecules that link sister chromatids distal to the crossover, tension can be generated on the meiosis I spindle. Although recombination exists to physically link homologous chromosomes, it has the important evolutionary consequence of generating gametes that are not genetically identical to either parent. Due to independent assortment and the swapping of genetic material between parental

chromosomes, new combinations of alleles are contributed to the next generation, leading to diversity within the population.

Regulation of meiotic chromosome segregation by CDK, polo kinase, and the APC/C

Among the targets of Ndt80 essential for progression through the meiotic divisions are the genes encoding the B-type cyclins *CLB1*, *CLB3*, and *CLB4*, and the polo kinase *CDC5*. Analogously to mitosis, CDK activity is required for progression through the meiotic divisions (Figure 10). However, the regulation of meiotic CDK activity exhibits important differences from the regulation of M-CDKs. First, the mitotic cyclin of central importance to mitosis, Clb2, is absent during meiosis (Grandin and Reed, 1993). Second, Clb1 kinase activity, but not protein, is restricted to meiosis I (Carlile and Amon, 2008). Third, Clb3 is translationally controlled, and produced only during meiosis II (Carlile and Amon, 2008). The intricate regulation of cyclins in meiosis is critical for proper chromosome segregation. Over-expression of *CLB3* or *CLB1*, but curiously not *CLB4*, prior to the first meiotic division leads to the separation of sister chromatids rather than homologs during meiosis I (Carlile and Amon, 2008; Miller et al., 2012). Unraveling in greater detail how B-type cyclins are regulated and how cyclin specificity contributes to meiotic progression are important questions for the future, as CDK activity drives meiotic progression across species.

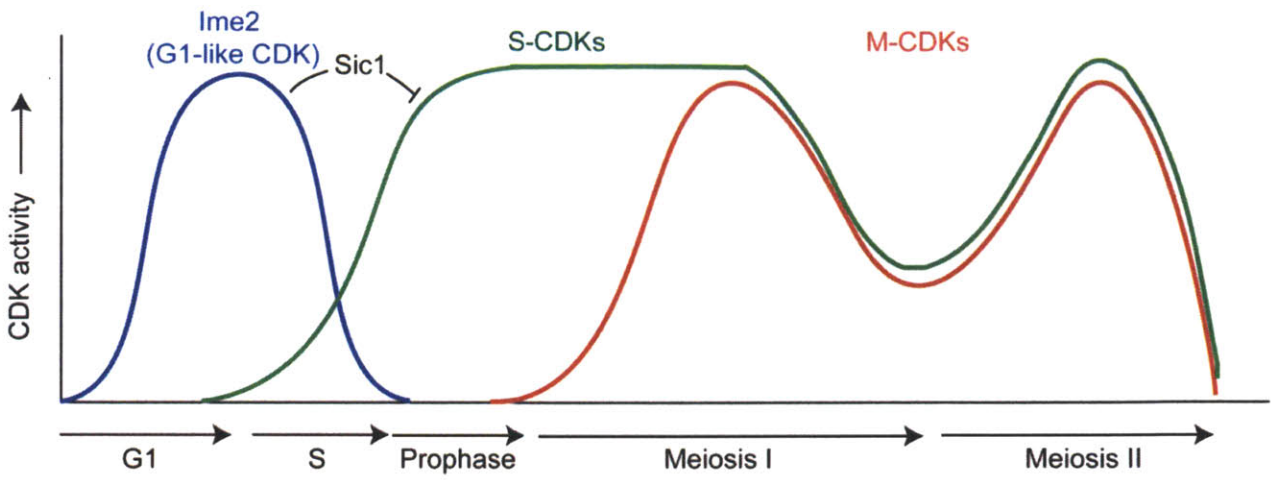

Figure 10. CDK activity in budding yeast meiosis

CDK activity drives progression through meiosis. Although G1-CDKs are absent in meiosis, the kinase Ime2 acts as a G1-like CDK, fulfilling the important role of triggering Sic1 degradation. S-CDKs drive progression through S phase and M-CDKs drive progression through the meiotic divisions. It is thought that CDK activity dips between the two meiotic divisions. Finally, how CDKs are inactivated at the end of meiosis is unclear.

The Ndt80 target, the polo kinase Cdc5, is also a central regulator of meiotic progression in all species analyzed. In budding yeast, cells lacking Cdc5 in meiosis arrest in metaphase I (Clyne et al., 2003; Lee and Amon, 2003). *CDC5*, unlike in mitosis, is an essential coordinator of nearly all aspects of the meiosis I division; it is required for chiasmata resolution, securin degradation, sister kinetochore co-orientation, cohesion removal, and Cdc14 release in anaphase I (Clyne et al., 2003; Lee and Amon, 2003). Precisely how Cdc5 functions during the meiotic divisions is less clear, and experiments addressing this question will be described in Chapter 3. The role of polo kinase in coordinating the meiotic divisions is conserved. For example, in *Drosophila*, *polo* is required for nuclear envelope breakdown and entry into the meiotic divisions. In

fact, a *polo* inhibitor called *matrimony* is present during prophase to ensure that *polo* is not activated prematurely (Xiang et al., 2007). Furthermore, in *Drosophila*, *polo* also functions in the regulation of cohesion (Clarke et al., 2005).

In addition to the conserved role for CDK activity and polo kinase activity in mitosis and meiosis, regulated proteolysis is also a conserved theme. Just as in mitosis, securin is degraded at the metaphase I-anaphase I and metaphase II-anaphase II transitions (Salah and Nasmyth, 2000). However, APC/C activity differs in several key ways. First, the APC/C^{Cdh1} does not appear to play a key role in the meiosis I-meiosis II transition, highlighted by the observation that Clb1, which is targeted by the APC/C^{Cdh1}, is not degraded at the meiosis I-meiosis II transition (Carlile and Amon, 2008). In contrast with mitosis, an additional meiosis-specific APC/C activator exists in meiosis called *AMA1*. The APC/C^{Ama1} primarily functions to degrade mitotic regulators during prophase I in order to ensure that mitotic regulators are repressed during prophase I (Okaz et al., 2012). An APC inhibitor called Mnd2 also exists in meiosis. Mnd2 ensures that securin is not degraded prematurely during the elongated meiotic prophase (Oelschlaegel et al., 2005; Penkner et al., 2005). Meiosis-specific APC/C activators are not specific to budding yeast. In *Drosophila*, a female-specific activator of the APC/C called *cortex* is required for proper meiosis, as it targets cyclin A for degradation prior to the metaphase I arrest in oocytes (Pesin and Orr-Weaver, 2007).

Specializations of Meiosis I

Co-orientation of sister chromatids

In order for homologous chromosomes to segregate during meiosis I, they must not only be linked to each other, but also linked properly to the meiosis I spindle. To achieve co-orientation, kinetochores from sister chromatids must be attached to microtubules emanating from the same spindle pole. Sister kinetochore co-orientation is best understood in budding yeast where it is achieved by the monopolin complex (Figure 11). Monopolin consists of Mam1, Csm1, Lrs4, and the casein kinase Hrr25. Mam1 is a meiosis I specific protein, whereas Csm1 and Lrs4 localize to the nucleolus during interphase. Late in prophase I, Csm1 and Lrs4 are released from the nucleolus. They associate, along with Mam1 and Hrr25, with kinetochores until metaphase I (Petronczki et al., 2006; Rabitsch et al., 2003; Toth et al., 2000), and *MAM1* is required for robust localization of Csm1 and Lrs4 to kinetochores (Rabitsch et al., 2003). The localization of Mam1 depends on *SPO13*, a gene encoding a meiosis I-specific protein of unknown function (Katis et al., 2004b; Lee et al., 2004). In addition, Cdc5 and DDK also control monopolin function as Mam1 localization and Lrs4 phosphorylation and nucleolar release depend on both *CDC5* and *CDC7* (Clyne et al., 2003; Lee and Amon, 2003; Matos et al., 2008). Cdc5 may act through Spo13, as the two proteins physically interact (Matos et al., 2008).

Finally, kinetochore co-orientation is regulated by temporal regulation of microtubule-kinetochore interactions. *NDC80*, an outer kinetochore component, is transcriptionally and translationally controlled such that Ndc80 protein is undetectable

during prophase I. Forced expression of *NDC80* and overexpression of the cyclin *CLB3* leads to the biorientation of sister chromatids during meiosis I, demonstrating that limiting outer kinetochore assembly to metaphase I promotes co-orientation of sister chromatids (Miller et al., 2012).

Recent structural studies have shed light on how the monopolin complex links sister kinetochores. The structure of the Csm1-Lrs4 complex indicates that these proteins form a Y-shaped complex. Moreover, Csm1 is able to bind to the kinetochore components Dsn1 and Mif2, suggesting that the monopolin complex may bind to and crosslink sister kinetochores (Corbett et al., 2010). Mam1 and kinetochore components are thought to bind at different sites on Csm1 (Corbett and Harrison, 2012). Electron microscopy data indicate that, in budding yeast, one microtubule binds to the pair of sister chromatids co-segregating in meiosis I (Winey et al., 2005), further suggesting that the monopolin complex clamps sister kinetochores such that sister chromatids can move together to the same spindle pole in meiosis I. Interestingly, in budding yeast the monopolin complex is sufficient to promote sister kinetochore co-orientation; overexpression of *MAM1* and *CDC5* in mitosis induces sister chromatid co-orientation in an *LRS4* and *CSM1* dependent manner (Monje-Casas et al., 2007).

Sister kinetochore co-orientation is mediated by different mechanisms in other eukaryotes. One main reason for this is likely to be that multiple microtubules are captured by larger kinetochores in other organisms: in fission yeast 2-4 microtubules are captured, and in metazoans 15-30 microtubules are captured (Ding et al., 1993; McEwen et al., 1997). In fission yeast, Moa1, a meiosis I-specific protein, mediates sister kinetochore co-orientation by a cohesion-mediated mechanism. Moa1 and Rec8

physically interact in the central core of the centromere, a location where Rec8's mitotic counterpart is not found (Yokobayashi and Watanabe, 2005). One model posits that Moa1 may aid Rec8 in establishing cohesion at the central core of the centromere, thus enabling sister kinetochores to be co-oriented (Yokobayashi and Watanabe, 2005). Homologs of Lrs4 and Csm1 exist in fission yeast, called Mde4 and Pcs1 respectively. These proteins also cycle between the nucleolus and kinetochores, but function only in mitosis to inhibit merotelic attachments (Gregan et al., 2007; Rabitsch et al., 2003). It has been proposed that Mde4 and Pcs1 promote proper biorientation in mitosis by aligning microtubule-binding sites (Gregan et al., 2007).

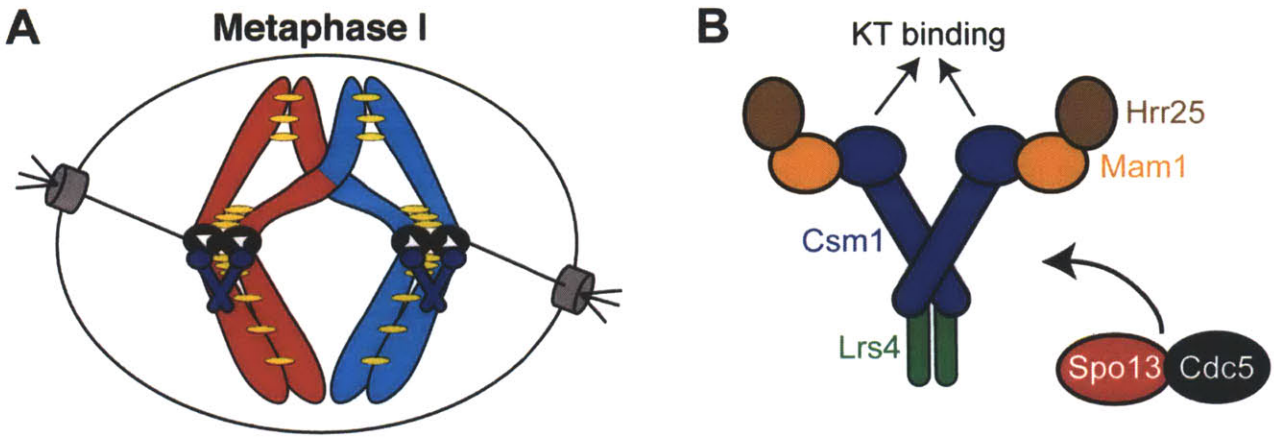

Figure 11. Schematic of monopolin complex

(A) The monopolin complex (Mam1, Lrs4, Csm1, Hrr25) co-orients sister kinetochores, likely by clamping together sister kinetochores. The Y-shaped cartoon represents the monopolin complex. (B) A cartoon representing all of the known monopolin components. Mam1 has been shown to interact with the globular domain of Csm1 and with the N-Terminus of Hrr25. The globular domain of Csm1 also interacts with kinetochore components. Spo13 and Cdc5 physically interact, and are required for monopolin function.

Stepwise loss of cohesion

Once homologous chromosomes and sister chromatids are linked and sister kinetochores attach to microtubules emanating from the same spindle pole, tension can be achieved across the meiotic spindle. The linkages between chromosomes, particularly cohesion distal to chiasmata, resist the pulling force of the microtubules. The spindle assembly checkpoint monitors tension in a manner similar to mitosis, with the APC/C^{Cdc20} inhibited until all homologs are under tension (Shonn et al., 2000). Similarly, kinetochore-microtubule attachments are regulated by the Aurora B kinase (Monje-Casas et al., 2007). When all homologous chromosomes are properly bioriented on the meiosis I spindle and tension is achieved, the metaphase I-anaphase I transition occurs in a manner similar to mitosis. The APC/C association with Cdc20 triggers the degradation of securin, freeing separase to cleave the α-kleisin subunit of the cohesin complex, Rec8 (Buonomo et al., 2000; Ciosk et al., 1998; Cohen-Fix et al., 1996). The removal of sister chromatid cohesion from chromosome arms during meiosis I allows the dissolution of chiasmata, thus freeing homologous chromosomes to segregate.

Importantly, cohesion is retained around centromeric regions during the meiosis I division. This remaining pool of cohesion is essential for holding sister chromatids together until anaphase II. Rec8 itself is required for the protection of centromeric cohesion because centromeric cohesion is not protected when *REC8* is replaced with its mitotic counterpart *SCC1* (Toth et al., 2000). Rec8 is a highly phosphorylated protein, and its phosphorylation is required for removal from chromosome arms (Brar et al., 2006; Katis et al., 2010). The regulation of Rec8 phosphorylation is thought to provide the mechanism for the protection of centromeric cohesion.

One factor required for the protection of centromeric cohesion is the shugoshin family member Sgo1/MEI-S332 (Katis et al., 2004a; Kerrebrock et al., 1995; Kitajima et al., 2004; Marston et al., 2004). Cells lacking Sgo1 in meiosis lose centromeric cohesion prematurely and segregate sister chromatids randomly in meiosis II, leading to the production of inviable spores. Moreover, Sgo1 and MEI-S332 localize to centromeric regions until metaphase II (Katis et al., 2004a; Kerrebrock et al., 1995; Kitajima et al., 2004). Sgo1 localizes to a 50kb region around centromeres, and protects cohesin from removal in this region (Kiburz et al., 2005). Sgo1 localization depends on the kinetochore components Bub1, Iml3, and Chl4 (Kiburz et al., 2008; Kiburz et al., 2005; Marston et al., 2004). In yeast, Sgo1 recruits the phosphatase PP2A to centromeric regions (Kitajima et al., 2006; Riedel et al., 2006). PP2A is thought to maintain Rec8 in a dephosphorylated state refractory to cleavage by separase.

In budding yeast, the balance of Rec8 phosphorylation and dephosphorylation is therefore thought to control the stepwise loss of cohesion. If dephosphorylation of Rec8 protects it from cleavage, kinases must promote Rec8 phosphorylation on chromosome arms (Figure 12). Several kinases have been implicated in the phosphorylation of Rec8: the Dbf4-dependent kinase (DDK), the casein kinase CK1 (Hrr25 in budding yeast), and Cdc5 (Brar et al., 2006; Katis et al., 2010). The contribution of each of these kinases in controlling the stepwise loss of cohesion is under debate and is examined in detail in Chapter 3. Finally, centromeric cohesion is also protected by *SPO13* through an unknown mechanism (Katis et al., 2004b; Lee et al., 2004).

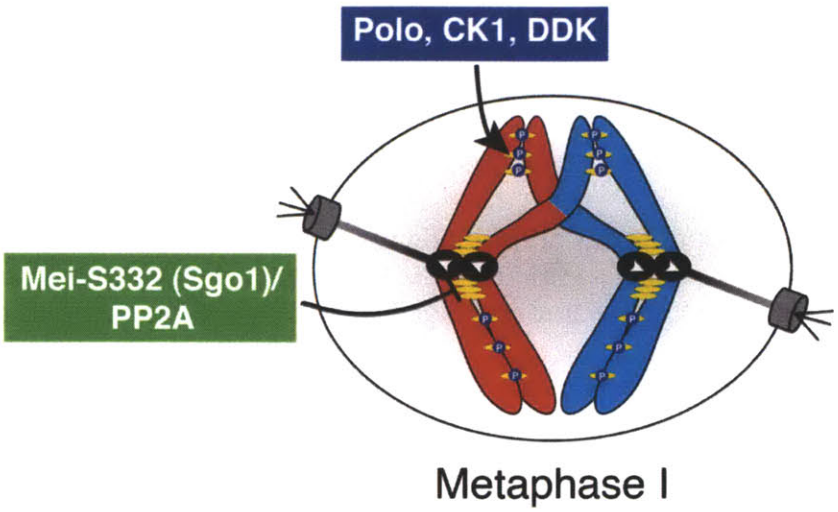

Figure 12. Control of stepwise loss of cohesion.
During meiosis, cohesion is lost from chromosome arms during meiosis I. Cohesion retained around centromeres holds sister chromatids together until meiosis II. The phosphorylation of Rec8 is critical for its efficient removal from chromosome arms. Polo kinase, casein kinase CK1, and DDK have been implicated in the phosphorylation of Rec8. Rec8 is protected at centromeres by shugoshin/MEI-S332-family proteins that localize to centromeric regions during meiosis I. Shugoshins recruit PP2A, which is thought to maintain Rec8 in its dephosphorylated state and thus refractory to cleavage during meiosis I. Figure used with permission from Elçin Ünal and Matt Miller.

The stepwise loss of cohesion is a conserved feature of meiosis, but utilizes different mechanisms in different organisms. For example, *C. elegans* lacks a single centromere, and must utilize a different mechanism to ensure the stepwise loss of cohesion. In *C. elegans*, the stepwise loss of cohesion is driven by the crossover, but remarkably still involves the control of REC-8 phosphorylation (Schvarzstein et al., 2010). Each chromosome usually undergoes one off-center recombination event, resulting in bivalents with long and short arms, and thus two distinct chromosomal domains. In late prophase, several chromosomal axis and SC components re-localize to

these chromosomal domains. Ultimately, the axial element components HTP-1 and HTP-2, and the nematode-specific protein LAB-1 localize to the long arm, where cohesion will be retained in meiosis I (de Carvalho et al., 2008; Martinez-Perez et al., 2008). In contrast, Aurora B localizes to the short arm. Aurora B is thought to phosphorylate REC-8 locally, leading to REC-8 removal, and segregation of homologous chromosomes (Kaitna et al., 2002; Rogers et al., 2002). HTP-1/2 and LAB-1 are thought to protect cohesin in a manner similar to, but independent of, shugoshins (de Carvalho et al., 2008; Martinez-Perez et al., 2008). Adding an additional layer of complexity, in worms, REC-8 is not the only α-kleisin subunit of cohesin; REC-8 is joined by COH-3 and COH-4-containing cohesin complexes to mediate cohesion (Severson et al., 2009).

The meiosis I-meiosis II transition

After homologous chromosomes segregate in meiosis I, the meiosis I spindle disassembles and cells prepare for the second meiotic division by degrading the meiosis I determinants Spo13 and Mam1 (Sullivan and Morgan, 2007; Toth et al., 2000), by duplicating SPBs, and by producing the cyclin Clb3 (Carlile and Amon, 2008). Importantly, cells cannot completely exit the meiotic program or create conditions permissible for re-replication. In Xenopus oocytes, CDK activity is reduced during the meiosis I-meiosis II transition, such that meiosis I exit can occur without risking complete meiotic exit or re-replication between the two meiotic divisions (Furuno et al., 1994; Huchon et al., 1993; Iwabuchi et al., 2000). In fission yeast, the *mes1+* gene controls the meiosis I-meiosis II transition by permitting retention of CDK activity

between the divisions. In cells lacking *mes1*, cells finish meiosis I but fail to enter meiosis II (Izawa et al., 2005). CDK activity is likely to be lowered between meiosis I and meiosis II in budding yeast as well, since overexpressing a stabilized version of the cyclin *CLB2* leads to a delay in anaphase I spindle disassembly (Marston et al., 2003). Finally, Ime2 kinase exhibits a second peak of activity in meiosis II, and may function at least in part to inhibit re-licensing of replication origins (Benjamin et al., 2003; Holt et al., 2007).

In budding yeast, exit from meiosis I, like exit from mitosis, is controlled by Cdc14 activity. Cdc14 is released from the nucleolus in both anaphase I and anaphase II (Buonomo et al., 2003; Marston et al., 2003). Cells containing a temperature-sensitive allele of *CDC14* at the restrictive temperature exhibit a severe delay in meiosis I spindle disassembly, and the FEAR network, but not the MEN, is responsible for Cdc14 release in anaphase I (Attner and Amon, 2012; Buonomo et al., 2003; Kamieniecki et al., 2005; Marston et al., 2003; Pablo-Hernando et al., 2007). In addition to *SPO12* and *SLK19*, which act positively within in the FEAR network, the phosphatase $PP2A^{CDC55}$ acts negatively to restrain Cdc14 release during meiosis I (Bizzari and Marston, 2011). Intriguingly, FEAR network and *CDC14* mutants revealed that events in the meiotic divisions are uncoupled. In these mutants, trapped chromosomes that do not segregate in meiosis I undergo an equational segregation at the time when meiosis II would occur (Buonomo et al., 2003; Marston et al., 2003). It seems that cells utilize the FEAR network, which promotes a transient burst of Cdc14 release, to ensure that meiosis I exit occurs without exit from the meiotic program.

Finally, meiosis II segregation can occur. Cells duplicate spindle pole bodies and prepare to segregate sister chromatids in a manner similar to mitosis. Cells increase CDK activity to drive progression through meiosis II. In yeast, *CLB3* message is relieved from translational control, and Clb3 protein is produced in meiosis II (Carlile and Amon, 2008). In Xenopus, cyclin B is produced (Hochegger et al., 2001). Proper biorientation of sister chromatids is again monitored by Aurora B and securin degradation is again triggered (Monje-Casas et al., 2007; Salah and Nasmyth, 2000). Exit from meiosis II is poorly understood. Surprisingly, the MEN, a pathway essential for exit from mitosis, is not essential for exit from meiosis I or II as judged by anaphase II spindle disassembly (Attner and Amon, 2012; Kamieniecki et al., 2005; Pablo-Hernando et al., 2007). The FEAR network may play this role, but much remains to be discovered.

Meiosis is coupled to a developmental program

Meiotic chromosome segregation yields gametes with half the genomic content of the progenitor cells, and is tightly coupled to the developmental programs governing gametogenesis. In females and males, meiosis is coupled to the oogenesis and spermatogenesis programs respectively. In yeast, meiosis is coupled to spore formation. Differences between these developmental programs are reflected in differences in meiotic progression. In worms, flies, and vertebrates, a prophase arrest in female meiosis is observed, in which maternal components are stockpiled. In vertebrates, hormonal signals trigger the resumption of meiosis. Different species exhibit differently secondary arrests, with vertebrates arresting in metaphase II (Von Stetina and Orr-Weaver, 2011). In vertebrates, the metaphase II arrest is maintained by

Mos and MAP kinase activity, which inhibits the APC/C (Tunquist and Maller, 2003). When the egg is fertilized, a calcium signal relieves inhibition of the APC (Rauh et al., 2005), leading to the completion of meiosis and the genesis of a new organism.

In human females, early stages of meiosis are carried out during fetal development. Human females are therefore born with their oocytes arrested in prophase/G2. Meiotic arrest is maintained over years and even decades, as meiosis is resumed upon ovulation. Indeed, human female meiosis is error-prone and age-dependent; meiotic missegregation in human females leads to miscarriages and Down's syndrome (Hassold and Hunt, 2001). Recombination errors, spindle assembly checkpoint defects, or deterioration of cohesion over time have all been proposed to lead to meiotic missegregation. Indeed, it has recently been shown that centromeric cohesion is weakened in oocytes from old mice (Chiang et al., 2010). Remarkably, human female meiosis is robust enough to provide a complete haploid genomic complement to the next generation.

Concluding Remarks

Cell division is essential for the propagation of life. Faithful transmission of the genome is thus intricately controlled in all cells. Furthermore, cell cycle control mechanisms are widely conserved among eukaryotes. Chromosome segregation alternates with DNA replication to produce two cells with an identical genomic complement to the progenitor cell. Sexually reproducing organisms undergo a second type of cell division, meiosis. Meiosis generates gametes with half the genomic complement as the parent cell, thus allowing the fusion of two gametes to produce an

embryo with the correct ploidy. During meiosis, two rounds of chromosome segregation follow one round of DNA replication. Homologous chromosomes split during meiosis I and sister chromatids separate during meiosis II. Both mitosis and meiosis are governed by oscillations in CDK activity and regulated proteolysis, and in both division types cohesion holds chromosomes together. Understanding how conserved cell cycle control mechanisms are differentially controlled in mitosis and meiosis has been a fruitful approach to understanding how different cell division types are established. Chapter 2 will describe how the Mitotic Exit Network, a pathway essential for mitotic exit, is controlled during meiosis. Chapter 3 will describe how the conserved polo kinase Cdc5 coordinates the events of meiosis I and prepares the cell for meiosis II chromosome segregation.

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CHAPTER 2:

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; Rock and Amon, 2009; Stegmeier et al., 2002; Yoshida et al., 2002), which contributes to the co-ordination 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; Valerio-Santiago and Monje-Casas, 2011; Visintin and Amon, 2001). 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 (Bardin et al., 2000; Pereira et al., 2000; Yeh et al., 1995). 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; Bertazzi et al., 2011; Chan and Amon, 2010; D'Aquino et al., 2005; Falk et al., 2011; Maekawa et al., 2007; Pereira et al., 2000). 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 12F). 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 2A). *MOB1-mn* cells progressed through the meiosis I-meiosis II transition with wild-type kinetics, but were delayed in exit from meiosis II (Figure 2B-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 2E; (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).

Figure 1

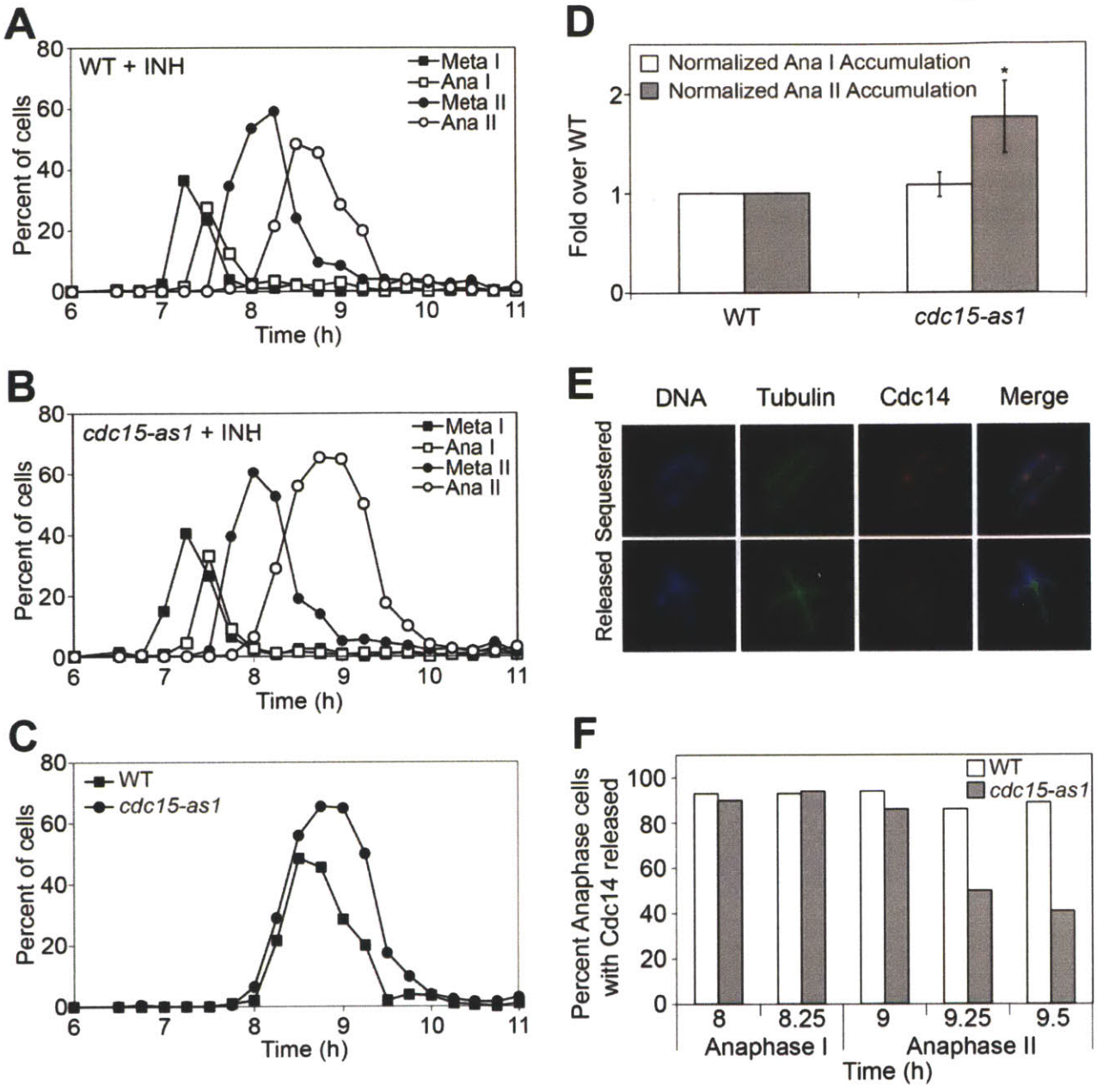
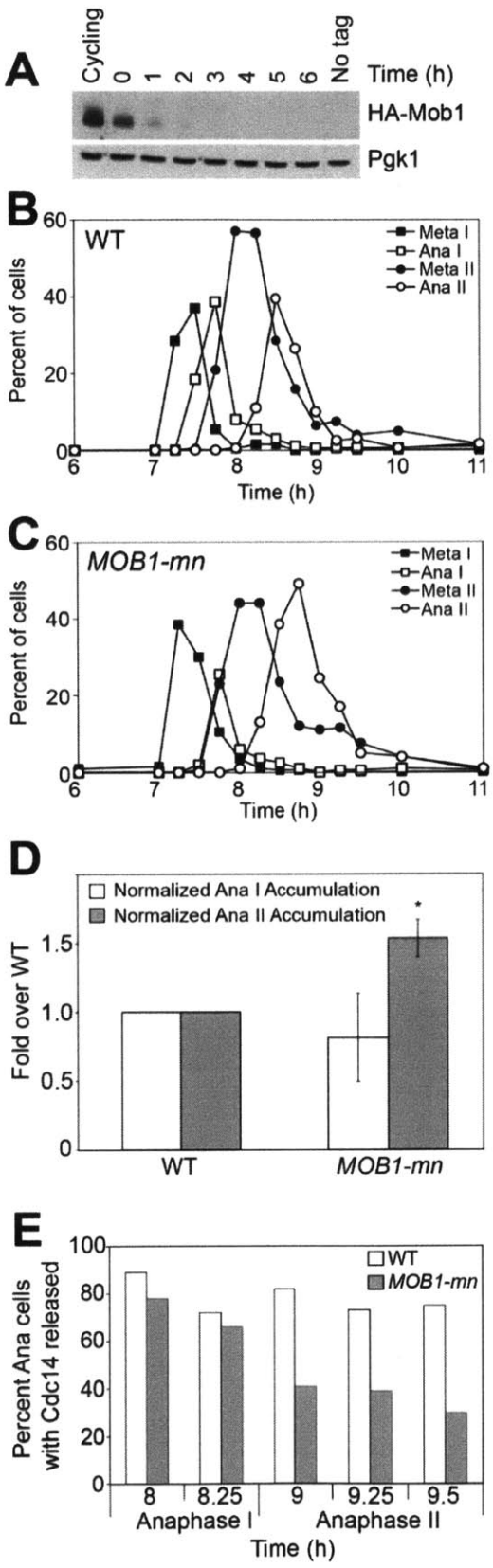

A
WT + INH
Meta I
Ana I
Meta II
Ana II
Percent of cells
Time (h)
B
cdc15-as1 + INH
C
WT
cdc15-as1
D
Normalized Ana I Accumulation
Normalized Ana II Accumulation
Fold over WT
WT
cdc15-as1
E
DNA
Tubulin
Cdc14
Merge
Sequestered
Released
F
Percent Anaphase cells with Cdc14 released
Anaphase I
Anaphase II

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. 1μM β-estradiol and 10μM 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).

Figure 2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1
2
3
4
5
6
No tag
Time (h)
HA-Mob1
Pgk1
B
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Meta I
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Meta II
Ana II
Percent of cells
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MOB1-mn
Meta I
Ana I
Meta II
Ana II
Percent of cells
Time (h)
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Normalized Ana II Accumulation
Fold over WT
WT
MOB1-mn
E
WT
MOB1-mn
Percent Ana cells with Cdc14 released
Anaphase I
Anaphase II
Time (h)

Figure 2. The MEN promotes timely exit from anaphase II.

(A) Mob1 is depleted during meiosis. *MOB1-mn* cells (A20239) were transferred to sporulation medium at t=0 and the amount of Mob1 protein was determined by Western blotting using α-HA antibodies. Pgk1 was used as a loading control.

(B - D) Wild-type (B, A14201) or *MOB1-mn* (C, A20239) cells were induced to sporulate via the Ndt80 block-release protocol. 1μM β-estradiol was added to cultures six hours after transfer to sporulation medium. The percentage of cells in metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), anaphase II (open circles) was determined at the indicated times (B, C; n=200 cells/time point). The graph in (D) shows the area under the line for anaphase I and anaphase II spindles and is expressed as fold change of *MOB1-mn* mutants over wild-type (n=3 experiments). For anaphase I accumulation, *MOB1-mn* fold change mean=0.813, SD=0.320, p=0.424 (two-tailed, paired t-test). For anaphase II accumulation, fold change mean=1.533, SD=0.136, p=0.040 (two-tailed, paired t-test). (E) Quantification of Cdc14 release in anaphase II cells. WT (A22130; white bars) or *MOB1-mn* (A25319; gray bars) cells carrying *CDC14-HA* fusions were induced to sporulate via the Ndt80 block-release protocol. At time points throughout meiosis, the percentage of anaphase I or anaphase II cells with Cdc14 released from the nucleolus was determined (n=100 cells for each time point).

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 3A-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 3E-H). This change in mobility is likely due to phosphorylation (Gruneberg et al., 2000; Keck et al., 2011; Luca and Winey, 1998) but the significance of this mobility shift is at present unclear.

Figure 3

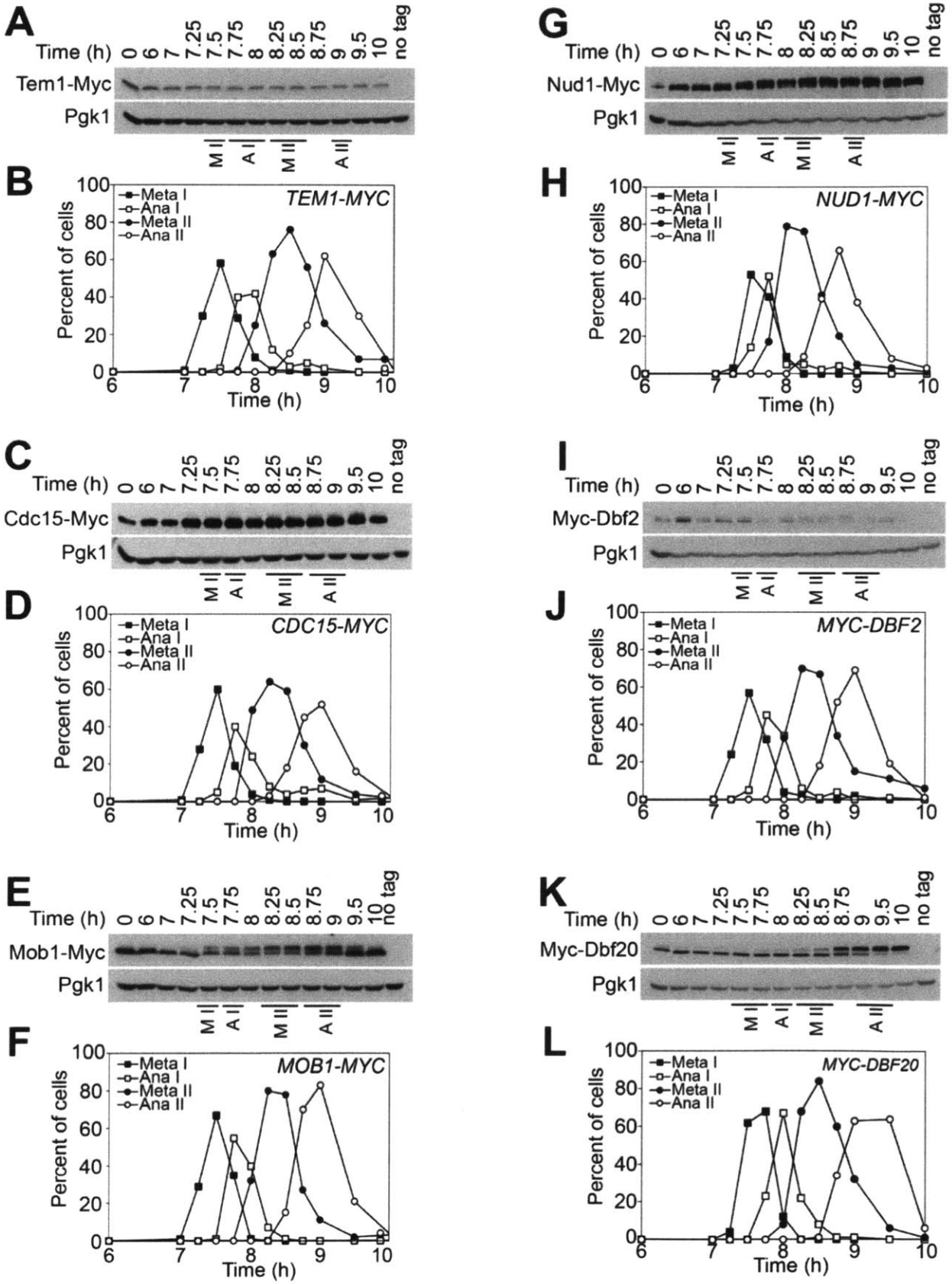
A
Time (h) 0 6 7 7.25 7.5 7.75 8 8.25 8.5 8.75 9 9.5 10 no tag
Tem1-Myc
Pgk1
M I A I M II A III
B
TEM1-MYC
Meta I
Ana I
Meta II
Ana II
Percent of cells
Time (h)
G
Nud1-Myc
H
NUD1-MYC
C
Cdc15-Myc
D
CDC15-MYC
I
Myc-Dbf2
J
MYC-DBF2
E
Mob1-Myc
F
MOB1-MYC
K
Myc-Dbf20
L
MYC-DBF20

Figure 3. MEN components are produced in meiosis.
Cells with MEN components epitope-tagged at the chromosomal locus were sporulated via the Ndt80 block-release protocol. Time points were taken throughout meiosis and the levels of the indicated MEN components was determined. The stages of meiosis are indicted by black bars (MI – metaphase I, AI – anaphase I, MII – metaphase II, AII – anaphase II). 100 cells were analyzed per timepoint. (A-B) Tem1-Myc (A18790), (C-D) Cdc15-Myc (A22416), (E-F) Mob1-Myc (A18787), (G-H) Nud1-Myc (A19443), (I-J) Myc-Dbf2 (A18949), (K-L) Dbf20-Myc (A18950).

Of the two Dbf2-like kinases present in *S. cerevisiae* only Dbf20 was expressed at appreciable levels during meiosis (Figure 3I-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 4A-B, Figure 5A; 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 4C; Figure 5B-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.

Figure 4

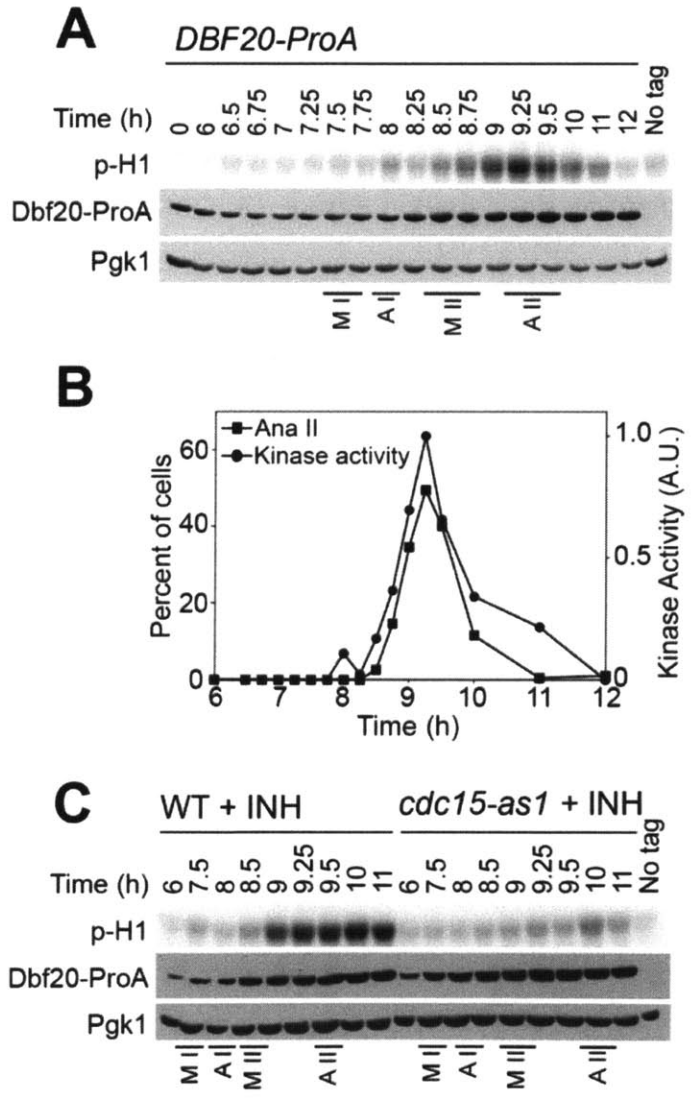

Figure 4. Dbf20 kinase activity peaks in anaphase II and depends on Cdc15 kinase activity.

(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 (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) 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.

Figure 5

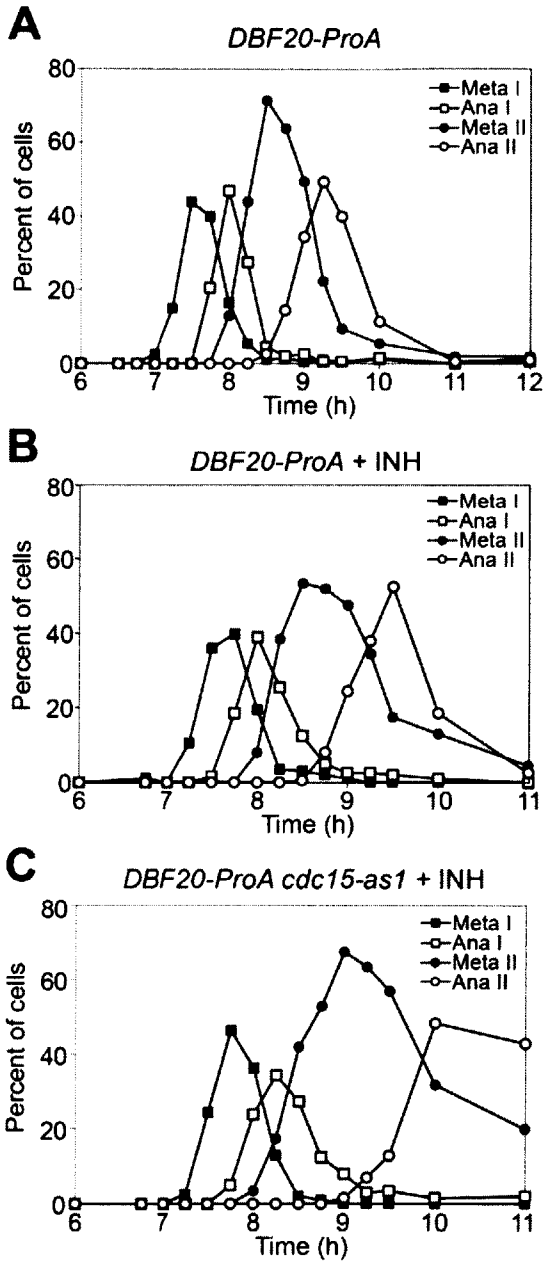

Figure 5. Dbf20 kinase activity peaks in anaphase II: meiotic kinetics.

Meiotic progression of cells analyzed in Figure 2. The percentage of cells in each stage of meiosis at time points following entry into the meiotic divisions is shown: metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), anaphase II (open circles). For each time point, n=200 cells. (A) Meiotic progression of experiment shown in Fig 2A. (B-C) Meiotic progression of experiment shown in Fig. 4.

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 6A-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 6B, bottom panels).

Figure 6

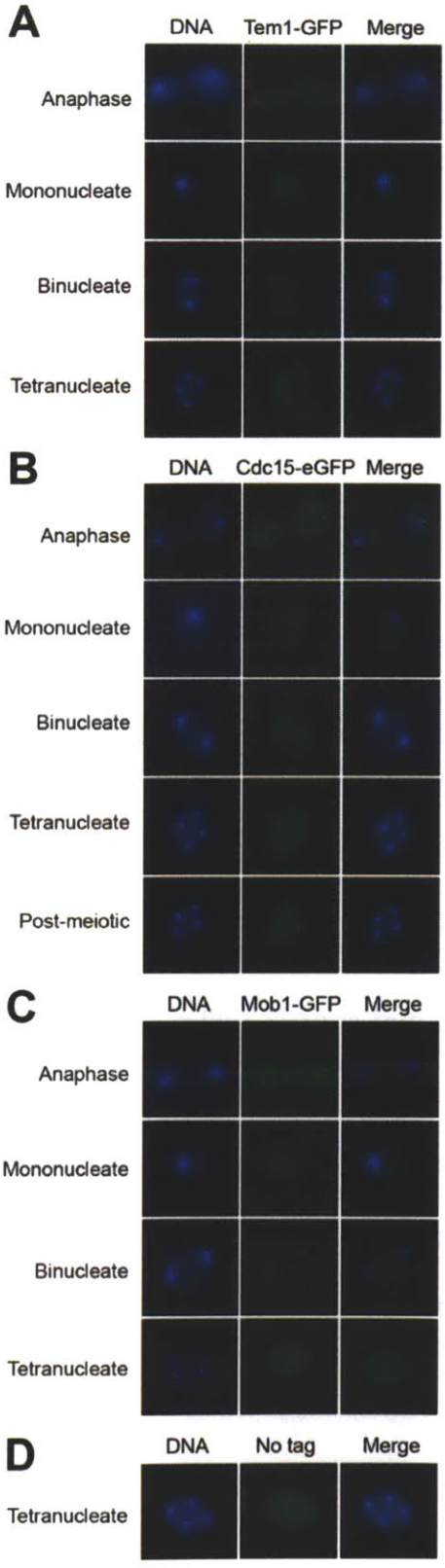
A
DNA
Tem1-GFP
Merge
Anaphase
Mononucleate
Binucleate
Tetranucleate
B
DNA
Cdc15-eGFP
Merge
Anaphase
Mononucleate
Binucleate
Tetranucleate
Post-meiotic
C
DNA
Mob1-GFP
Merge
Anaphase
Mononucleate
Binucleate
Tetranucleate
D
DNA
No tag
Merge
Tetranucleate

Figure 6. Tem1-GFP, Cdc15-eGFP and Mob1-GFP are not detected on SPBs during meiosis.

(A-C) Cells carrying a *TEM1-GFP* fusion (A22854; A), *CDC15-eGFP* (A21815; B), or *MOB1-GFP* (A22269; C) were cultured in YEPD medium or sporulated. Top panels show a representative cell in anaphase. The panels in the second, third, and fourth row show representative cells at the mononucleate, binucleate, and tetranucleate stage of meiosis. In addition, a post-meiotic cell is shown in (B). (D) A no tag control (A4962) illustrates background fluorescence.

Interestingly, the protein required for tethering these proteins to SPBs during mitosis, Nud1, was present at all SPBs throughout meiosis (Figure 7A-B). Furthermore, 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 7C-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 (Molk et al., 2004; Pereira et al., 2000). 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.

Figure 7

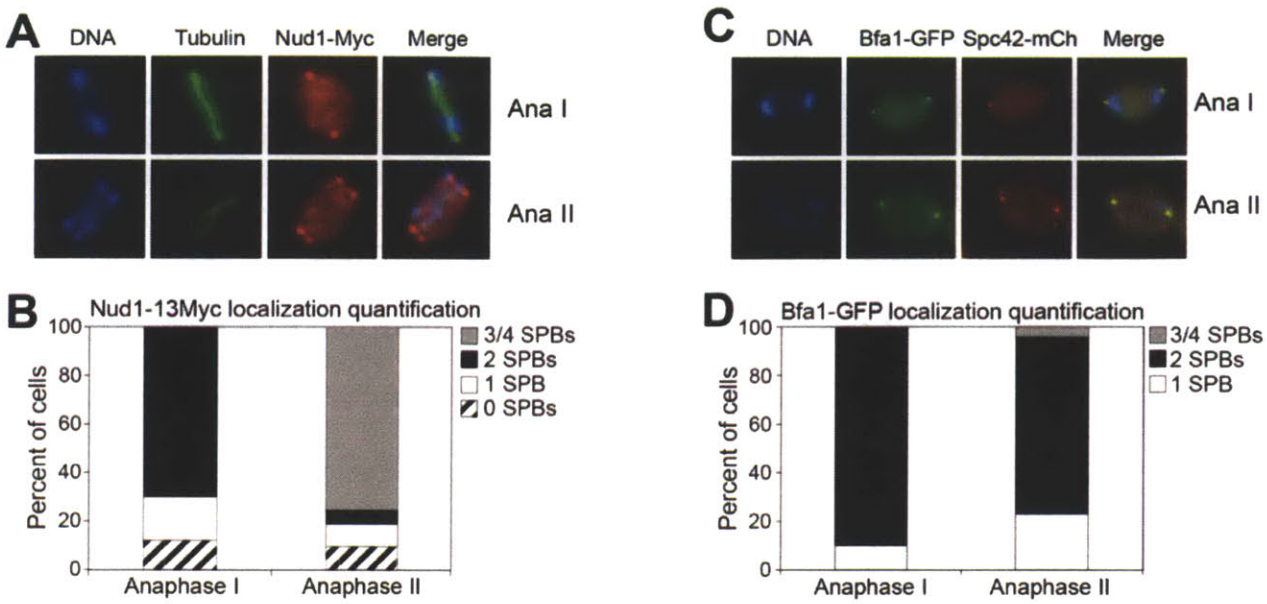

Figure 7. Nud1-Myc and Bfa1-GFP localize to spindle pole bodies in meiosis. (A, B) Cells carrying a *NUD1-MYC* fusion (A19443) were sporulated. The top panel shows a representative anaphase I cell; the bottom panel a representative anaphase II cell. The quantification of Nud1-Myc localization is shown in (B). Percent of anaphase I and anaphase II cells with Nud1-Myc on 0 SPBs (striped bar), 1 SPB (white bar), 2 SPBs (black bar), or 3/4 SPBs (gray bar) is shown. (C, D) Cells carrying *BFA1-GFP* and *SPC42-mCherry* fusions (A24035) were sporulated. Top panel shows a representative anaphase I cell; the bottom panel a representative anaphase II cell. Spc42-mCh is an integral component of the SPB. Quantification of Bfa1-GFP localization to SPBs is shown in (D). The percent of anaphase I and anaphase II cells with Bfa1-GFP on 1 SPB (white bar), 2 SPBs (black bar), or 3/4 SPBs (gray bar) is shown.

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 8A-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 8D-F).

Figure 8

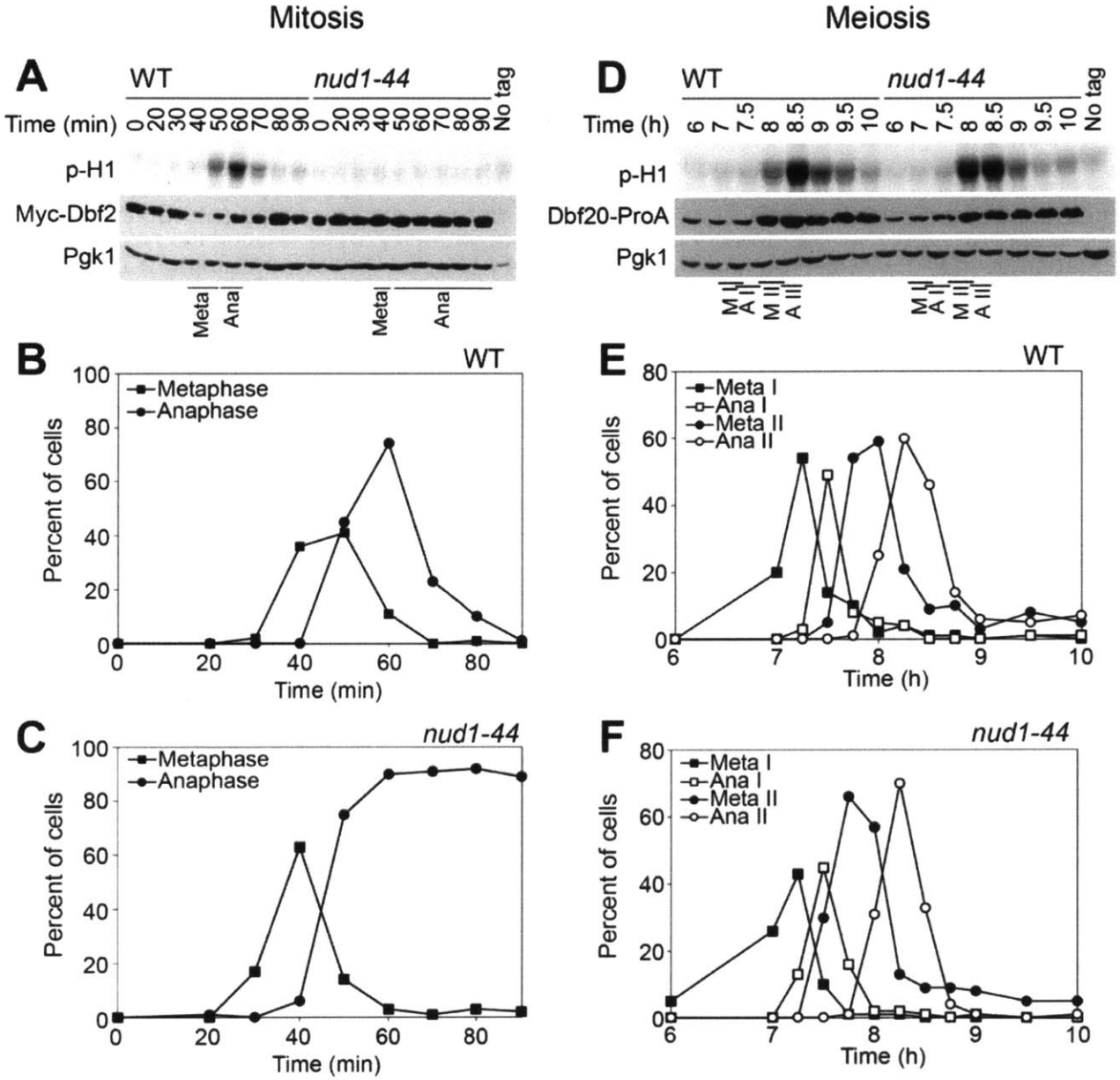
Mitosis
Meiosis
A
WT
nud1-44
No tag
Time (min)
0 20 30 40 50 60 70 80 90 0 20 30 40 50 60 70 80 90
p-H1
Myc-Dbf2
Pgk1
Meta
Ana
Meta
Ana
D
WT
nud1-44
No tag
Time (h)
6 7 7.5 8 8.5 9 9.5 10 6 7 7.5 8 8.5 9 9.5 10
p-H1
Dbf20-ProA
Pgk1
M I
A I
M II
A III
M I
A I
M II
A III
B
WT
Metaphase
Anaphase
Percent of cells
Time (min)
E
WT
Meta I
Ana I
Meta II
Ana II
Percent of cells
Time (h)
C
nud1-44
Metaphase
Anaphase
Percent of cells
Time (min)
F
nud1-44
Meta I
Ana I
Meta II
Ana II
Percent of cells
Time (h)

Figure 8. 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 (5μg/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).

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 9A-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.

Figure 9

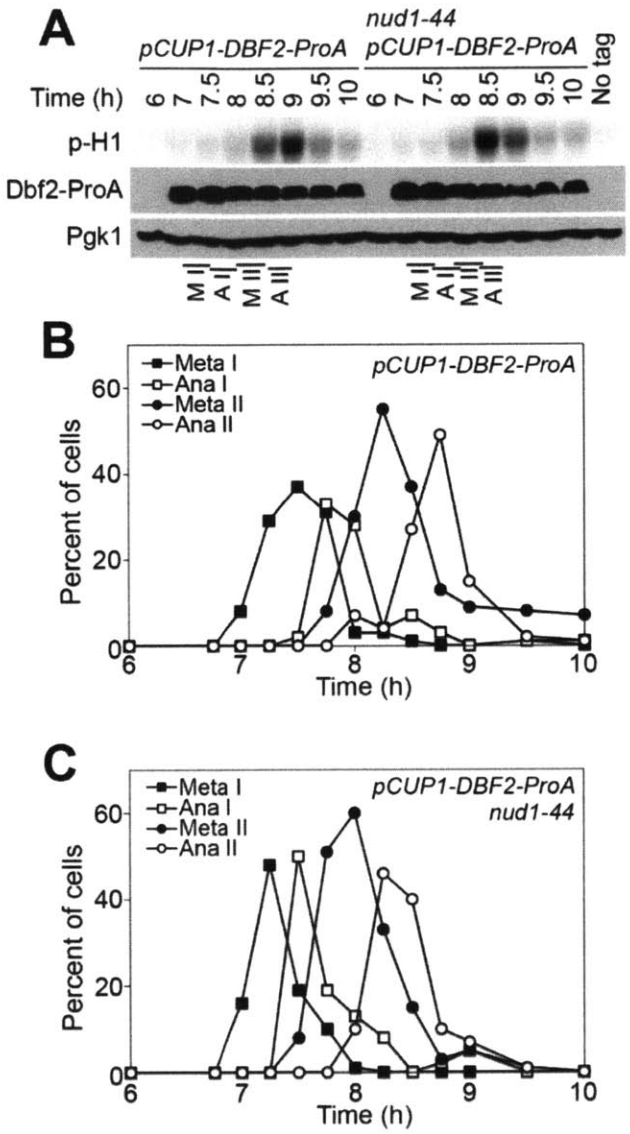

Figure 9. Dbf2 kinase activity does not require *NUD1* during meiosis.
pCUP1-DBF2-ProA (A30919) or *pCUP1-DBF2-ProA nud1-44* (A30890) cells were sporulated as in Fig. 8, with the addition of 50μM $CuSO_4$ at six hours after transfer to sporulation medium. Dbf2 kinase activity, Dbf2-ProA protein (A) and meiotic progression (B, C) were analyzed at the indicated times.

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 10A, 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 10A-D). Expression of Dbf2 during the meiotic divisions led to Dbf2 kinase activity that is regulated similarly to that of Dbf20 (Figure 10E-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 10A-D, (Visintin and Amon, 2001)), but Dbf20 is largely inactive throughout the mitotic cell cycle (Figure 10A-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.

Figure 10

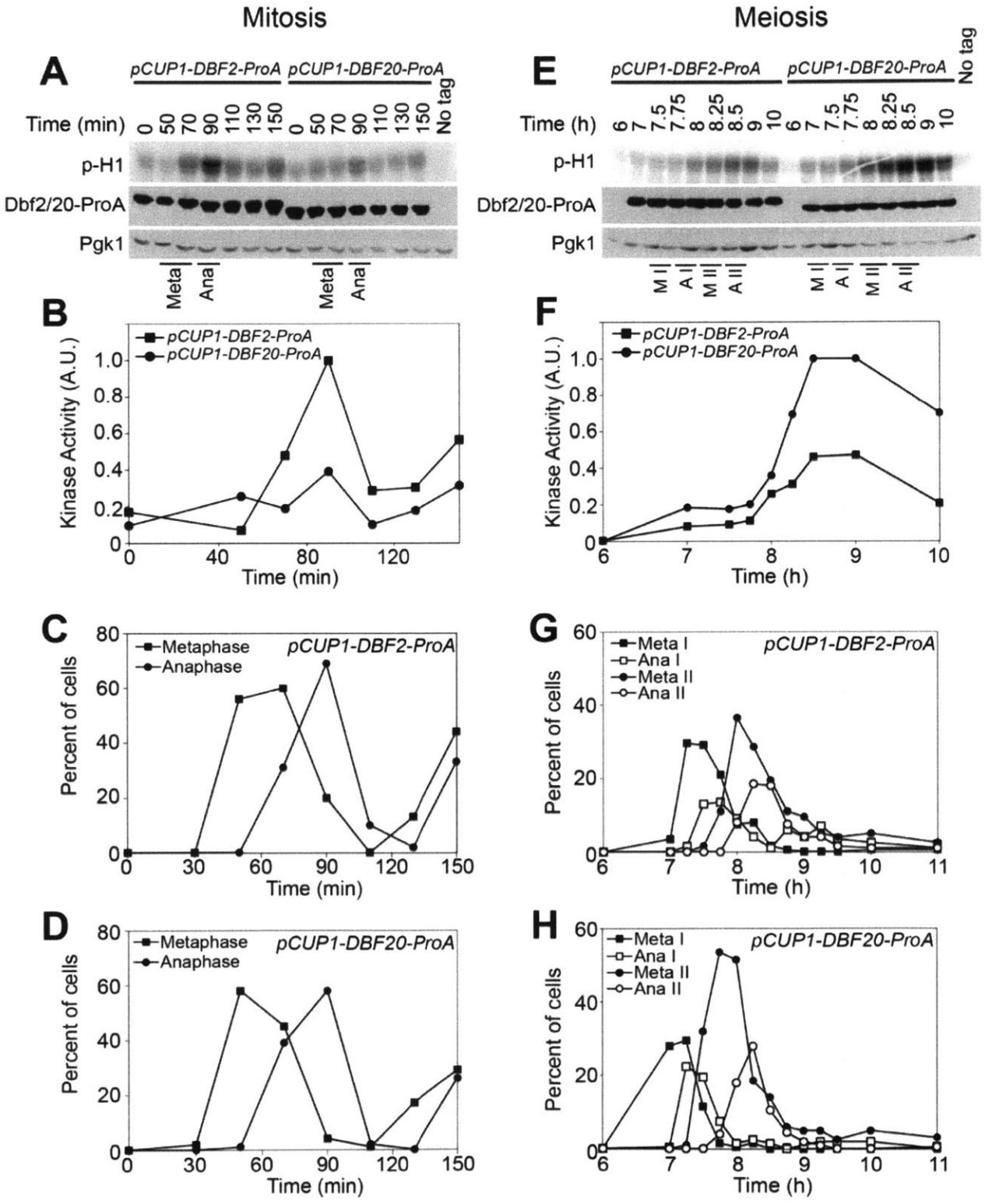
Mitosis
Meiosis
A
pCUP1-DBF2-ProA
pCUP1-DBF20-ProA
No tag
Time (min)
0 50 70 90 110 130 150 0 50 70 90 110 130 150
p-H1
Dbf2/20-ProA
Pgk1
Meta
Ana
E
Time (h)
6 7 7.5 7.75 8 8.25 8.5 9 10 6 7 7.5 7.75 8 8.25 8.5 9 10
M I
A I
M II
A II
B
Kinase Activity (A.U.)
Time (min)
F
Time (h)
C
Percent of cells
Metaphase
Anaphase
G
Meta I
Ana I
Meta II
Ana II
D
H

Figure 10. 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 (5μg/ml) in YEPD medium. 50μM $CuSO_4$ 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 50μM $CuSO_4$. 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 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. 1μM β-estradiol and 50μM $CuSO_4$ 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).

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 11A-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 11C-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 10A), 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 11E-F).

Figure 11

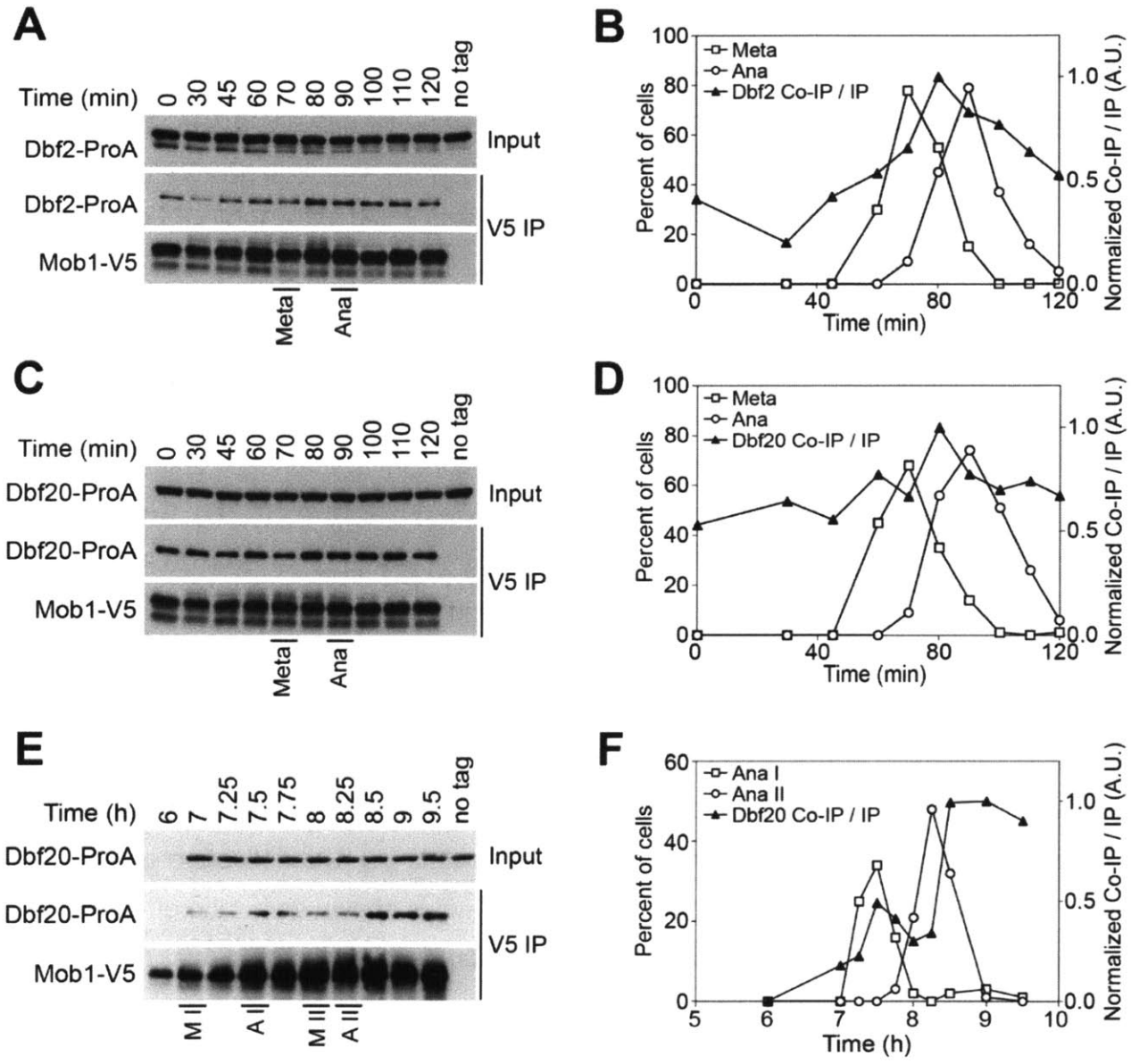
A
Time (min) 0 30 45 60 70 80 90 100 110 120 no tag
Dbf2-ProA
Input
Dbf2-ProA
V5 IP
Mob1-V5
Meta
Ana
B
Meta
Ana
Dbf2 Co-IP / IP
Percent of cells
Normalized Co-IP / IP (A.U.)
Time (min)
C
Time (min) 0 30 45 60 70 80 90 100 110 120 no tag
Dbf20-ProA
Input
Dbf20-ProA
V5 IP
Mob1-V5
Meta
Ana
D
Meta
Ana
Dbf20 Co-IP / IP
Percent of cells
Normalized Co-IP / IP (A.U.)
Time (min)
E
Time (h) 6 7 7.25 7.5 7.75 8 8.25 8.5 9 9.5 no tag
Dbf20-ProA
Input
Dbf20-ProA
V5 IP
Mob1-V5
M I
A I
M II
A II
F
Ana I
Ana II
Dbf20 Co-IP / IP
Percent of cells
Normalized Co-IP / IP (A.U.)
Time (h)

Figure 11. 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 (5μg/ml) in YEPD medium. 50μM $CuSO_4$ 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 50μM $CuSO_4$. 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-immunoprecitation 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 1μM β-estradiol and 50μM $CuSO_4$ 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.

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 12A-C). In contrast, the Dbf20-Mob1 interaction occurred independently of *CDC15* during mitosis (Figure 12D-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.

Figure 12

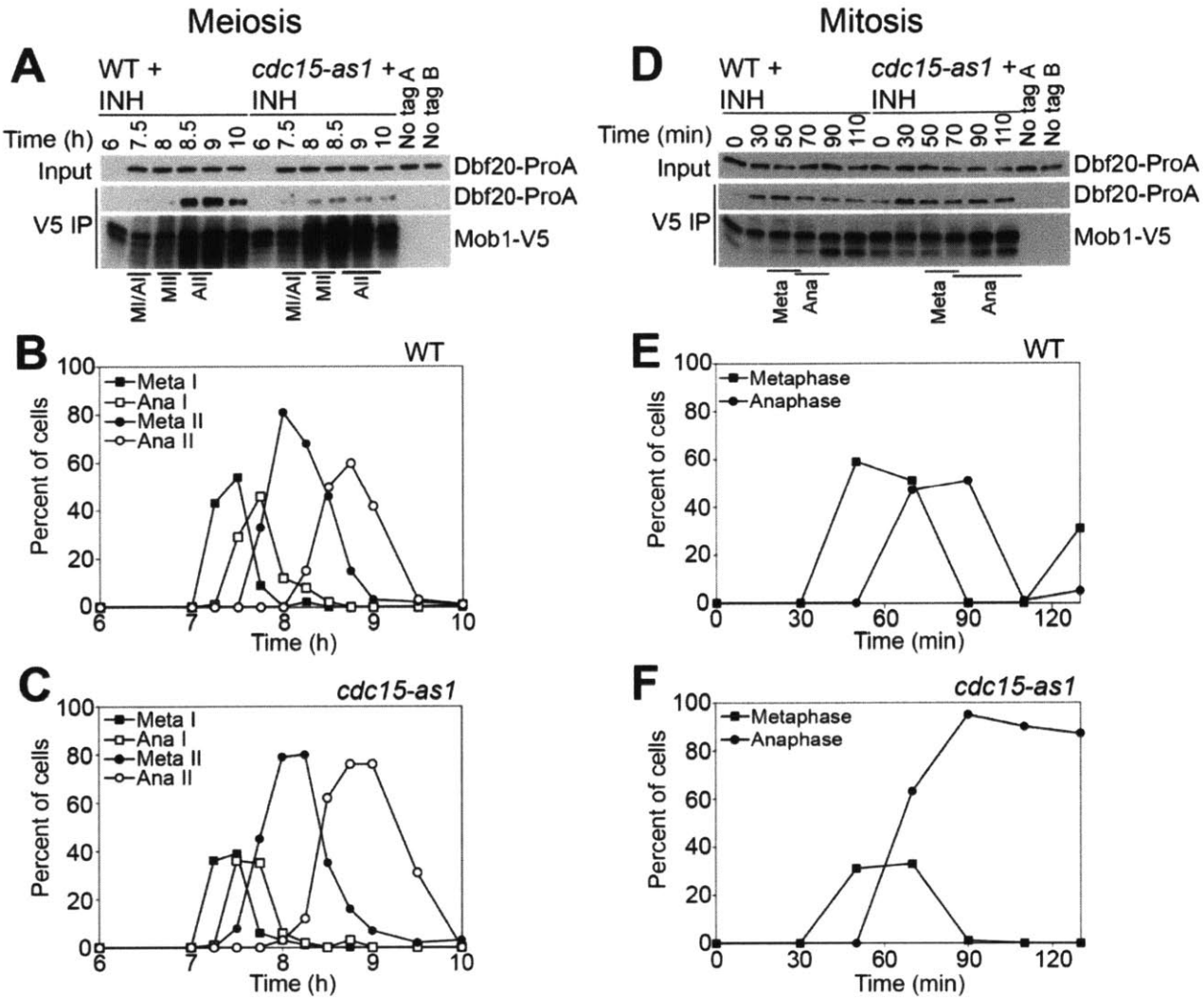
Meiosis
A
WT + INH
cdc15-as1 + INH
No tag A
No tag B
Time (h)
6 7.5 8 8.5 9 10 6 7.5 8 8.5 9 10
Input
Dbf20-ProA
Dbf20-ProA
V5 IP
Mob1-V5
MI/AI
MII
AII
MI/AI
MII
AII
B
WT
Meta I
Ana I
Meta II
Ana II
Percent of cells
Time (h)
C
cdc15-as1
Meta I
Ana I
Meta II
Ana II
Percent of cells
Time (h)
Mitosis
D
WT + INH
cdc15-as1 + INH
No tag A
No tag B
Time (min)
0 30 50 70 90 110 0 30 50 70 90 110
Input
Dbf20-ProA
Dbf20-ProA
V5 IP
Mob1-V5
Meta
Ana
Meta
Ana
E
WT
Metaphase
Anaphase
Percent of cells
Time (min)
F
cdc15-as1
Metaphase
Anaphase
Percent of cells
Time (min)

Figure 12. 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 10μM NA-PP1 and 50μM $CuSO_4$ 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 (5μg/ml) in YEPD medium as in Figure 10A. 50μM $CuSO_4$ 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 10μM NA-PP1 and 50μM $CuSO_4$. Total Dbf20-ProA protein (input), Co-immunoprecipitated Dbf20-ProA, and immumoprecipitated 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).

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 (Buonomo et al., 2003; Marston et al., 2003; Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). 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*, (Chan and Amon, 2010; D'Aquino et al., 2005; Pereira and Schiebel, 2005) 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 3I). When we expressed Dbf2 from an inducible promoter in meiosis, the protein was readily expressed and was active as a kinase (Figure 10E). 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 10E). 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 3K; 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 1. *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, 1μM β-estradiol was added to the medium allowing cells to progress through the meiotic divisions in a synchronous

manner. For *cdc15-as1* experiments, 10μM 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 0.05μg/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 100μl 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 150µl). 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 200µl 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 500µg (mitosis) of extract were used in immunopreciptiation experiments (volume of 150µl) 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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TABLE 1. Yeast strains used in this study. All strains are in the SK1 strain background.

A4841	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG*
A4962	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG,*
A8499	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG 3MYC-DBF2*
A14201	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1*
A18787	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, MOB1-13MYC:KanMX6/MOB1-13MYC:KanMX6*
A18790	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, TEM1-9MYC:TRP1/TEM1-9MYC:TRP1*
A18949	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, 3MYC-DBF2/3MYC-DBF2*
A18950	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, 3MYC-DBF20/3MYC-DBF20*
A19440	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, cdc15-as1:URA3/cdc15-as1:URA3*
A19443	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, NUD1-13MYC:KanMX6/NUD1-13MYC:KanMX6*
A20239	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, KanMX6:pCLB2-3HA-MOB1/KanMX6:pCLB2-3HA-MOB1*
A21815	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, CDC15-eGFP:KanMX6/CDC15-eGFP:KanMX6*
A22129	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, cdc15-as1:URA3/cdc15-as1:URA3, CDC14-3HA/CDC14-3HA*

A22130	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3:pGPD1-GAL4(848).ER::URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, CDC14-3HA/CDC14-3HA*
A22269	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, MOB1-GFP:KanMX6/MOB1-GFP:KanMX6*
A22416	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, CDC15-9MYC:TRP1/CDC15-9MYC:TRP1*
A22854	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, TEM1-GFP:HIS3MX6/TEM1-GFP:HIS3MX6*
A23162	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, DBF20-TEV-ProA-7HIS:HIS3MX6/DBF20-TEV-ProA-7HIS:HIS3MX6*
A23733	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, DBF20-TEV-ProA-7HIS:HIS3MX6/DBF20-TEV-ProA-7HIS:HIS3MX6, cdc15-as1:URA3/cdc15-as1:URA3*
A24035	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, BFA1-GFP:HIS3MX6/BFA1-GFP:HIS3MX6, SPC42-mCherry:NatMX6/SPC42-mCherry:NatMX6*
A25020	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG, KanMX6:pCUP1-DBF2-TEV-ProA-7HIS:HIS3MX6*
A25028	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, KanMX6:pCUP1-DBF2-TEV-ProA-7HIS:HIS3MX6/KanMX6:pCUP1-DBF2-TEV-ProA-7HIS:HIS3MX6*
A25191	*MATa, ho::LYS2, lys2, leu2::hisG, his3::hisG, trp1::hisG, ura3::pGPD1-GAL4(848).ER::URA3, GAL-NDT80:TRP1, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6*
A25193	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6*
A25195	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6/KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6*
A25319	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, KanMX6:pCLB2-3HA-*

	MOB1/KanMX6:pCLB2-3HA-MOB1, CDC14-3HA/CDC14-3HA
A27370	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6/KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6, MOB1-3V5:KanMX6/MOB1-3V5:KanMX6*
A27371	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6/KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6, MOB1-3V5:KanMX6/MOB1-3V5:KanMX6*
A27367	*MATa, ho::LYS2, lys2, leu2::hisG, his3::hisG, trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1, MOB1-3V5:KanMX6, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6*
A27687	*MATa, ho::LYS2, lys2, leu2::hisG, his3::hisG, trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1, MOB1-3V5:KanMX6, KanMX6:pCUP1-DBF2-TEV-ProA-7HIS:HIS3MX6*
A27697	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, DBF20-TEV-ProA-7HIS:HIS3MX6/DBF20-TEV-ProA-7HIS:HIS3MX6, nud1-44:TRP1/nud1-44:TRP1, nud1::HIS5/nud1::HIS5*
A28757	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG nud1-44:TRP1, nud1::HIS5, 3MYC-DBF2*
A29124	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3, cdc15-as1:URA3, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6*
A29125	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3, cdc15-as1:URA3, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6, MOB1-3V5:KanMX6*
A29149	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6/KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6, MOB1-3V5:KanMX6/MOB1-3V5:KanMX6, cdc15-as1:URA3/cdc15-as1:URA3*
A29150	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6/KanMX6:pCUP1-DBF20-TEV-ProA-7HIS:HIS3MX6, cdc15-as1:URA3/cdc15-as1:URA3*
A30890	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, nud1-44:TRP1/nud1-44:TRP1, nud1::HIS5/nud1::HIS5, MOB1-3V5:KanMX6, KanMX6:pCUP1-DBF2-TEV-*

	ProA-7HIS:HIS3MX6, 3MYC-DBF20
A30919	*MATa/alpha, ho::LYS2/ ho::LYS2, lys2/ lys2, leu2::hisG/ leu2::hisG, his3::hisG/ his3::hisG, trp1::hisG/ trp1::hisG, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, GAL-NDT80:TRP1/ GAL-NDT80:TRP1, nud1-44:TRP1/nud1-44:TRP1, nud1::HIS5/nud1::HIS5, MOB1-3V5:KanMX6, 3MYC-DBF20*

CHAPTER 3:

The Polo kinase Cdc5 is a central regulator of meiosis I.

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The experiments in figures 1-6, 7A-B, 7E, 8A-B, 9, 10A-C, 11A, 12 were performed by MAA
The experiments in figures 10D-G, 11B-D were performed by MPM
The experiments in figures 7C and 8C were performed by LE
The experiment in figure 7D was performed by SKE

ABSTRACT

During meiosis, two consecutive rounds of chromosome segregation yield four haploid gametes from one diploid cell. The Polo kinase Cdc5 is required for meiotic progression, but how Cdc5 coordinates multiple cell cycle events during meiosis I is not understood. Here we show that *CDC5*-dependent phosphorylation of Rec8, a subunit of the cohesin complex that links sister chromatids, is required for efficient cohesin removal from chromosome arms, which is a prerequisite for meiosis I segregation. *CDC5* also establishes conditions for centromeric cohesin removal during meiosis II by promoting the degradation of Spo13, a protein that protects centromeric cohesin during meiosis I. Despite *CDC5*'s central role in meiosis I, *CDC5* is dispensable during meiosis II and does not even phosphorylate its meiosis I targets during the second meiotic division. We conclude that Cdc5 is not only a central regulator of mitosis, but has evolved into a master regulator of the unique meiosis I chromosome segregation pattern.

INTRODUCTION

Polo-like kinases are central regulators of chromosome segregation. Members of this family of protein kinases have been implicated in centrosome duplication, recovery from DNA damage checkpoint arrest, nuclear envelope break-down, chromosome morphogenesis and segregation, exit from mitosis and cytokinesis (Archambault and Glover, 2009). Budding yeast contains a single Polo-like kinase, *CDC5*. Unlike in higher eukaryotes, *CDC5* primarily regulates post-metaphase events, during which its essential function is to trigger exit from mitosis. Exit from mitosis is the final cell cycle transition during which the mitotic spindle disassembles, chromosomes decondense and cytokinesis occurs. Cdc5 performs multiple functions during this transition (Stegmeier and Amon, 2004). *CDC5* activates two signaling pathways, the Mitotic Exit Network (MEN) and the FEAR network, that in turn trigger exit from mitosis by activating the protein phosphatase Cdc14 (Hu et al., 2001; Jaspersen et al., 1998; Pereira et al., 2002; Rock and Amon, 2011; Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999; Yoshida et al., 2002). In addition to its essential function in exit from mitosis, *CDC5* also contributes to the efficient inactivation of cohesins, the protein complexes that hold sister chromatids together until the onset of chromosome segregation. Cdc5 phosphorylates the cohesin subunit Scc1/Mcd1 to facilitate its cleavage by the protease Separase (Alexandru et al., 2001; Hornig and Uhlmann, 2004).

CDC5 also regulates the specialized cell division that gives rise to gametes, known as meiosis (Sharon and Simchen, 1990). Meiosis produces four haploid gametes from one diploid progenitor. This reduction in ploidy is accomplished by two consecutive rounds of chromosome segregation following one round of DNA replication. During

meiosis I, homologous chromosomes segregate; during meiosis II, sister chromatids split (Marston and Amon, 2004). During this division *CDC5* acquires novel functions to bring about this unique chromosome segregation pattern. To facilitate meiosis I, chromosomes are modified in three fundamental ways. First, homologous chromosomes must be physically linked in order to be accurately segregated. This linking is mediated by homologous recombination, where programmed double strand-breaks created throughout the genome catalyze the exchange of DNA strands between homologous chromosomes. Cohesin complexes distal to the resulting cross-overs link homologs. Second, sister chromatids of each homolog must be segregated to the same pole rather than to opposite poles as they are during mitosis. This requires the fusion of sister kinetochores by co-orientation factors (called the monopolin complex in yeast) to facilitate the attachment of microtubules emanating from one spindle pole. Third, to facilitate two rounds of chromosome segregation, cohesin complexes must be lost in a step-wise manner from chromosomes. During meiosis I cohesin complexes are lost from chromosome arms to bring about the segregation of homologous chromosomes (Buonomo et al., 2000). The residual cohesins at centromeres facilitate the accurate attachment and segregation of sister chromatids during metaphase II.

Cdc5 has been implicated in the execution of all three meiosis I-specific events. *CDC5* is required for the resolution of double Holliday junctions during homologous recombination (Clyne et al., 2003; Sourirajan and Lichten, 2008). Cdc5 also controls the attachment of sister chromatids to microtubules emanating from the same pole by promoting the association of the monopolin complex with kinetochores (Clyne et al., 2003; Lee and Amon, 2003). Finally, *CDC5* has been implicated in controlling the

stepwise loss of cohesins (Brar et al., 2006; Clyne et al., 2003; Lee and Amon, 2003). The balance of phosphorylation and dephosphorylation of the cohesin subunit Rec8, a meiosis-specific cohesin subunit that replaces Scc1/Mcd1 in the meiotic cohesin complex, is thought to control the stepwise loss of cohesins from chromosomes. Rec8 is a highly phosphorylated protein, and its phosphorylation is critical for its proteolytic cleavage and removal from chromosome arms during meiosis I (Brar et al., 2006; Katis et al., 2010). Maintaining Rec8 in a dephosphorylated form around centromeric regions protects it from cleavage. This is accomplished by the shugoshin/MEI-S332 family of proteins. The budding yeast shugoshin/MEI-S332, Sgo1, recruits the phosphatase PP2A to centromeric regions (Katis et al., 2004a; Kerrebrock et al., 1995; Kitajima et al., 2004; Kitajima et al., 2006; Riedel et al., 2006). Our studies have implicated Cdc5 as one, but not the only, protein kinase phosphorylating Rec8 to target it for proteolytic cleavage by Separase (Brar et al., 2006). A subsequent study identified Dbf4-dependent kinase (DDK) and the casein kinase Hrr25 as Rec8 kinases important for Rec8 cleavage, but concluded that Rec8 phosphorylation by Cdc5 was dispensable for Rec8 cleavage (Katis et al., 2010).

In addition to controlling meiosis I specific events, *CDC5* also regulates general cell cycle functions during meiosis I that it does not affect during mitosis. During meiosis I, *CDC5* controls Separase activity. Separase is kept inactive during early stages of the cell cycle by Securin (Pds1 in yeast). Pds1 degradation, which is mediated by the ubiquitin ligase APC/C-Cdc20, liberates Separase to trigger anaphase (Ciosk et al., 1998; Cohen-Fix et al., 1996; Salah and Nasmyth, 2000). During meiosis I, but not during mitosis, *CDC5* is required for Pds1 degradation, as cells depleted for Cdc5

exhibit a delay in Pds1 degradation (Clyne et al., 2003; Lee and Amon, 2003). How Cdc5 controls multiple cell cycle events and takes on new functions during meiosis I is not understood. Cdc5 has been proposed to carry out some of its meiosis I functions via its physical interaction with Spo13, a meiosis I-specific protein of unknown function essential for sister kinetochore co-orientation and the protection of centromeric cohesins during meiosis I (Katis et al., 2004b; Lee et al., 2004; Matos et al., 2008). However, the interplay between Spo13 and Cdc5 is not well understood. Similarly, little is known about whether and how Cdc5 functions during meiosis II because cells depleted for Cdc5 arrest in metaphase I (Clyne et al., 2003; Lee and Amon, 2003).

Producing gametes with the correct chromosome number is essential for sexual reproduction. Chromosome mis-segregation during meiosis is the leading cause of miscarriages and congenital birth defects in humans (Hassold and Hunt, 2001). Thus, understanding how conserved cell cycle regulators such as Polo kinases shape the meiotic divisions and are themselves modulated to bring about meiosis-specific events is not only critical for understanding the basic processes of sexual reproduction but also for gaining insight into the molecular basis of infertility and developmental disabilities such as Down Syndrome. Here we define the role of the Polo kinase Cdc5 in establishing the meiotic chromosome segregation pattern. We show that *CDC5* controls cohesin removal in multiple ways. Cdc5-dependent phosphorylation of Rec8 is essential for efficient Rec8 cleavage and timely meiosis I chromosome segregation. Furthermore, Cdc5 triggers the degradation of Spo13. By promoting Spo13 degradation and therefore disrupting the cohesin-protective domain around centromeres, *CDC5* establishes permissive conditions for the removal of cohesins during meiosis II. Our data further

show that despite its central role in meiosis I chromosome segregation, *CDC5* is dispensable during meiosis II. Execution point studies show that *CDC5* is not required for meiotic progression after exit from meiosis I and in fact does not phosphorylate its meiosis I targets during meiosis II. Our findings indicate that the evolution of additional layers of *CDC5* mediated regulation is a central aspect of establishing the unique meiotic chromosome segregation pattern.

RESULTS

Phosphorylation of Rec8 residues S136 and S179 is *CDC5*-dependent

The step-wise loss of cohesins is mediated by the regulated phosphorylation of the cohesin complex subunit Rec8. We and others previously mapped the phosphorylation sites in Rec8, and determined that phosphorylation of Rec8 is crucial for its cleavage and removal from chromosome arms in meiosis I (Brar et al., 2006; Katis et al., 2010). Phosphorylation of many of the sites depended on *CDC5*, which led us to conclude that *CDC5*-dependent phosphorylation contributes to Rec8 cleavage (Brar et al., 2006). However, substituting a subset of the *CDC5*-dependent phosphorylation sites in *REC8* with alanine does not prevent its cleavage (Brar et al., 2006), suggesting that Cdc5 is not the only kinase controlling Rec8 cleavage and removal. Indeed, Katis et al (2010) identified two additional kinases regulating Rec8: Hrr25 and DDK. This study also came to the conclusion that *CDC5*-dependent phosphorylation of Rec8 did not contribute to Rec8 removal from chromosomes. This conclusion was at odds with our finding that many of the Rec8 phosphorylation sites we (Brar et al., 2006) and Katis et al. (2010) identified as required for cohesin removal were

phosphorylated in a *CDC5*-dependent manner in our mass spectrometry analyses (Brar et al., 2006). To address this discrepancy we first wished to verify that the phosphorylation sites in Rec8 that we determined to be *CDC5* dependent by mass-spectrometry were indeed phosphorylated in a *CDC5*-dependent manner. We raised phospho-specific antibodies against three putative *CDC5*-dependent phosphorylation sites, S136, S179 and S197 and one site, S521, whose phosphorylation was predicted to be *CDC5*-independent.

We succeeded in generating phospho-specific antibodies against S136, S179 and S521 (Figure 1A-C; Brar et al., 2006). The antibody recognized wild-type Rec8 but not a Rec8 mutant protein in which these residues were mutated to sites that can no longer be phosphorylated. We were not able to generate phospho-specific antibodies against S197. Consistent with the mass-spectrometry results, we found that phosphorylation of S136 and S179 depended on *CDC5*, but phosphorylation of S521 did not (Figure 1D, Figure 2A-B; (Brar et al., 2006)). S136 and S179 were phosphorylated in cells depleted for the APC/C activator Cdc20, which arrests cells in metaphase I but not in cells depleted for Cdc5 which also arrests cells in metaphase I (Figure 1D, Figure 2A-B; (Brar et al., 2006); (Lee and Amon, 2003)). Phosphorylation of S521 was *CDC5* independent (Brar et al., 2006).

Our analyses further showed that expression of *CDC5* was sufficient to induce S136 and S179 phosphorylation. To regulate *CDC5* expression we placed the gene under the control of the copper-inducible *CUP1* promoter. We arrested cells in prophase I, when Cdc5 is normally not expressed by preventing expression of the gene encoding the transcription factor Ndt80 and then induced *CDC5* expression. Both S136 and S179

phosphorylation were induced upon Cdc5 expression (Figure 1E; 2C-D). We conclude that *CDC5* is necessary and sufficient for the phosphorylation of Rec8-S136 and Rec8-S179. Moreover, because the *CDC5*-dependence of S136, S179, and S521 was accurately defined by our mass spectrometry analysis, phosphorylation of S197 and the other sites defined as *CDC5*-dependent by our mass spectrometry analysis most likely depend on *CDC5* as well.

Figure 1

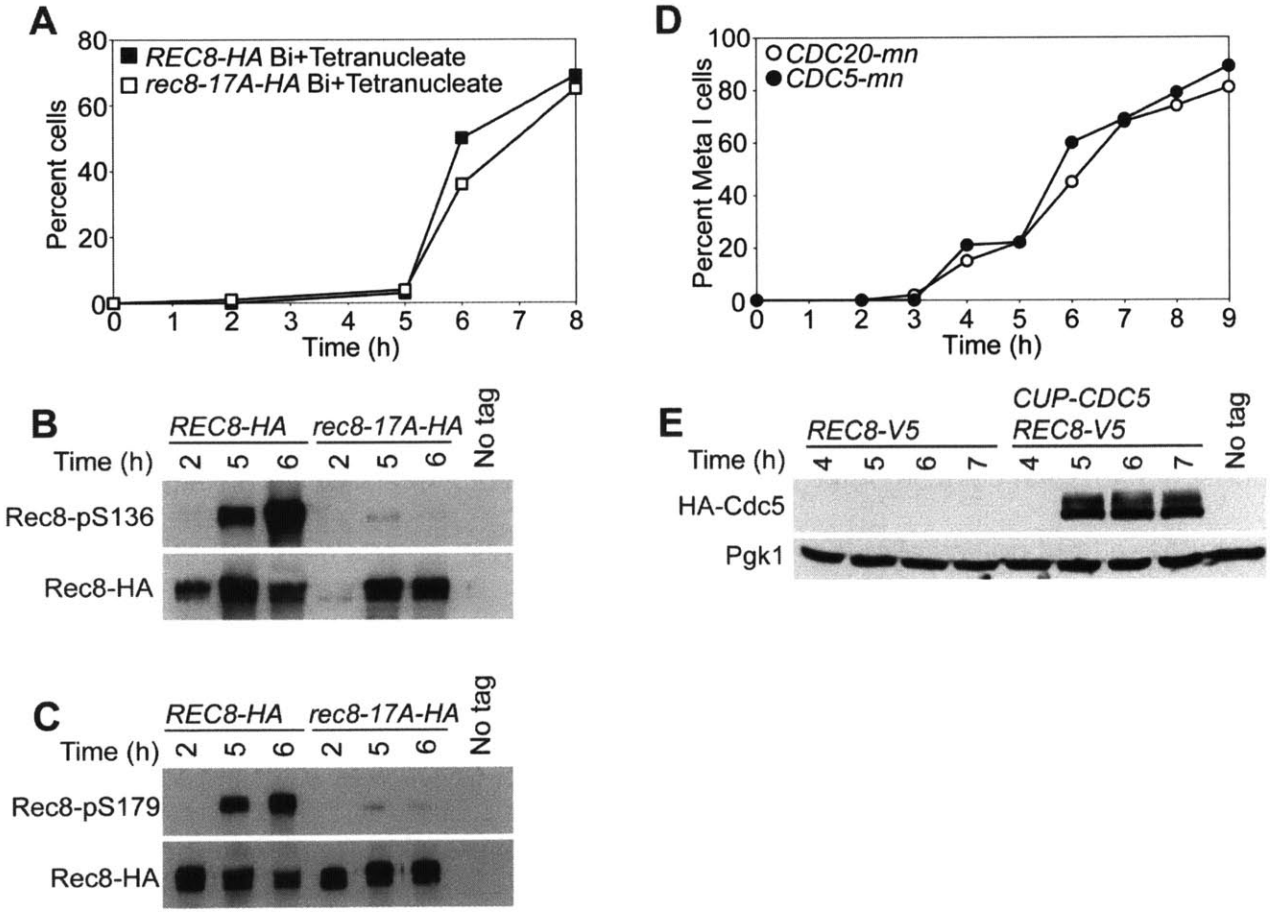


Figure 1. Phosphorylation of Rec8 residues S136 and S179 is *CDC5*-dependent.
(A-C) Cells containing *REC8-HA* (A1972) or *rec8-17A-HA* (A13559) were induced to sporulate. The percentage of cells with two and four nuclei (A; n=100 cells/time point), total Rec8-HA protein levels and S136 (B) and S179 (C) phosphorylation were determined at the indicated times. A no tag control (A4962) is shown in the last lane.
(D) Cells containing a *REC8-HA* fusion were arrested in metaphase I either by depletion of *CDC20* (*CDC20-mn*; A27808) or *CDC5* (*CDC5-mn*; A27809). The percentage of cells with metaphase I spindles was determined (n=100 cells/time point).
(E) Degree of *CDC5* expression in the experiment shown in Figure 1C and D: Cells containing *NDT80* under the control of the *GAL1-10* promoter (*GAL-NDT80*), a *REC8-V5* fusion, and *CDC5* (A33368) or *CDC5* under the control of the copper-inducible promoter *pCUP1 (CUP-CDC5*; A32851*)* were induced to sporulate. Cultures were induced with 50 μM $CuSO_4$ after 4 hours, and cultures remained arrested in prophase I due to the lack of *NDT80* expression. Cdc5 levels were assessed. Pgk1 was used as a loading control.

Figure 2

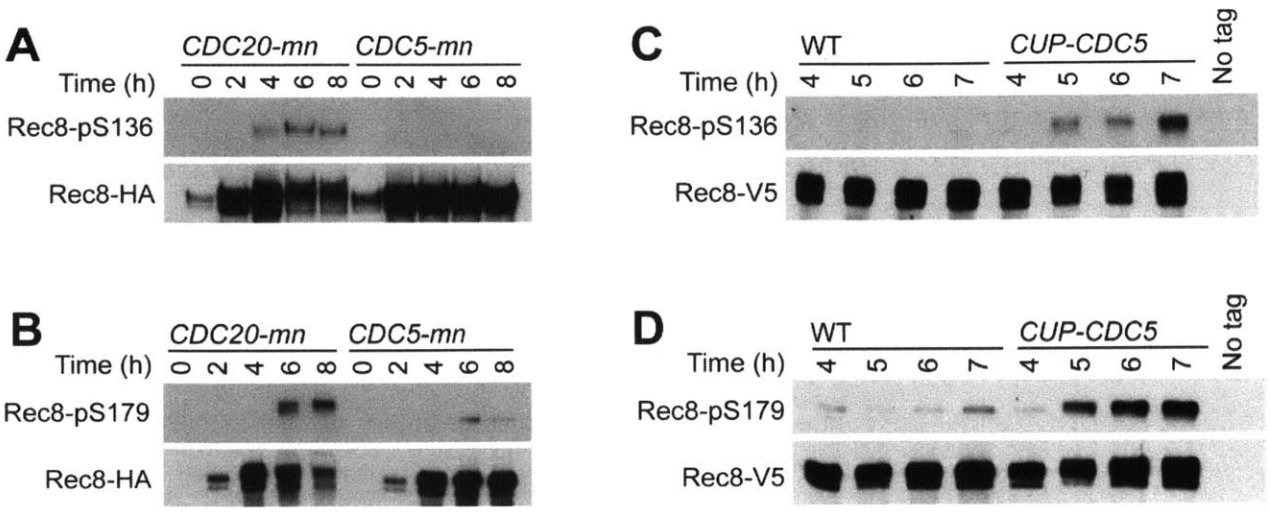


Figure 2. Phosphorylation of Rec8 residues S136 and S179 is *CDC5*-dependent.

(A-B) Cells containing *REC8-HA* were induced to sporulate and arrested in metaphase I either by meiotic depletion of *CDC20* (*CDC20-mn*; A27808) or *CDC5* (*CDC5-mn;* A27809). The phosphorylation state of Rec8-S136 (A) and Rec8-S179 (B) as well as total levels of Rec8-HA were determined at the indicated times.

(C-D) Cells containing *NDT80* under the control of the *GAL1-10* promoter (*GAL-NDT80*), a *REC8-V5* fusion, and *CDC5* (A33368) or *CDC5* under the control of the copper-inducible promoter *pCUP1 (CUP-CDC5*; A32851*)* were induced to sporulate. Cultures were induced with 50μM $CuSO_4$ after 4 hours, and cultures remained arrested in prophase I due to the lack of *NDT80* expression. Phosphorylation of Rec8-S136 (C) and Rec8-S179 (D), and total Rec8-HA protein were assessed at the indicated times. A vegetative no-tag control (A4962) is shown in the last lane.

Phosphorylation of S136 and S179 contributes to cohesin removal.

By examining the effects of mutating phosphorylated residues to amino acids that mimic phosphorylation, Katis et al (2010) showed that phosphorylation of S136, S179, S197 and T209 are critical for cohesin removal. If phosphorylation of S136, S179, S197 and T209 is important for cohesin cleavage, Rec8 mutants that mimic phosphorylation of these sites should no longer be protected from cleavage around centromeres during meiosis I and centromeric cohesins are lost prematurely during meiosis I. Premature loss of centromeric cohesins can be assayed because it leads to the random segregation of sister chromatids during meiosis II. Katis, et al. (2010) found that a *REC8* mutant in which S136, S179, S197 and T209 were mutated to aspartic acid (henceforth *rec8-S136D S179D S197D T209D)* causes premature loss of centromeric cohesion and nearly random meiosis II chromosome segregation.

We observed a similar result. We monitored chromosome segregation utilizing a tandem array of TetO sequences integrated close to the centromere of one copy of chromosome V. Cells also expressed a tetR-GFP fusion, allowing visualization of the tetO arrays (Michaelis et al., 1997; Tanaka et al., 2002). In wild-type cells carrying these heterozygous GFP dots, meiosis I yields two nuclei, with two GFP dots in one of the two nuclei. After meiosis II, two of the four nuclei contain one GFP dot each. If centromeric cohesins are lost prematurely, chromosome segregation will appear normal during meiosis I. However, since sister chromatids are no longer linked, they will segregate randomly during meiosis II, causing 50% of tetranucleates harboring one GFP dot in two of the four nuclei and 50% of tetrads harboring two GFP dots in one of the four nuclei. *rec8* phospho-mimetic mutants segregated homologous chromosomes normally during

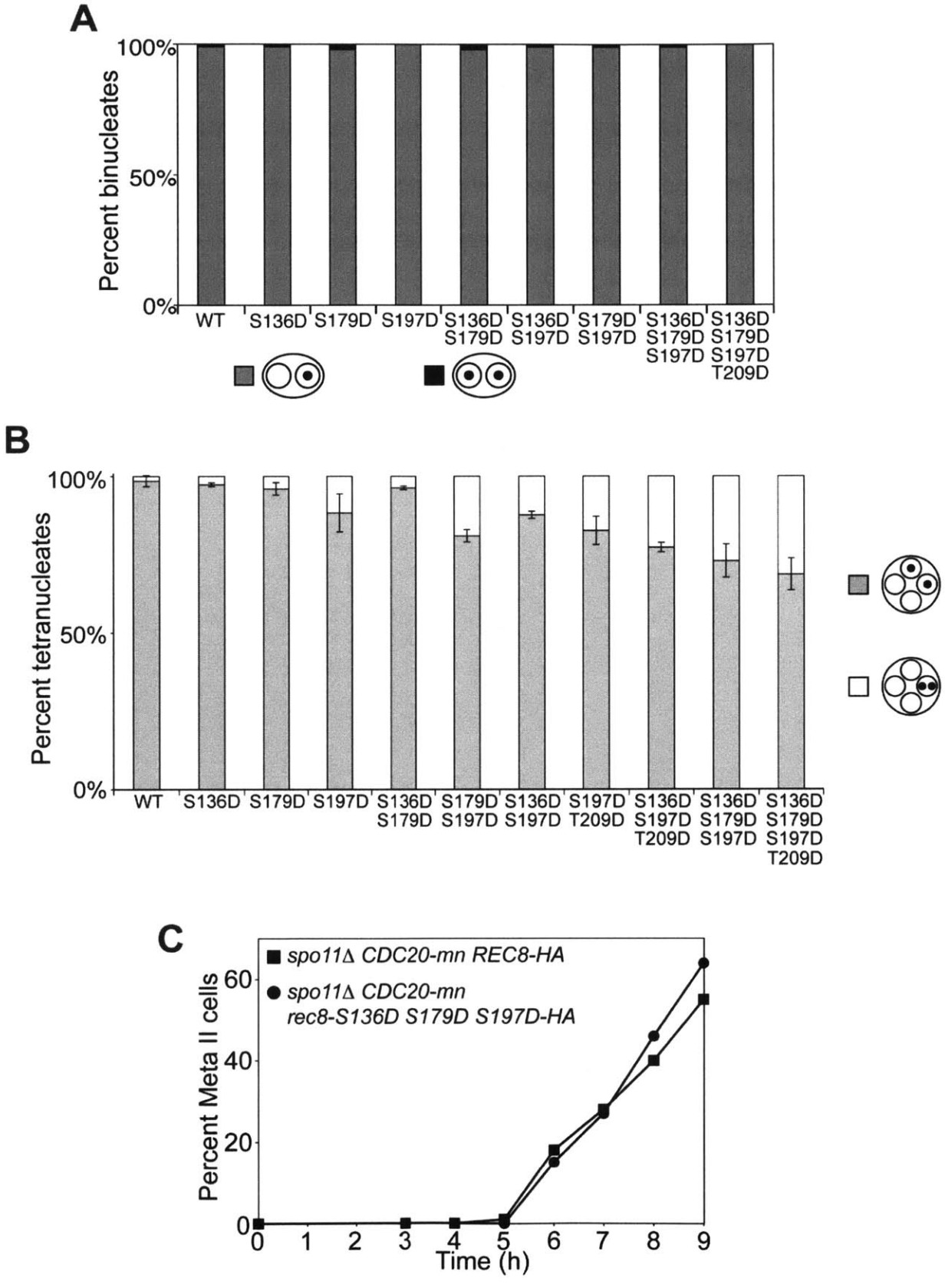
meiosis I (Figure 3A). Meiosis II chromosome segregation was, however, almost random in the *rec8-S136D S179D S197D T209D* mutant. 31% of cells harbored the two GFP dots in one of the four nuclei (Figure 3B; Katis et al., 2010). The *rec8-S136D S179D S197D* mutant mis-segregated chromosomes in meiosis II to the same extent as the *rec8-S136D S179D S197D T209D* mutant, indicating that mimicking phosphorylation of T209 does not significantly contribute to the observed phenotype in the context of the *rec8-S136D S179D S197D* mutant.

To address the possibility that the premature loss of centromeric cohesins was a consequence of the *rec8-S136D S179D S197D* mutant being unable to establish functional cohesion, we examined the ability of the mutant allele to maintain a metaphase II arrest under conditions in which cohesin removal is impaired. Cells depleted for the APC/C activator Cdc20 (*CDC20-mn*) and lacking the double-strand break inducing endonuclease Spo11 arrest in metaphase II. In this mutant, unlinked homologs segregate randomly during meiosis I, but cells arrest in metaphase II because they lack the ability to degrade the Separase inhibitor Securin, thus preventing Separase from cleaving cohesin (Salah and Nasmyth, 2000). Replacing *REC8* with the *rec8-S136D S179D S197D* allele did not affect the ability of *CDC20-mn spo11Δ* cells to arrest in metaphase II (Figure 3C) excluding the possibility that the meiosis II chromosome segregation defect observed in the *rec8-S136D S179D S197D* mutant is due to an inability to establish functional cohesion.

We next addressed the contribution of individual phospho-mimetic residues to the regulation of cohesin removal. With the exception of *rec8-S197D*, which showed a subtle phenotype, none of the single mutants exhibited a significant meiosis II

chromosome segregation defect. However, mutating both S136 and S179 to aspartic acid significantly enhanced the *rec8-S197D* phenotype (11.6% versus 27% of cells with co-segregated sisters, p=0.016), whereas the T209D mutation did not significantly enhance the *rec8-S197D* phenotype (Figure 3B). Our results demonstrate that residues whose phosphorylation depends on *CDC5* are important for cohesin removal control. Thus, *CDC5*-dependent phosphorylation contributes to cohesin removal.

Figure 3


A
100%
50%
0%
Percent binucleates
WT
S136D
S179D
S197D
S136D S179D
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S179D S197D
S136D S179D S197D
S136D S179D S197D T209D
B
100%
50%
0%
Percent tetranucleates
WT
S136D
S179D
S197D
S136D S179D
S179D S197D
S136D S197D
S197D T209D
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S136D S179D S197D
S136D S179D S197D T209D
C
spo11Δ CDC20-mn REC8-HA
spo11Δ CDC20-mn rec8-S136D S179D S197D-HA
Percent Meta II cells
0
20
40
60
0 1 2 3 4 5 6 7 8 9
Time (h)

Figure 3. Phosphorylation of Rec8 residues S136 and S179 contributes to cohesin removal.

(A) Cells containing heterozygous, centromeric GFP dots, and *rec8*-phosphomimetic mutants (A30411, A30407, A30408, A32252, A30409, A32256, A32250, A30410, A30406), were induced to sporulate. Six hours after transfer to sporulation medium, samples were taken, and GFP dot distribution in binucleate cells was determined (n=100 cells/time point).

(B) *HA*-tagged *REC8* phosphomimetic mutants (A30411, A30407, A30408, A32252, A30409, A32254, A32256, A32250, A32258, A30410, A30406) containing heterozygous centromeric GFP dots on chromosome V were induced to sporulate. After 10 hours in sporulation medium, the distribution of GFP in tetranucleate cells was counted. 100 cells were counted for each of three independent experiments. The difference between the *rec8-S197D-HA* mutant (mean missegregation=11.7%, SD=6.0) and the *rec8-S136D S179D S197D-HA* mutant (mean missegregation=27%, SD=5.3) was significant (p=0.016, Student's t-test). The difference between the *rec8-S197D* mutant and the *rec8-S136D S197D T209D-HA* mutant (mean missegregation=22.7%, SD=1.5) was significant (p=0.038, Student's t-test).

(C) *spo11Δ CDC20-mn* mutants containing *REC8-HA* (A33493; closed squares) or *rec8-S136D S179D S197D-HA* (A33491; closed circles) were induced to sporulate. The percentage of cells in metaphase II was quantified at the indicated times (n=100 cells/time point).

CDC5 is required for Rec8 cleavage

Although the above phospho-mutant analysis unequivocally demonstrates that *CDC5*-dependent phosphorylation contributes to cohesin removal, the large number of *CDC5*-dependent phosphorylation sites within Rec8 (11 sites were identified by mass spectrometry, and an additional nine sites fit the *CDC5*-consensus motif but were not covered in the mass spectrometry analysis; (Brar et al., 2006)) makes it difficult to assess the degree to which *CDC5* is needed for cohesin removal. To address this question we examined the consequences of depleting Cdc5 on cohesin cleavage. We depleted Cdc5 during meiosis by placing the *CDC5* gene under the control of the mitosis-specific *CLB2* promoter (*CDC5-mn*). This allele supports vegetative growth but Cdc5 becomes rapidly degraded upon entry into sporulation (Lee and Amon, 2003).

CDC5-mn cells arrest in metaphase I (Clyne et al., 2003; Lee and Amon, 2003). Securin is partially stabilized in this arrest, precluding our ability to look directly at Rec8 cleavage (Clyne et al., 2003; Lee and Amon, 2003). To bypass the requirement of *CDC5* for securin degradation, we deleted the meiosis-specific APC/C activator *AMA1*, a mutant in which securin degradation is *CDC5*-independent (Katis et al., 2010). We confirmed that Pds1 was more readily degraded in *CDC5-mn* cells in the absence of *AMA1*. Both *CDC5-mn* and *CDC5-mn ama1*Δ cells arrest in metaphase I (Figure 4A). However, whereas Pds1 was found in the nucleus of 80% of *CDC5-mn* metaphase I cells, this number was reduced to 50% in *CDC5-mn ama1*Δ cells (Figure 4B). Therefore, Pds1 is more readily degraded in cells lacking *AMA1*.

To assess the ability of *CDC5-mn ama1*Δ cells to cleave Rec8 we examined the appearance of the Rec8 cleavage product by Western blot analysis in cells induced to

undergo a synchronous meiosis. Rec8 cleavage was greatly delayed in the *CDC5-mn ama1*Δ double mutant even though at least half the cell population had degraded Pds1 (Figure 4C). The residual Rec8 cleavage observed in *CDC5-mn ama1*Δ cells could be due to a partial activation of the *CLB2* promoter. The *CLB2* promoter used to drive *CDC5* in this experiment is derepressed in *ama1*Δ mutants (Okaz et al., 2012). Indeed we found that in *CDC5-mn ama1*Δ cells, Clb2 protein accumulates (Figure 5). We conclude that *CDC5* is required for Rec8 cleavage. Cdc5 is not the only kinase required for Rec8 cleavage, but acts in addition to Casein kinase and DDK.

Figure 4

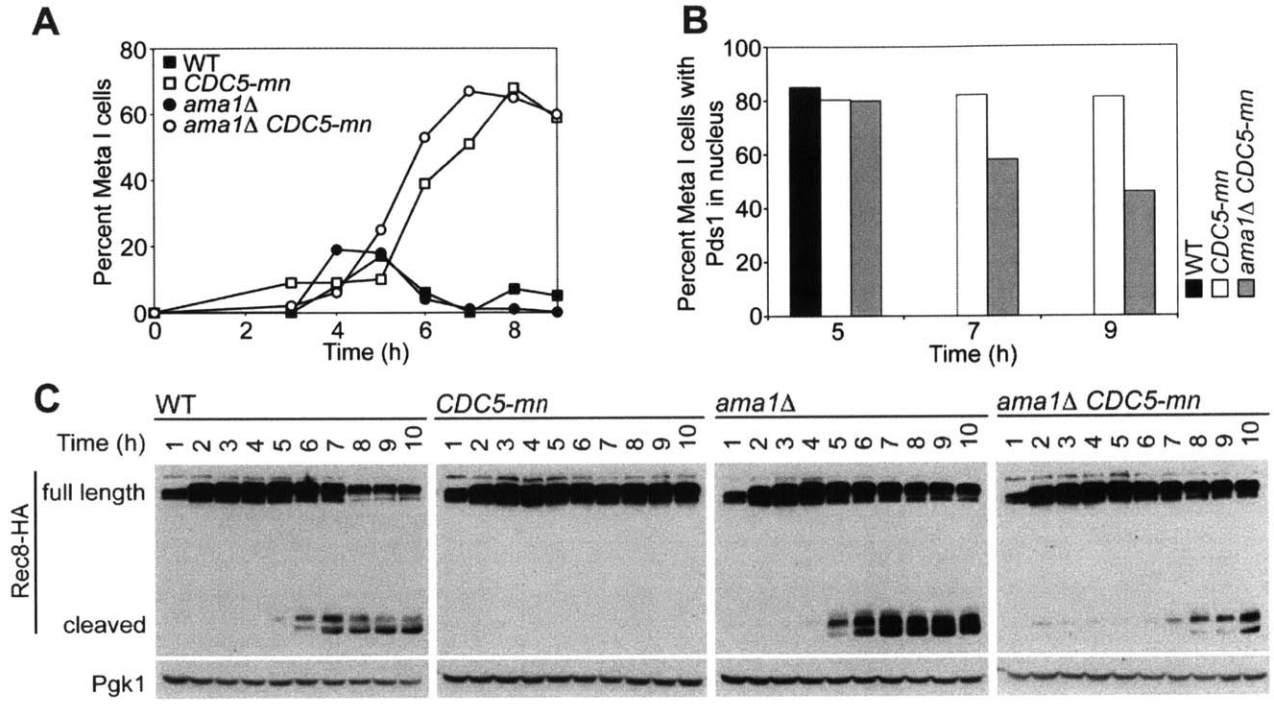


Figure 4. *CDC5* is required for Rec8 cleavage.

Wild-type (A33293; closed quares), *ama1Δ* (A33119; closed circles), *CDC5-mn* (A33292; open squares), or *ama1Δ CDC5-mn* (A33118; open circles) cells containing *PDS1-MYC*, *REC8-HA* and *ubr1Δ* (to allow detection of the Rec8 cleavage product) were induced to sporulate. The percentage of cells in metaphase I (A; n=100 cells/time point) and the percentage of metaphase I cells with Pds1 in the nucleus (B; n>50 cells/time point) was quantified at the indicated times. (C) Rec8-HA and Pgk1 (loading control) protein levels were analyzed by Western blotting at the indicated times.

Figure 5

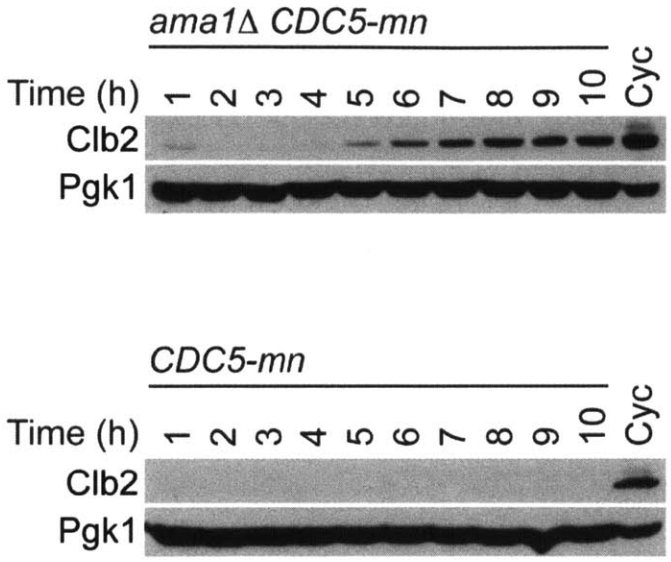


Figure 5. The *CLB2* promoter is de-repressed in *ama1*Δ cells.
Samples from the *CDC5-mn* (A33292) and *ama1*Δ *CDC5-mn* (A33118) strains shown in Figure 4 were analyzed for Clb2 protein levels. A wild-type vegetative control is used in the last lane. Pgk1 is used a loading control.

Meiosis I is suppressed in cells overexpressing *CDC5*.

If *CDC5* is indeed critical for the control of cohesin removal, over-expressing *CDC5* in meiosis I could lead to increased Rec8 phosphorylation at centromeric regions, thus over-riding the cohesin protective machinery, and inducing the premature loss of centromeric cohesion. To test this possibility, we examined the effects of overexpressing *CDC5* from the *CUP1* promoter on meiotic progression using a synchronization procedure that produces highly synchronized meiotic cultures. We reversibly arrested cells at the end of prophase I (in pachytene), by placing *NDT80*, the

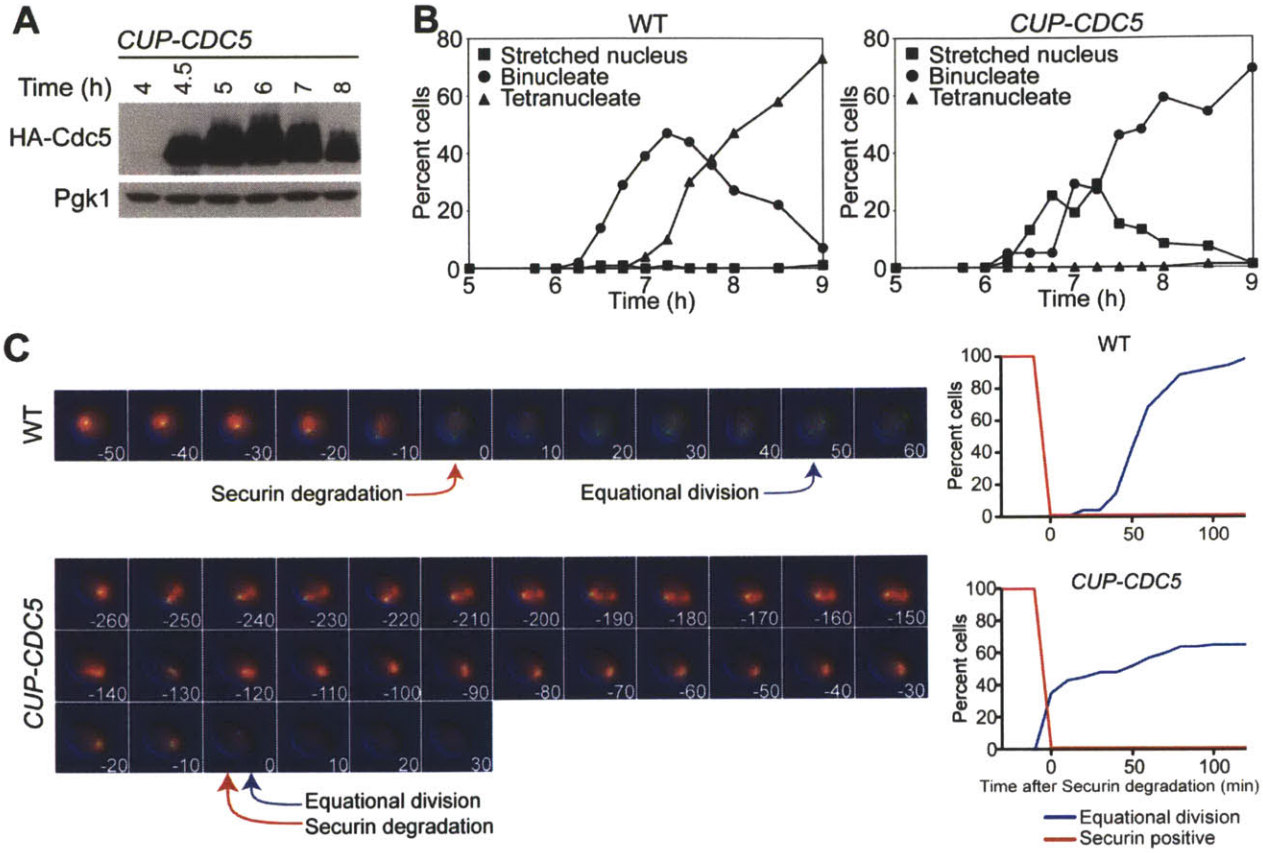
gene encoding a transcription factor required for entry into the meiotic divisions, under the control of the *GAL1-10* promoter. Cells also contained a *GAL4*-estrogen receptor (*GAL4-ER*) fusion. Upon addition of β-estradiol to the medium, cells exit the prophase I block and progress synchronously through the meiotic divisions (Carlile and Amon, 2008). *CDC5* expression was induced one hour prior to release from the pachytene block leading to high levels of Cdc5 at the time of release into the divisions without producing high levels of Cdc5 during pre-meiotic S phase, when *CDC5* is normally not expressed (Figure 6A; (Clyne et al., 2003)).

Cells overexpressing *CDC5* (*CUP-CDC5*) underwent a single meiotic division. This division was delayed compared to the first meiotic division in wild-type. The nucleus was stretched for prolonged periods of time and ultimately resolved into two nuclei (Figure 6B). To determine whether the single meiotic division in *CUP-CDC5* cells was meiosis I or meiosis II-like we assessed the segregation of heterozygous GFP dots. At the same time we monitored Pds1 degradation using a Pds1-tdTomato fusion (Miller et al., 2012). In wild-type cells, heterozygous GFP dots separated during meiosis II, 50 minutes after Pds1 degradation at the metaphase I – anaphase I transition (Figure 6C). In *CUP-CDC5* cells Pds1 degradation was significantly delayed (approximately 80 minutes; data not shown). However, once Pds1 was degraded, 48% of cells split sister chromatids immediately (Figure 6C; median=10 minutes after Pds1 degradation). 20% of *CDC5*-overexpressing cells underwent meiosis I following Pds1 degradation and 32% never underwent chromosome segregation. This phenotypic heterogeneity could be due to differential overexpression from the *CUP1* promoter, which we observe (unpublished observations). It is also possible that this "mixed segregation" reflects the true effect of

high levels of *CDC5* on chromosome segregation. Cells lacking the meiosis-specific factor *SPO13*, for example, exhibit a mixed chromosome segregation pattern, where during a single meiotic chromosome segregation, some chromosomes segregate in a meiosis I-like pattern whereas others in a meiosis II-like fashion (Hugerat and Simchen, 1993; Katis et al., 2004b; Lee et al., 2004). Irrespective of the origin of this heterogeneity, the observation that sister chromatids segregate prematurely during meiosis I in a fraction of cells overexpressing *CDC5* indicates that these cells are undergoing a meiosis II-like division.

To further investigate the effects of high levels of Cdc5 on cohesin removal from chromosomes, we analyzed Rec8 localization on chromosome spreads from *CUP-CDC5* cells. Rec8 should be lost from centromeric regions in binucleate cells if sister chromatids have segregated. Consistent with premature sister chromatid segregation in *CUP-CDC5* cells we found that Rec8 was absent around centromeres in 24% of binucleate cells (Figure 6D). In 26% of binucleate cells we observed an unusual Rec8 staining pattern. Rec8 decorated chromosomes and the space around the DNA (Figure 6D). This unusual Rec8 localization pattern likely reflects the high percentage of metaphase I-arrested cells with extremely stretched nuclei that we observe in *CUP-CDC5* cultures. After spreading, such stretched metaphase I nuclei could be mis-categorized as binucleate anaphase I or metaphase II nuclei. Our results suggest that overexpression of *CDC5* induces premature loss of centromeric cohesin and identify *CDC5* as an important regulator of cohesin loss in meiosis I.

Figure 6


A
CUP-CDC5
Time (h)
4
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5
6
7
8
HA-Cdc5
Pgk1
B
WT
CUP-CDC5
Stretched nucleus
Binucleate
Tetranucleate
Percent cells
Time (h)
C
WT
CUP-CDC5
Securin degradation
Equational division
Time after Securin degradation (min)
Equational division
Securin positive
D
DNA
Rec8
Ndc10
Merge
Colocalized
Mislocalized
Not colocalized
Percent binucleates
Rec8 not colocalized
Rec8 mislocalized
Rec8 colocalized

Figure 6. Cells overexpressing *CDC5* in meiosis undergo a mixed segregation.

(A, B) Wild-type (A31020) or *CUP-CDC5* (A32746) cells harboring a *GAL-NDT80* fusion as the sole source of *NDT80* and a *GAL4-ER* fusion were induced to sporulate. Cells were induced with 50 μM $CuSO_4$ 4 hours after transfer to sporulation medium to induce *CDC5* expression, and with 1 μM β-estradiol after 5 hours to release cells synchronously from the prophase I block into the meiotic divisions. Cdc5 levels after copper induction are shown in (A). The percentage of WT or *CUP-CDC5* cells with stretched nuclei (squares), two nuclei (circles), and four nuclei (triangles) is shown in (B; n=100 cells/time point).

(C) Wild-type (A31020) and *CUP-CDC5* (A32746) cells harboring heterozygous, centromeric GFP dots and a Pds1-tdTomato fusion were induced to sporulate and followed by live cell microscopy (n=75 cells). A representative montage for wild type (top) and *CUP-CDC5* (bottom) is shown on the left. The time of Pds1 degradation was set to t=0 and the percent of cells with split GFP dots were plotted as a Kaplan-Meier curve on the right. Note that Pds1 accumulation in meiosis II is not observed with the Pds1-tdTomato construct, due to delayed maturation of the fluorophore (Katis et al., 2010).

(D) Wild-type (A33791) and *CUP-CDC5* (A33459) cells containing *GAL-NDT80*, *GAL4-ER*, *REC8-V5*, and *NDC10-MYC* fusions were sporulated as in Figure 6A. Centromeric Rec8 localization was determined in spread nuclei. Ndc10-Myc was used to identify centromeric regions. Examples of chromosome spreads of binucleate cells with Rec8 localized to centromeric regions (colocalized; top), present throughout the nucleus and on chromosomes (mislocalized; middle), or absent from nuclei (not localized; bottom) are shown on the left. DNA is shown in blue, Rec8 in green, and Ndc10 in red. The quantification of the three classes is shown on the right. Wild-type cells were counted from the 7.5 hour time point, and *CUP-CDC5* cells were counted from the 9 hour time point (n>50 cells/time point).

***CDC5* regulates the stability of Spo13.**

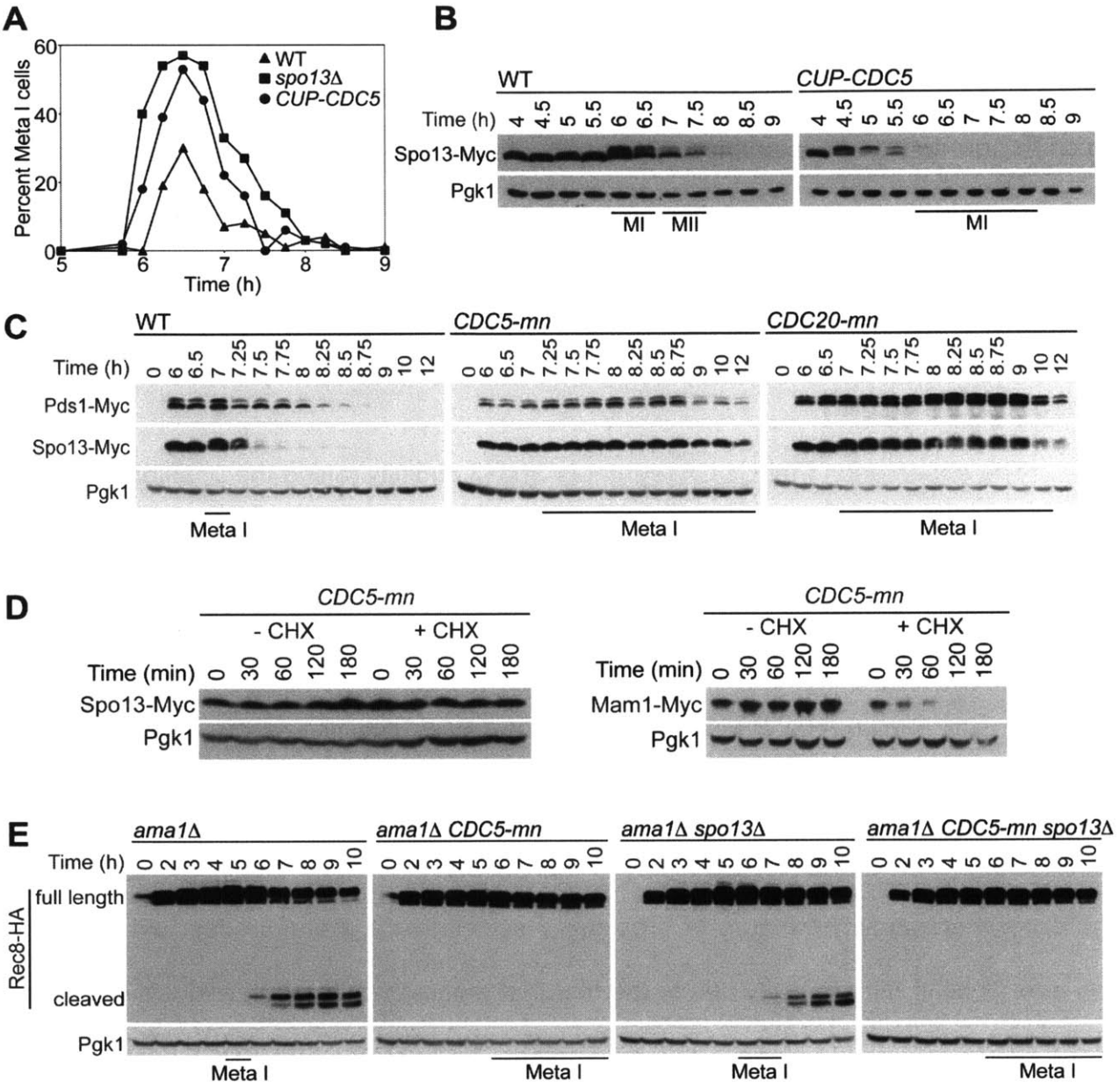
If cells segregate sister chromatids rather than homologous chromosomes during meiosis I, sister kinetochores must be bi-oriented. As approximately half of the *CUP-CDC5* cells segregate sister chromatids during meiosis I, co-orientation must at least be partially defective in *CUP-CDC5* cells. The phenotype of *CUP-CDC5* cells resembles that of cells lacking *SPO13*. Spo13 is a meiosis I-specific protein required for preventing cohesin removal around centromeres during meiosis I and for sister kinetochore co-orientation (Katis et al., 2004b; Lee et al., 2004). *spo13*Δ cells, like *CUP-CDC5* cells, undergo a single meiotic division in which some chromosomes segregate in a meiosis I-like manner and others in a meiosis II-like manner. Additionally, both *CUP-CDC5* and *spo13*Δ mutants exhibit a metaphase I delay (Figure 7A). These findings raised the possibility that high levels of Cdc5 interfere with *SPO13* activity thereby affecting both centromeric cohesin protection and sister kinetochore co-orientation.

To determine whether Cdc5 affects *SPO13* function we first analyzed Spo13 protein levels in *CUP-CDC5* cells. We arrested cells in prophase I and induced *CDC5* expression one hour prior to release from the prophase I block. In wild-type cells Spo13 is degraded at the metaphase I – anaphase I transition by the APC/C-Cdc20 ((Sullivan and Morgan, 2007); Figure 7B, 8A-B). In *CUP-CDC5* cells, Spo13 levels declined prematurely prior to anaphase I onset (Figure 7B, Figure 8A-B). The decline in Spo13 levels was preceded by the appearance of slower migrating forms of Spo13, which is consistent with *CDC5*-dependent phosphorylation of Spo13 seen later during meiosis I (Matos et al., 2008). This finding indicates that *CDC5* promotes Spo13 degradation. Consistent with this observation is the finding that *CDC5* is required for the decline in

Spo13 levels observed in anaphase I. Spo13 levels remained as high in the metaphase I arrest caused by the depletion of Cdc5 as they did in cells depleted for the APC/C activator Cdc20 (Figure 7C, Figure 8C). In order to directly assess the role of *CDC5* in Spo13 degradation, we examined the half-life of Spo13 in cells lacking Cdc5 (*CDC5-mn*). Spo13 was stable in Cdc5 depleted cells (Figure 7D). In contrast, another meiosis I-specific protein, Mam1, was not stabilized in *CDC5-mn* cells, indicating that *CDC5* does not affect the stability of all meiosis I proteins (Figure 7D). We conclude the *CDC5* controls Spo13 stability.

The observation that *CDC5* regulates Spo13 stability raised the possibility that the premature loss of centromeric cohesin observed in *CUP-CDC5* cells is a consequence of premature loss of Spo13. If *CDC5* was regulating cohesin removal solely by affecting *SPO13* function, deleting *SPO13* should suppress the Rec8 cleavage defect of cells depleted for Cdc5. This was not the case. Deleting *SPO13* did not suppress the cohesin cleavage defect of *CDC5-mn ama1*Δ cells (Figure 7E, 8D) indicating that *CDC5* regulates Rec8 cleavage in additional ways. We note that unlike in the experiment shown in Figure 4C, residual Rec8 cleavage was not observed in *CDC5-mn ama1*Δ cells. This is likely due to the fact that progression through meiosis was slow in the *CDC5-mn ama1*Δ strain in this experiment. We conclude that *CDC5* controls cohesin removal in at least three ways. First, *CDC5* is required for Pds1 degradation, and thus separase activation. Second, Cdc5 phosphorylates Rec8 to render it susceptible to Separase cleavage. Third, *CDC5* promotes the degradation of the centromeric cohesin protector Spo13.

Figure 7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Percent Meta I cells
Time (h)
WT
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CUP-CDC5
B
WT
CUP-CDC5
Time (h)
Spo13-Myc
Pgk1
MI
MII
MI
C
WT
CDC5-mn
CDC20-mn
Time (h)
Pds1-Myc
Spo13-Myc
Pgk1
Meta I
Meta I
Meta I
D
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- CHX
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ama1Δ spo13Δ
ama1Δ CDC5-mn spo13Δ
Time (h)
Rec8-HA
full length
cleaved
Pgk1
Meta I
Meta I
Meta I
Meta I

Figure 7. Cdc5 regulates Spo13 stability.

(A) Wild-type (A31020), *CUP-CDC5* (A32746) or *spo13Δ* (A30960) cells were induced to sporulate as in Figure 6A. The percentage of metaphase I cells in wild-type (closed triangles), *spo13Δ* (closed squares) and *CUP-CDC5* (closed circles) was determined at the indicated times (n=100 cells/time point).

(B) Wild-type (A33501) or *CUP-CDC5* (A33497) cells containing *GAL-NDT80*, *GAL4-ER*, and *SPO13-MYC* fusions were induced to sporulate as in Figure 6A. Samples were taken at the indicated times to determine Spo13 protein levels. The peaks of meiosis I (MI) and meiosis II (MII) are indicated under the blots. Graphs of meiotic progression are shown in Figure 8A and B.

(C) Wild-type (A23405), *CDC5-mn* (A23757), and *CDC20-mn* (A23664) cells containing *PDS1-MYC*, *SPO13-MYC*, *GAL-NDT80*, and *GAL4-ER* fusions were induced to sporulate. 1 μM β-estradiol was added to the cultures at 6 hours after transfer to sporulation medium. Samples were taken at the indicated times to determine Spo13-Myc and Pds1-Myc protein levels by Western blotting. The peak of metaphase I is indicated below the blots. Graph of meiotic progression is shown in Figure 8C.

(D) *CDC5-mn* cells carrying a *SPO13-MYC* fusion (A6139; left panel) or a *MAM1-MYC* fusion (A17131; right panel) were induced to sporulate and allowed to progress into a metaphase I arrest for 6 hours. Cells were then treated with 0.5 mg/ml cycloheximide (CHX) to halt translation, and Spo13-Myc or Mam1-Myc protein levels were examined at the indicated time points after addition of the translation inhibitor.

(E) *ama1Δ* (A33767), *ama1Δ CDC5-mn* (A33766), *ama1Δ spo13Δ* (A33768), *ama1Δ CDC5-mn spo13Δ* (A33765) mutants were induced to sporulate as in Figure 4. All strains contained *REC8-HA* and *ubr1Δ*. Rec8-HA was analyzed by Western blotting at the indicated times. The peak of metaphase I is indicated below the blots. Graph of meiotic progression is shown in Figure 8D.

Figure 8

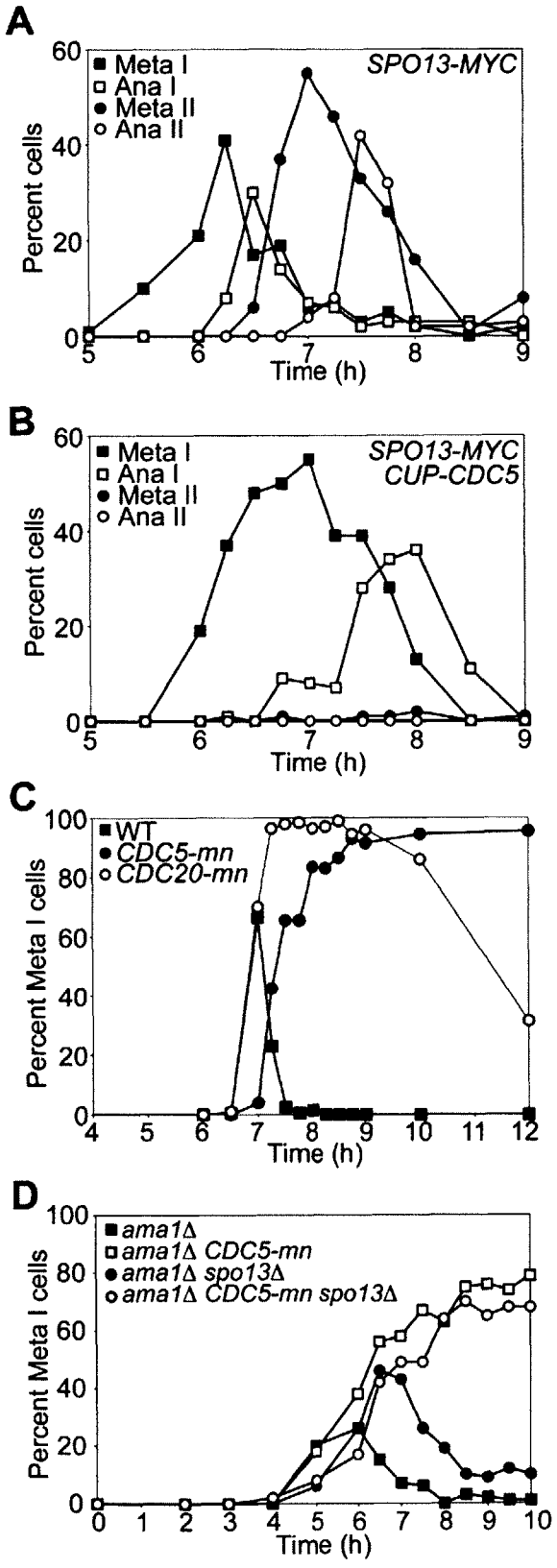
A
Meta I
Ana I
Meta II
Ana II
SPO13-MYC
Percent cells
Time (h)
B
Meta I
Ana I
Meta II
Ana II
SPO13-MYC
CUP-CDC5
Percent cells
Time (h)
C
WT
CDC5-mn
CDC20-mn
Percent Meta I cells
Time (h)
D
ama1Δ
ama1Δ CDC5-mn
ama1Δ spo13Δ
ama1Δ CDC5-mn spo13Δ
Percent Meta I cells
Time (h)

Figure 8. *CDC5* regulates Spo13 abundance: meiotic progression.
(A-B) Meiotic progression for the experiment shown in Figure 7B. The percentage of cells in metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), and anaphase II (open circles) was determined for wild-type (A) and *CUP-CDC5* (B) cells (n=100 cells/time point).
(C) The percentage of wild-type (closed squares), *CDC5-mn* (closed circles), and *CDC20-mn* (open circles) in metaphase I was determined for the experiment shown in Figure 7C.
(D) The percentage of *ama1Δ* (A33767, closed squares), *ama1Δ CDC5-mn* (A33766, open squares), *ama1Δ spo13Δ* (A33768, closed circles), and *ama1Δ CDC5-mn spo13Δ* (A33765, open circles) cells in metaphase I was determined for the experiment shown in Figure 7E (n=100 cells/time point).

CDC5 is dispensable during meiosis II.

CDC5 is a central regulator of meiosis I chromosome segregation. Is *CDC5* also a critical regulator of meiosis II? To address this question we examined the consequences of inactivating *CDC5* after meiosis I. We employed an inhibitor-sensitive allele of *CDC5*, *cdc5-as1*, allowing us to inhibit Cdc5 kinase activity at any time, while leaving other cellular kinase activities intact (Snead et al., 2007). We arrested wild-type and *cdc5-as1* cells in prophase I and added inhibitor at various times after release from the prophase I block. As expected, addition of inhibitor to meiotic cultures prior to metaphase I (1 hour after release from the prophase I block) resulted in a robust metaphase I arrest (Figure 9A-B). Adding inhibitor 15 minutes later (1.25 hours after release from the prophase I block) revealed that in addition to being critical for the metaphase I – anaphase I transition *CDC5* is also required for exit from meiosis I. We observed an accumulation of anaphase I cells in these cultures (Figure 9C). This finding is consistent with the observation that the FEAR network, in which *CDC5* plays a critical

role, is essential for exit from meiosis I (Buonomo et al., 2003; Marston et al., 2003; Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002).

In contrast, when we added inhibitor after cells had completed meiosis I (1.5 hours after release from the prophase block), we only observed a subtle delay in meiosis II progression (Figure 9D-F). We were able to exclude the possibility that the inhibitor was ineffective when added at late time points in meiosis. Cdc5 activates the protein phosphatase Cdc14 by promoting its release from the nucleolus during mitotic anaphase as well as during anaphase I and II (Buonomo et al., 2003; Lee and Amon, 2003; Marston et al., 2003; Stegmeier et al., 2002). Cdc14 release from the nucleolus did not occur during anaphase II in *cdc5-as1* cells when the kinase was inhibited 1.5 hours after release from the prophase I block (Figure 9G-H). These findings indicate that *CDC5* is largely dispensable for meiosis II chromosome segregation and exit from meiosis II. Furthermore, these results show that unlike during mitosis and meiosis I, release of Cdc14 from the nucleolus is not required for exit from meiosis II.

Figure 9

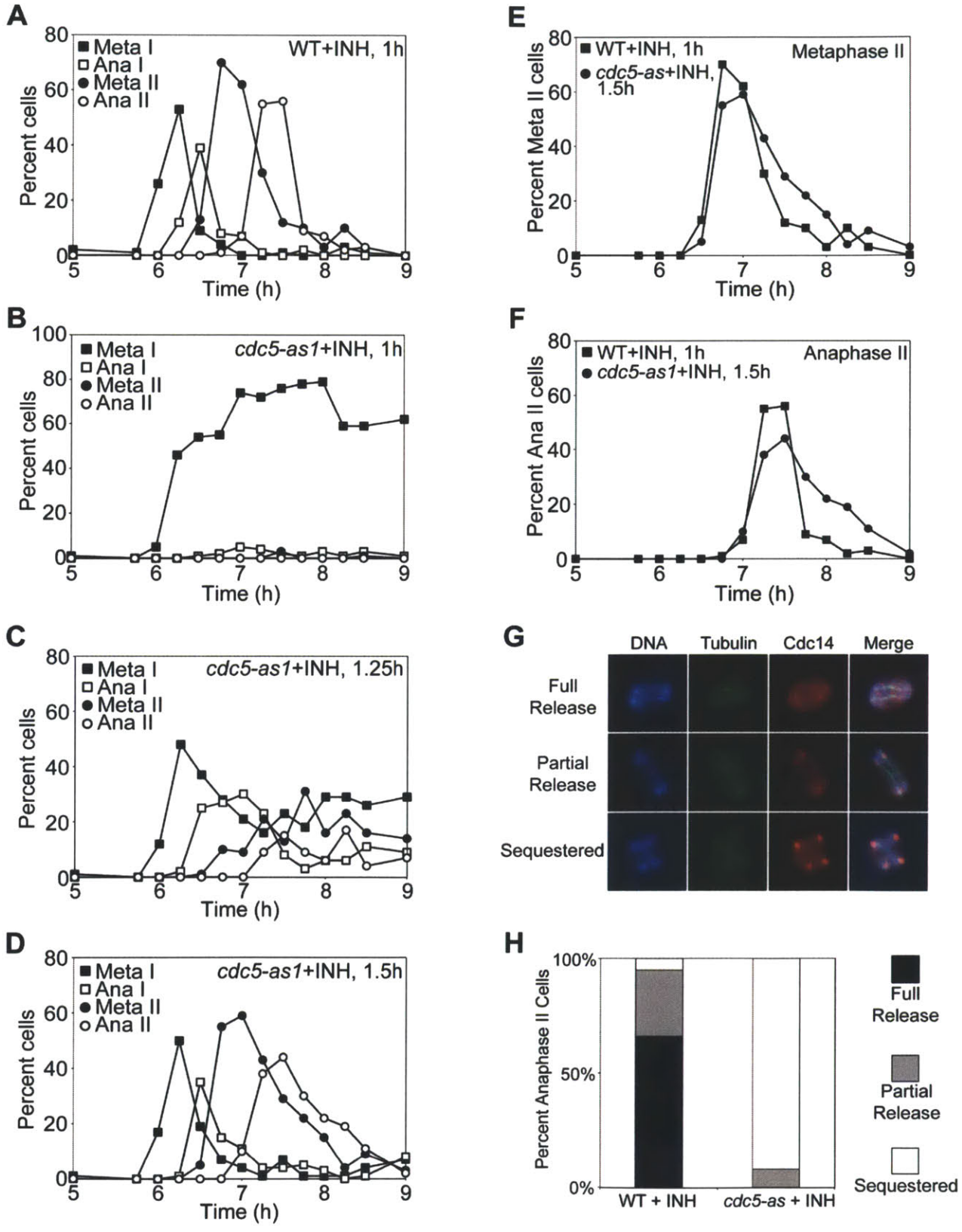
A
Meta I
Ana I
Meta II
Ana II
WT+INH, 1h
Percent cells
Time (h)
B
cdc5-as1+INH, 1h
C
cdc5-as1+INH, 1.25h
D
cdc5-as1+INH, 1.5h
E
WT+INH, 1h
cdc5-as+INH, 1.5h
Metaphase II
Percent Meta II cells
F
Anaphase II
Percent Ana II cells
G
DNA
Tubulin
Cdc14
Merge
Full Release
Partial Release
Sequestered
H
Percent Anaphase II Cells
100%
50%
0%
WT + INH
cdc5-as + INH
Full Release
Partial Release
Sequestered

Figure 9. *CDC5* is dispensable during meiosis II.

(A-D) Wild-type (A22132) or *cdc5-as1* (A33513) cells containing *GAL-NDT80*, *GAL4-ER*, and *CDC14-HA* fusions were induced to sporulate. 1 μM β-estradiol was added to cultures 5 hours after transfer to sporulation medium to release cells into the meiotic divisions. The percentage of metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), and anaphase II (open circles) was determined at the indicated time points (n=100 cells/ time point) CMK (5μM) inhibitor was added at the following times: wild-type: 1 hour after release into the meiotic divisions (A), *cdc5-as1:* 1 hour after release (B), *cdc5-as1:* 1.25 hours (C), and *cdc5-as1*: 1.5 hours after release into the meiotic divisions (D).

(E-F) Quantification of the number of wild-type cells with inhibitor added 1 hour after release from the prophase I block (closed squares) and *cdc5-as1* cells with inhibitor added 1.5 hours after release (closed circles) in metaphase II (E) and anaphase II (F; n=100 cells/time point) of the experiment described in (A-D).

(G, H) Examples of anaphase II cells with Cdc14 fully released from the nucleolus (top; G), partially released (middle; G), or sequestered in the nucleolus (bottom; G). DNA is shown in blue, tubulin in green, and Cdc14 in red. The percentage of anaphase II cells with Cdc14 fully released (black bars), partially released (gray bars), or sequestered (white bars) is shown in (H). Wild-type cells with inhibitor added 1 hour after release from the prophase I block and *cdc5-as1* cells with inhibitor added 1.5 hours after release into the meiotic divisions (n=100 cells).

Many Cdc5 substrates are only phosphorylated during meiosis I.

Our execution point studies indicate that *CDC5* is dispensable for meiosis II Pds1 degradation and cohesin removal as well as exit from meiosis II. Analysis of two *CDC5*-dependent phosphorylation sites in Rec8, S136 and S179, further showed that *CDC5* is not only dispensable for cohesin removal during meiosis II, but that residues in Rec8 known to be phosphorylated in a *CDC5*-dependent manner during meiosis I are in fact not phosphorylated during meiosis II. We followed S136 and S179 phosphorylation in cells progressing through meiosis and found that phosphorylation was restricted to

meiosis I (Figure 10A-C; 11A). Because Rec8 levels are much lower in metaphase II than in metaphase I, differences in Rec8 phosphorylation are most clearly seen when similar amounts of Rec8 from meiosis I and meiosis II time points were analyzed (Figure 10B, C). These comparisons show that *CDC5*-dependent phosphorylation of Rec8 S136 and S179 is absent during meiosis II. This lack of *CDC5*-dependent phosphorylation of Rec8 S136 and S179 is not due to the absence of Cdc5 in meiosis II. In fact, Cdc5 protein is produced throughout meiosis, with levels higher during meiosis II than during meiosis I (Figure 10D; 11B).

Rec8 is not the only Cdc5 substrate whose phosphorylation is greatly reduced during meiosis II. Lrs4, a component of the monopolin complex, is phosphorylated in a *CDC5*-dependent manner, which is detected as slower migrating forms on SDS PAGE (Clyne et al., 2003; Rabitsch et al., 2003). Slower migrating forms of Lrs4-Myc were present during prophase I and metaphase I but absent during meiosis II (Figure 10E; 11C). We also analyzed the phosphorylation state of the B-type cyclin Clb1. Clb1 protein undergoes dramatic changes in mobility during meiosis I (Carlile and Amon, 2008). The meiosis I-specific slower migrating forms of Clb1 depend on *CDC5,* as they are lost upon *CDC5* inactivation (Figure 10F). Similar to Lrs4, the slower migrating forms of Clb1 were only present during meiosis I (Figure 10G; 11D). Our results indicate that phosphorylation of at least some Cdc5 substrates is high in meiosis I but largely absent during meiosis II.

Figure 10

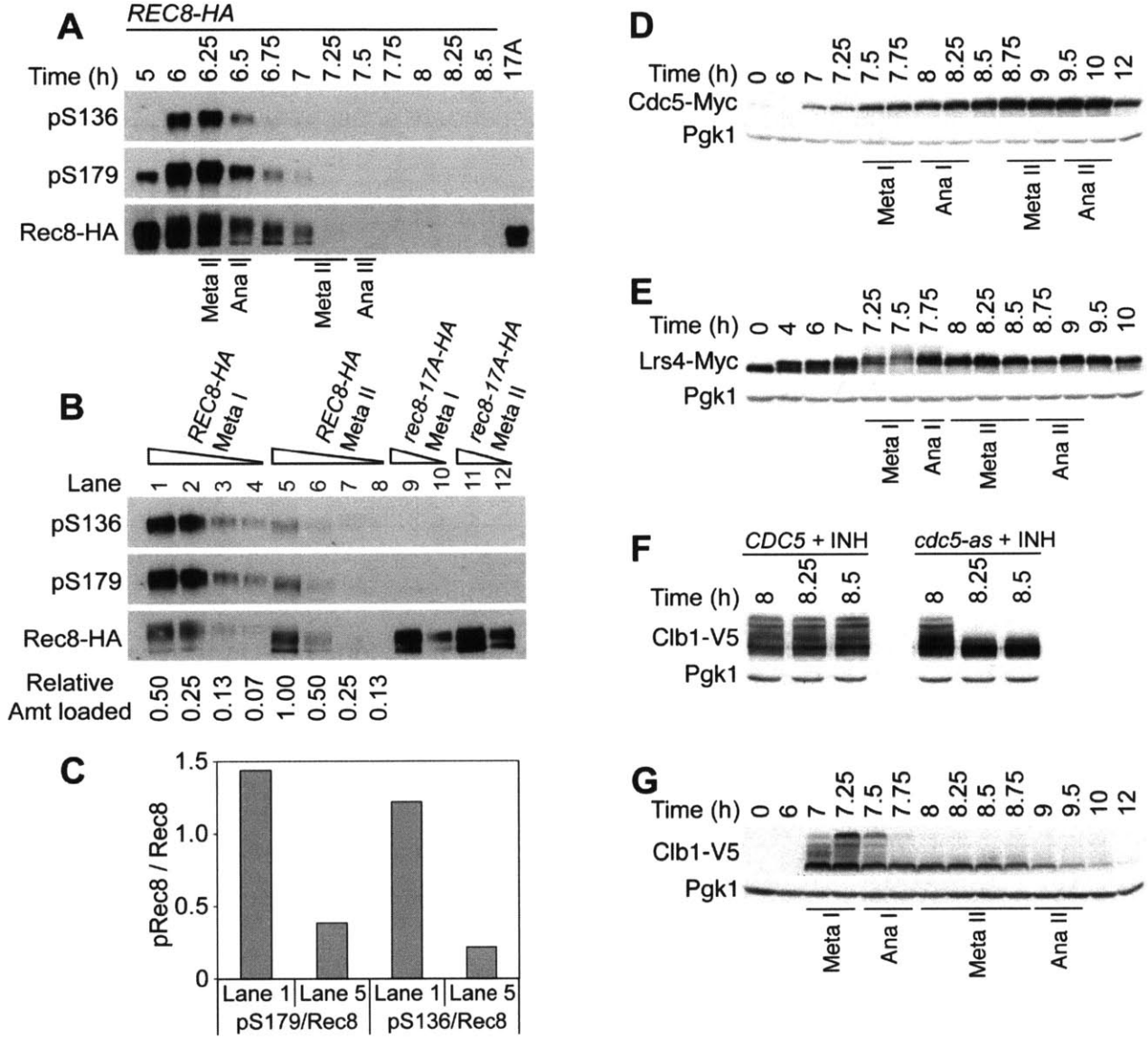
A
REC8-HA
Time (h)
5 6 6.25 6.5 6.75 7 7.25 7.5 7.75 8 8.25 8.5 17A
pS136
pS179
Rec8-HA
Meta II
Ana II
Meta II
Ana III
B
REC8-HA Meta I
REC8-HA Meta II
rec8-17A-HA Meta I
rec8-17A-HA Meta II
Lane
1 2 3 4 5 6 7 8 9 10 11 12
pS136
pS179
Rec8-HA
Relative Amt loaded
0.50 0.25 0.13 0.07 1.00 0.50 0.25 0.13
C
pRec8 / Rec8
1.5
1.0
0.5
0
Lane 1
Lane 5
Lane 1
Lane 5
pS179/Rec8
pS136/Rec8
D
Time (h)
0 6 7 7.25 7.5 7.75 8 8.25 8.5 8.75 9 9.5 10 12
Cdc5-Myc
Pgk1
Meta I
Ana I
Meta II
Ana II
E
Time (h)
0 4 6 7 7.25 7.5 7.75 8 8.25 8.5 8.75 9 9.5 10
Lrs4-Myc
Pgk1
Meta I
Ana I
Meta II
Ana II
F
CDC5 + INH
cdc5-as + INH
Time (h)
8 8.25 8.5 8 8.25 8.5
Clb1-V5
Pgk1
G
Time (h)
0 6 7 7.25 7.5 7.75 8 8.25 8.5 8.75 9 9.5 10 12
Clb1-V5
Pgk1
Meta I
Ana I
Meta II
Ana II

Figure 10. Cdc5 targets are phosphorylated in meiosis I but not meiosis II.

(A) Cells containing *REC8-HA*, *GAL-NDT80*, and *GAL4-ER* fusions (A21230) were induced to sporulate, and 1 μM β-estradiol was added to cultures at 5 hours after transfer to sporulation medium. Samples were taken at the indicated time points to analyze total Rec8 protein, and Rec8-S136 and Rec8-S179 phosphorylation. A *rec8-17A-HA* strain (17A; A21235) arrested in metaphase I was used as a control for the phospho-antibodies, as both S136 and S179 are mutated to alanine in this strain. The peaks of the stages of meiosis are indicated below the blot. Graph of meiotic progression is shown in Figure 11A.

(B, C) A two-fold dilution series of samples from the 6.25 hour time point (lanes 1 - 4), 7 hour time point (lanes 5-8) from (A) and *rec8-17A-HA* metaphase I (lanes 9, 10) and metaphase II (lanes 11, 12) controls were analyzed for total Rec8 levels and Rec8-S136 and Rec8-S179 phosphorylation. The relative amount of sample loaded is indicated below the blot. Twice as much sample was loaded in the meiosis II (lanes 5-9) than the meiosis I dilution series (lanes 1-4). Quantifications of lanes 1 and 5 are shown in (C).

(D) Cells carrying *GAL4-ER*, *GAL-NDT80* and *CDC5-MYC* fusions (A24758) were induced to sporulate. Cells were released from the *NDT80* block 6 hours after transfer to sporulation medium. Samples were taken at indicated time points to analyze Cdc5-Myc levels. Graph of meiotic progression is shown in Figure 11B.

(E) Cells carrying *GAL4-ER*, *GAL-NDT80* and *LRS4-MYC* fusions (A24760) were induced to sporulate. Cells were released from the *NDT80* block 6 hours after transfer to sporulation medium. Samples were taken at the indicated time points to analyze Lrs4-Myc levels and mobility shift by Western blot. Graph of meiotic progression is shown in Figure 11C.

(F) Wild-type (A24207) or *cdc5-as* (A26176) cells carrying the *CLB1-V5* fusion and also harboring a Cdc20 depletion allele (*CDC20-mn*) were arrested in metaphase I. 8 hours after induction of sporulation, when the majority of cells had arrested in metaphase I, cells were treated with 5 μM CMK. Samples were taken at the indicated time points to analyze Clb1-3V5 levels and mobility.

(G) Cells carrying *GAL-NDT80*, *GAL4-ER*, and *GAL-CLB1-V5* (A23650) fusions were induced to sporulate. Cells were induced with 1 μM β-estradiol 6 hours after transfer to sporulation medium. Samples were taken at the indicated times to analyze Clb1-V5 protein levels and mobility. Graph of meiotic progression is shown in Figure 11D.

Figure 11

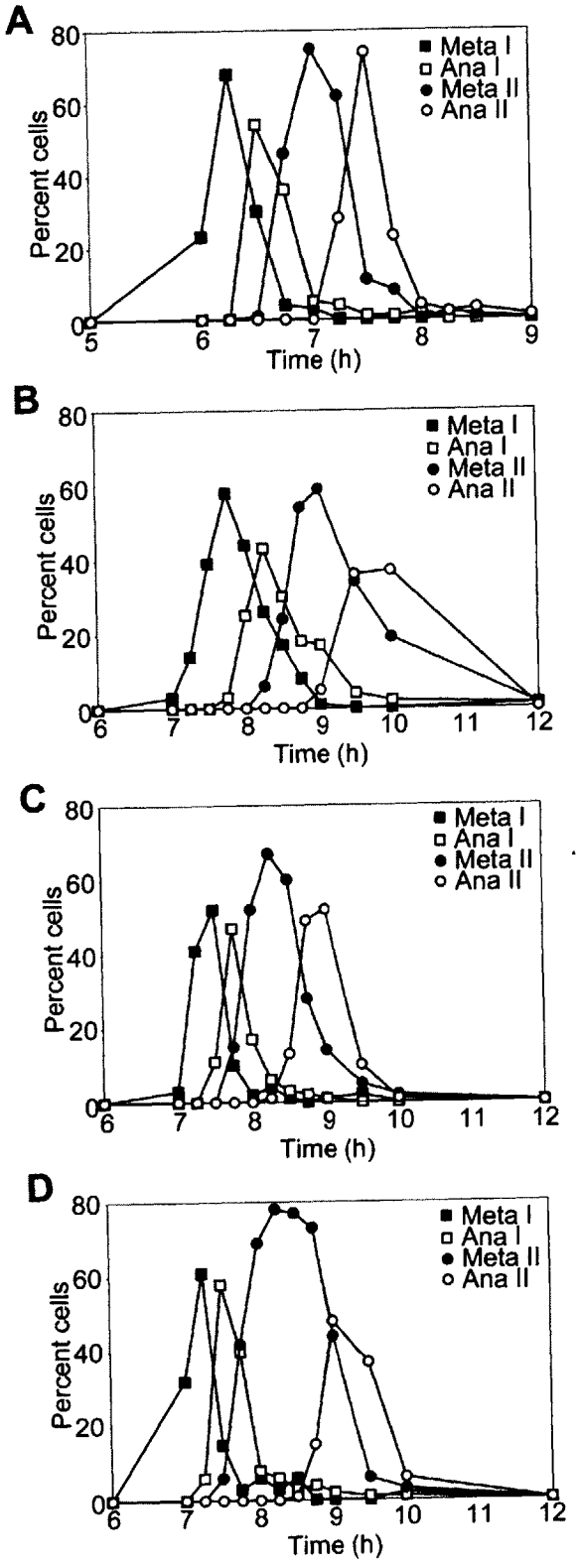
A
B
C
D
Meta I
Ana I
Meta II
Ana II
Percent cells
Time (h)
0
20
40
60
80
5
6
7
8
9
10
11
12

Figure 11. Cdc5 targets are phosphorylated in meiosis I: meiotic progression.
(A-D) Meiotic progression for the experiments shown in Figure 10. The percentage of cells in metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), and anaphase II (open circles) was determined at the indicated times (n=100 cells/time point). Meiotic progressions are shown for the experiments shown in Figure 10A (A), Figure 10D (B), Figure 10E (C), and Figure 10G (D).

Rec8 phosphorylation is dispensable for anaphase II entry.

Our results indicate that despite Cdc5's central role in cohesin removal during meiosis I, it is dispensable for this process during meiosis II. This difference could be due to other kinases, i.e. Hrr25 or DDK, bringing about phosphorylation-dependent removal of Rec8 from chromosomes. Alternatively, Rec8 phosphorylation may not be essential for centromeric cohesin removal and hence meiosis II chromosome segregation. To distinguish between these possibilities we examined the effects of mutating 29 phosphorylation sites in Rec8 required for efficient cleavage to alanine (*rec8-29A*; (Brar et al., 2006)) on meiosis II chromosome segregation. The *rec8-29A* mutant exhibits a prophase I delay, due to delayed repair of double strand breaks during homologous recombination and a metaphase I delay due to impairment of Rec8 cleavage (Figure 12A, B; (Brar et al., 2009). We were able to circumvent both cell cycle delays by deleting *SPO11*. Deleting *SPO11* eliminates double strand break formation and hence bypasses the double strand break repair defect of *rec8-29A* mutants. Furthermore, it bypasses the metaphase I delay in *rec8-29A* cells because preventing recombination prevents the formation of linkages between homologous chromosomes and hence the need for cohesin removal for meiosis I chromosome segregation to

occur. Remarkably, *rec8-29A spo11*Δ double mutants entered anaphase II with kinetics indistinguishable from that of the *spo11*Δ single mutants (Figure 12C). This finding indicates that while essential for anaphase I onset, Rec8 phosphorylation is dispensable for anaphase II onset. Taken together, our results show that meiosis I and meiosis II chromosome segregation are regulated in fundamentally different ways and that *CDC5* is a significant contributor to shaping the unique meiosis I chromosome segregation pattern.

Figure 12

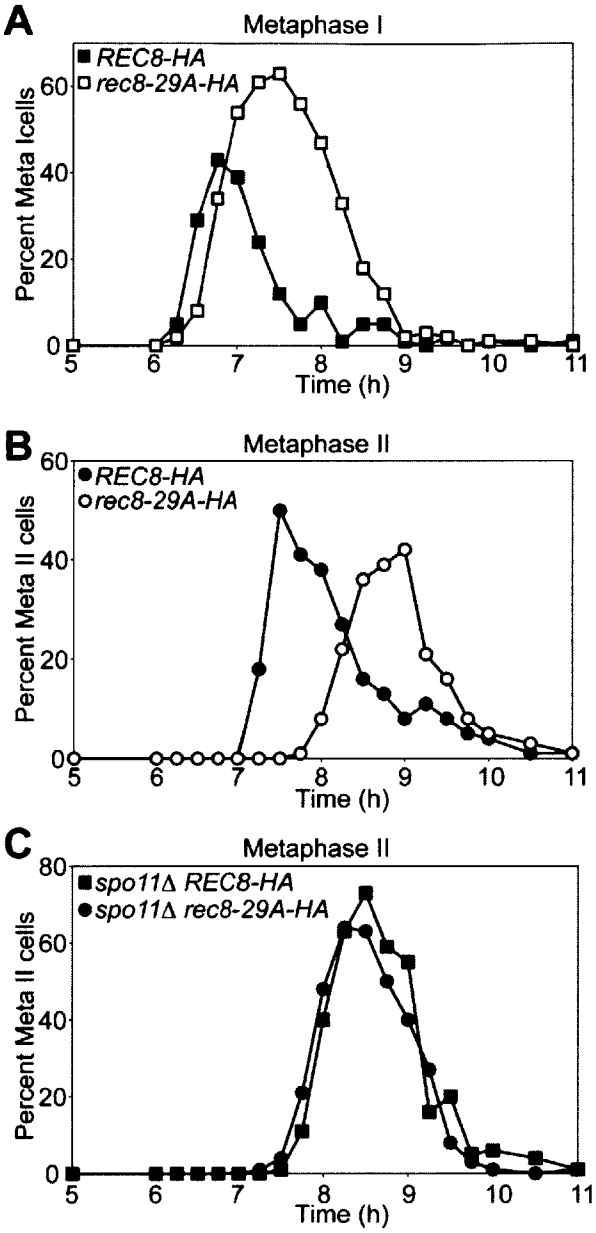

Figure 7. Rec8 phosphorylation is dispensable for anaphase II onset.

(A, B) *REC8-HA* (A22804) or *rec8-29A-HA* (A22803) mutants containing *GAL-NDT80 GAL4-ER* fusions were induced to sporulate. 1 μM β-estradiol was added to cultures 5 hours after transfer to sporulation medium to release cells into the meiotic divisions. The percentage of *REC8-HA* (closed symbols) and *rec8-29A-HA* cells (open symbols) in metaphase I (A) and in metaphase II (B) was determined.

(C) *REC8-HA spo11Δ* (A33469) and *rec8-29A-HA spo11Δ* (A33453) mutants containing *GAL-NDT80 GAL4-ER* fusions were induced to sporulate. 1 μM β-estradiol was added to cultures at 5 hours to release cells into the meiotic divisions. The percentage of cells in metaphase II was determined for *REC8-HA spo11Δ* (closed squares), and *rec8-29A-HA spo11Δ* (closed circles) mutants (n=100 cells/time point).

DISCUSSION

Our studies on the Polo kinase *CDC5* revealed functions shared between mitosis and meiosis and roles unique to meiosis. Thus, *CDC5* is an integral aspect of the evolution of the meiotic chromosome segregation program. A key aspect of Cdc5's role in meiosis is its ability to regulate the function of meiosis-specific proteins. Cdc5 phosphorylation of the meiosis-specific cohesin subunit Rec8 is critical to cohesin removal during meiosis I. Its role in regulating the stability of the meiosis I-specific protein Spo13, establishes conditions for cohesin removal during meiosis II. Finally, Cdc5 regulates a number of proteins critical for the co-segregation of sister chromatids during meiosis I. Thus, Cdc5 has acquired novel functions to facilitate meiosis I chromosome segregation.

CDC5's multiple roles in cohesin removal.

Although *CDC5* is dispensable for cohesin removal and anaphase entry during mitosis, it is essential for this process during meiosis I. Cdc5 controls meiotic cohesin removal in multiple ways. *CDC5* is required for the timely degradation of Securin, although we do not understand how Cdc5 contributes to Securin degradation. *CDC5* could be needed for APC/C activity in meiosis I but not in mitosis. In mammals, polo

kinase is important for APC/C activation (Eckerdt and Strebhardt, 2006; Hansen et al., 2004; Moshe et al., 2004). We favor the idea that during meiosis I, substrate phosphorylation may be important for targeting APC/C substrates for ubiquitylation. The APC/C substrate Spo13 is degraded prematurely in cells overexpressing *CDC5*, which is preceded by its hyper-phosphorylation. Perhaps during meiosis I, ubiquitylation by the APC/C is more similar to ubiquitylation mediated by the related ubiquitin ligase SCF. Substrate recognition by the SCF is controlled by substrate phosphorylation (Willems et al., 2004).

Our results indicate that the metaphase I arrest observed in Cdc5-depleted cells is not only due to a defect in degrading Securin but also due to a defect in Rec8 cleavage. Our previous studies have identified 11 phosphorylation sites in Rec8 whose phosphorylation was absent in cells depleted for Cdc5. As the mass spectrometry analyses were not performed in a quantitative manner it was possible that the results obtained using this method did not accurately reflect the dependencies that occurred *in vivo*. This concern is unwarranted. Examination of the phosphorylation state of three phosphorylation sites using phospho-specific antibodies showed that the mass spectrometry analysis accurately identified *CDC5* dependent and *CDC5* independent phosphorylation sites in Rec8. The study by Katis *et al.* (2010) and our data further indicate that the phosphorylation sites identified to be *CDC5* dependent are indeed important for Rec8 cleavage and meiosis I chromosome segregation. Katis *et al.* (2010) showed that a *REC8* allele in which S136, S179, S197 and T209 were mutated to aspartic acid caused premature loss of centromeric cohesin. Three of these four sites (S136, S179, and S197) were found to be *CDC5*-dependent by our studies. Among the

single mutants only the *rec8-S197D* mutant exhibited a subtle defect in centromeric cohesion protection. However, mutating S136 and S179 to aspartic acid significantly enhanced the phenotype of *rec8 S197D*. Similarly, mutating T209 to aspartic acid did not enhance the defect of the *rec8-S136D S179D S197D* mutant but enhanced the phenotype of the *rec8-S136D S197D* mutant. The fact that several sites in Rec8 must be mutated to aspartic acid to cause premature loss of centromeric cohesion is consistent with the previous proposal that bulk Rec8 phosphorylation is required for its cleavage (Brar et al., 2006).

The conclusion that *CDC5*-dependent phosphorylation of Rec8 is important for its cleavage and removal from chromosomes is not only supported by our phospho-mutant analysis but also by the observation that Rec8 cleavage is greatly delayed in *CDC5-mn ama1*Δ mutants. A previous study examined this very strain and concluded that *CDC5* was not required for Rec8 cleavage (Katis et al., 2010). We suspect that the dynamic range of the assay used to assess the role of *CDC5* in Rec8 cleavage, the presence of Rec8 in the nucleus, was limited, precluding the observation of the Rec8 cleavage defect of *CDC5-mn ama1*Δ mutants.

Cdc5 is clearly not the only kinase that can promote Rec8 cleavage. Mutating a subset of the *CDC5*-dependent phosphorylation sites in Rec8 to residues that can no longer be phosphorylated does not lead to a delay in anaphase I onset (Brar et al., 2006). Furthermore, cells lacking the APC/C inhibitor *MND2* cleave some Rec8 when arrested in prophase I, a cell cycle stage in which *CDC5* is not expressed (Oelschlaegel et al., 2005; Penkner et al., 2005). Katis et al., (2010) identified DDK and/or Hrr25 as kinases phosphorylating Rec8. We propose that in prophase I-arrested cells

phosphorylation by these kinases is sufficient to bring about some Rec8 cleavage. However our results also clearly show that at the metaphase I – anaphase I transition *CDC5* is critical for the timely cleavage of Rec8.

CDC5's role in meiosis II.

While Cdc5 is critical for the metaphase I – anaphase I transition, it is not needed for the equivalent transition during meiosis II. When Cdc5 is inactivated after the completion of meiosis I, *CDC5* appears dispensable for meiosis II chromosome segregation. In fact, Rec8 phosphorylation in general is not needed for meiosis II chromosome segregation. *spo11Δ* cells carrying a *REC8* allele with 29 putative phosphorylation sites mutated to alanine do not exhibit any delay in entry into anaphase II, even though *spo11Δ* cells harbor, in addition to centromeric cohesins, a substantial amount of arm cohesins in meiosis II (Shonn et al., 2000). It has been suggested that bulk Rec8 phosphorylation may be required to make Rec8 a better substrate for separase. If separase activity is low at the metaphase I-anaphase I transition, possibly due to low APC/C activity, but high at the metaphase II-anaphase II transition, Rec8 phosphorylation may not be needed in meiosis II. It is also possible that centromeric cohesin differs from chromosome arm cohesin. Perhaps cohesins loaded onto chromosomes during homologous recombination require phosphorylation for their removal but cohesins loaded during S phase do not. In this scenario, eliminating homologous recombination would eliminate the need for Rec8 phosphorylation in its cleavage.

Although *CDC5* activity is not needed during meiosis II, Cdc5 is likely to affect meiosis II chromosome segregation by functions it executes during meiosis I. *CDC5* is required for Spo13 degradation at the metaphase I – anaphase I transition. By triggering Spo13 degradation, Cdc5 eliminates meiosis I-specific chromosome properties: a cohesin protective domain around centromeres and co-oriented sister chromatids. Thus, Cdc5 not only promotes meiosis I chromosome morphogenesis, it ensures that the meiosis I chromosome characteristics are eliminated prior to meiosis II when sister chromatids and not homologs must be segregated.

Cdc5 activity is not only dispensable for meiosis II chromosome segregation but also for exit from meiosis II. This is however not due to the fact that exit from mitosis is regulated differently than exit from meiosis II, but because Cdc14 release from the nucleolus appears dispensable for exit from meiosis II. Cdc5 inactivation during meiosis II prevented the release of Cdc14 from the nucleolus, yet exit from meiosis II as judged by meiosis II spindle disassembly occurs with only a slight delay. Cdc14 brings about exit from mitosis by triggering the inactivation of B-type cyclin Clb-CDKs (Stegmeier and Amon, 2004). Perhaps CDK inactivation at the end of meiosis II does not require Cdc14 activity. Indeed the bulk of Clb-CDK inactivation occurs before exit from meiosis II. Clb2 is not expressed during meiosis and Clb1-CDK and Clb4-CDK inactivation takes place prior to anaphase II (Carlile and Amon, 2008; Grandin and Reed, 1993). Only Clb3-CDK activity persists until exit from meiosis II. Inactivation of this small pool of Clb-CDK activity may not require *CDC14* to activate the APC/C complexed with its activator Cdh1. Alternatively, meiosis II spindle disassembly may not be triggered by CDK inactivation. Although not needed for the final meiotic cell cycle transition, we did note

that Cdc5 is required for spore wall formation. How Cdc5 controls this process is not known. Cdc5 is a regulator of the Mitotic Exit Network, whose key function during meiosis is spore wall formation (Attner and Amon, 2012; Kamieniecki et al., 2005; Pablo-Hernando et al., 2007). The inability of cells lacking *CDC5* function to build spore walls could originate from this requirement.

Cdc5 – a versatile protein kinase.

Cdc5 sets itself apart from most other cell cycle kinases through its ability to phosphorylate different substrates at different cell cycle stages. In meiosis this versatility is extreme. Several Cdc5 substrates, Rec8 S136 and S179, Lrs4 and Clb1, are phosphorylated in meiosis I but significantly less so in meiosis II. We do not yet know whether this reflects a general down-regulation in Cdc5 activity during meiosis II or whether Cdc5's ability to phosphorylate a subset of substrates is decreased during meiosis II. Prior phosphorylation of substrates by CDKs can target Polo kinases to their substrates (Elia et al., 2003). It is thus possible that, lowered CDK activity in meiosis II could lead to a general decrease in *CDC5*-dependent phosphorylation in meiosis II. We favor the idea that Cdc5's ability to phosphorylate a subset of substrates changes during meiosis. During mitosis it is clear that Cdc5 phosphorylates different substrates at different times during the cell cycle. Cdc5 phosphorylates the cohesin subunit Scc1 at the onset of mitosis, but does not phosphorylate the MEN component Bfa1 until anaphase (Alexandru et al., 2001; Hu et al., 2001). During meiosis, Cdc5 is needed for the release of Cdc14 from the nucleolus during meiosis II suggesting that some Cdc5 activity is present during meiosis II. The critical Cdc5 targets in Cdc14 activation have

not yet been identified, precluding us from directly comparing Cdc5 activity in MEN regulation with its ability to phosphorylate Rec8, Lrs4 and Clb1. *In vitro* kinase assays are unlikely to provide insight into this question either as in budding yeast Cdc5 *in vitro* kinase activity merely reflects Cdc5 protein levels.

Polo-like kinases have been implicated in meiotic regulation in many species. Polo-like kinases are responsible for nuclear envelope breakdown and entry into the meiotic divisions and regulation of the cohesin-protective protein MEI-S332 (Clarke et al., 2005; Xiang et al., 2007). Our results identify a dramatic differential requirement for Polo kinases between meiosis I and meiosis II. It will be interesting to determine whether a similar differential requirement also exists in other eukaryotes and how it is established.

MATERIALS AND METHODS

Yeast Strains

All yeast strains used in this study are derivatives of SK1 and listed in Table 1. *REC8-3V5*, *ama1Δ*, *pCUP1-3HA-CDC5*, *SPO13-13MYC*, *CDC5-13MYC*, *LRS4-13MYC*, *CLB1-3V5*, and *pGAL-CLB1-3V5* were constructed using a PCR-based method (Longtine et al., 1998). *pCLB2-CDC20* and *pCLB2-CDC5* were previously described (Lee and Amon, 2003). *spo13Δ* was previously described (Lee et al., 2004). *PDS1-18MYC* was a gift from K. Nasmyth. *rec8-17A-3HA*, *rec8-29A-3HA*, and *ubr1Δ* are described in (Brar et al., 2006). *PDS1-tdTomato* was a gift from W. Zachariae. *CDC14-3HA* was previously described (Marston et al., 2003). *REC8-3HA*, GFP dots and *spo11Δ::URA3* are described in (Klein et al., 1999). The *cdc5-as1* allele (Snead et al.,

2007) was backcrossed 7X into SK1. pA498 was generated by cloning *REC8-3HA* into Yiplac128. Plasmid (pHG40) containing the *CUP1* promoter was a gift from Hong-Guo Yu. 3V5 tagging plasmids were provided by Vincent Guacci.

rec8-phosphomimetic mutants

Plasmids based on pA498 were mutated with a Stratagene Quikchange kit and then integrated at the *LEU2* locus into the *rec8Δ* strain A16004. Single-copy insertion was verified by Southern blot analysis. All mutants contained a 3HA tag at the C-terminus.

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. For the experiment shown in Figure 7, sporulation medium was supplemented with 0.02% raffinose. Sporulation experiments were performed at 30°C. For certain experiments indicated in the text, 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=5 or 6 hours, 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 (Carlile and Amon, 2008).

Fluorescence microscopy

Indirect in situ immunofluorescence was performed as described previously (Kilmartin and Adams, 1984). Anti-tubulin antibodies were used to stain spindle microtubules, and DAPI (4',6-diamidino-2-phenylindole) was used to stain DNA. Cdc14-3HA and Pds1-18Myc immunofluorescence was performed as described in (Marston et al., 2003). Cells were imaged with a Zeiss Axioplan 2 microscope and a Hamamatsu ORCA-ER digital camera. GFP dots were analyzed as described previously (Miller et al., 2012). Live cell imaging analysis was described previously (Miller et al., 2012).

Immunoblot analysis

For immunoblot analysis of HA-Cdc5, Clb2, Rec8-HA, Pds1-18Myc, Spo13-13Myc, Cdc5-13Myc, Lrs4-13Myc, Clb1-V5, 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 100µl 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. V5-tagged proteins were detected with anti-V5 (Invitrogen) antibodies at a dilution of 1:2000. Clb2 was detected with anti-Clb2 antibodies (a gift from Fred Cross) at a 1:2000 dilution. Pgk1 was detected with an anti-

Pgk1 antibody (Invitrogen, Carlsbad, CA) using a 1:5000 dilution. Quantification was performed using ImageQuant software.

Half-life measurements

For meiotic half-life assays, cells were sporulated for 6-8 hours, and protein synthesis was blocked by addition of 0.5mg/ml cycloheximide. Samples were taken at the indicated times after repression and analyzed by western blot.

Denaturing immunoprecipitation assays

Rec8-3HA, Rec8-17A-3HA, and Rec8-3V5 were immunoprecipitated as described previously with several modifications (Brar et al., 2006). After adding SDS to 1% and boiling samples, 7.5 volumes of NP40 buffer (150mM NaCl, 1% NP40, 50mM Tris-HCl pH 7.5, 2mg/ml BSA) were added. Extracts were incubated for 1.5 hours at 4°C with 25µl of either anti-HA affimatrix beads (Roche) or anti-V5 agarose (Sigma) pre-equilibrated in NP40 buffer. Samples were washed according to Brar et al., (2006), resuspended in 3X SDS sample buffer, and boiled. Samples were subjected to immunoblot analysis with antibodies recognizing Rec8-pS136 or Rec8-pS179. Anti-HA (1:1000) and anti-V5 (1:2000) antibodies were used to recognize total Rec8.

Antibodies were custom-made by Abgent Technologies. The Rec8-S136 antibody was previously described (Brar et al., 2006). The phospho-S179 antibody was raised using the peptide QDNV(pS)FIEEAKSIRRC. To detect Rec8 with this phospho-S179 antibody, blots were blocked for 2-4 hours at room temperature in 3.5%BSA/TBST, and

incubated overnight with the rabbit phospho-S179 antibody (1:1000) in 1%BSA/TBST overnight. Secondary antibody, goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP; Jackson Immunoresearch) was used at a 1:5000 dilution.

Chromosome spreads

Chromosome spreads were described previously (Miller et al., 2012). Ndc10-Myc was detected using a pre-absorbed Myc antibody (9E10 epitope, Covance, Princeton NJ) at a concentration of 1:200. Rec8-V5 was detected using an anti-V5 antibody (Abcam) at a 1:200 concentration. Preabsorbed anti-mouse-Cy3 and anti-rabbit-FITC secondary antibodies were used at a concentration of 1:400.

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Table 1. Yeast strains used in this study.
All strains are in the SK1 background.

A1972	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, ura3/ura3, TRP1/trp1::hisG, his4X/his4X, REC8-3HA:URA3/REC8-3HA:URA3*
A4962	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG*
A6139	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, cdc5::pCLB2-CDC5:KanMX6/cdc5::pCLB2-CDC5:KanMX6, SPO13/SPO13-13MYC:KANMX6*
A13559	*MATa/alpha ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, ura3/ura3, trp1::hisG/TRP1, HIS4/his4X, rec8::KanMX4/rec8::KanMX4::Rec8S197AS386AS387AS136AT173AS199AS245AT249AS521AS522AS314AS410AS179AS215AS465AS466AS285A-3HA*
A17131	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, cdc5::pCLB2-CDC5:KanMX6/cdc5::pCLB2-CDC5:KanMX6, NDC10/NDC10-6HA:HIS3MX6, MAM1/MAM1-9MYC:TRP1*
A21230	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, rec8::KanMX4:Rec8-3HA/rec8::KanMX4:Rec8-3HA*
A21235	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his::hisG, trp1::hisG/trp1::hisG GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, REC8S197AS386AS387AS136AT173AS199AS245AT249A-S521AS522AS314AS410AS179AS215AS465AS466AS285A-3HA/ REC8S197AS386AS387AS136AT173AS199AS245AT249A-S521AS522AS314AS410AS179AS215AS465AS466AS285A-3HA*
A22132	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ ura3::pGPD1-GAL4(848).ER:URA3, CDC14-3HA/CDC14*
A22803	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp::hisG, GAL-NDT80:TRP1/GAL-NDT80, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, rec8::KanMX4:REC8S197AS386AS387AS136AT173AS199AS245AT249AS521AS522AS314AS410AS179AS215AS465AS466AS285AS494AS421AY14AS552AT18AT19AS292AS425AS404AS125AT126AS224A-3HA/rec8::KanMX4:REC8S197AS386AS387AS136AT173AS199AS245AT249AS521AS522AS314AS410AS179AS215AS465AS466AS285AS494AS4*

	21AY14AS552AT18AT19AS292AS425AS404AS125AT126AS224A-3HA, ubr1::HIS3/ubr1::HIS3
A22804	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3,rec8::KanMX4:Rec8-3HA/rec8::KanMX4:Rec8-3HA, ubr1::HIS3/ubr1::HIS3*
A23405	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, PDS1-18MYC:LEU2/PDS1-18MYC:LEU2, spo13::SPO13-13MYC:KanMX6/spo13::SPO13-13MYC:KanMX6*
A23650	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, HIS3MX6:pGAL-CLB1-3V5:KanMX/HIS3MX6:pGAL-CLB1-3V5:KanMX*
A23664	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, PDS1-18MYC:LEU2/PDS1-18MYC:LEU2, spo13::SPO13-13MYC:KanMX6/spo13::SPO13-13MYC:KanMX6 cdc20::pCLB2-CDC20:KanMX6/cdc20::pCLB2-CDC20:KanMX6*
A23757	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, PDS1-18MYC:LEU2/PDS1-18MYC:LEU2, spo13::SPO13-13MYC:KanMX6/spo13::SPO13-13MYC:KanMX6, cdc5::pCLB2-CDC5:KanMX6/cdc5::pCLB2-CDC5:KanMX6*
A24207	*MATa/alpha, ho::LYS2/ho::LYS2, ura3/ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, cdc20::pCLB2-CDC20::KanMX6/cdc20::pCLB2-CDC20::KanMX6, CLB1-3V5:KanMX/CLB1-3V5:KanMX*
A24758	*MATa/alpha, ho::LYS2/ho::LYS2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, cdc5::CDC5-13MYC:KanMX6/cdc5::CDC5-13MYC:KanMX6*
A24760	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, LRS4-13MYC:KanMX6/LRS4-13MYC:KanMX6*
A26176	*MATa/alpha, ho::LYS2/ho::LYS2, ura3/ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, cdc20::pCLB2-CDC20:KanMX6/cdc20::pCLB2-CDC20:KanMX6, cdc5-as1 (cdc5L158G)/cdc5-as1 (cdc5L158G), CLB1-3V5:KanMX/CLB1-3V5:KanMX*

A27808	*MATa/alpha, ho::LYS2/ho::LYS2, ura3/ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, cdc20::pCLB2-CDC20:KanMX6/cdc20::pCLB2-CDC20:KanMX6, REC8-3HA:URA3/REC8-3HA:URA3*
A27809	*MATa/alpha, ho::LYS2/ho::LYS2, ura3/ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, cdc5::pCLB2-CDC5:KanMX6/cdc5::pCLB2-CDC5:KanMX6, REC8-3HA:URA3/REC8-3HA:URA3*
A30406	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S136DS179DS197DT209D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A30407	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S136D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A30408	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S179D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A30409	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S136DS179D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A30410	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S136DS179DS197D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A30411	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/REC8-3HA:URA3, leu2::hisG/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A30960	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, leu2::hisG/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3, spo13::hisG/spo13::hisG*
A31020	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3/ura3::pGPD1-GAL4(848).ER:URA3, leu2::hisG/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3, PDS1-tdTomato-KITRP1/PDS1-tdTomato-KITRP1*
A32250	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S197DT209D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*

A32252	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S197D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A32254	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S179DS197D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A32256	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S136DS179D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A32258	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S136DS197DT209D-3HA:LEU2/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3*
A32746	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3/ura3::pGPD1-GAL4(848).ER:URA3, leu2::hisG/leu2::pURA3-TetR-GFP:LEU2, CENV::TetOx224:HIS3, PDS1-tdTomato-KITRP1/PDS1-tdTomato-KITRP1, KanMX6:pCUP1-3HA-CDC5/KanMX6:pCUP1-3HA-CDC5*
A32851	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, KanMX6:pCUP1-3HA-CDC5/KanMX6:pCUP1-3HA-CDC5, REC8/REC8-3V5:HISMX*
A33118	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, ama1::HISMX6/ama1::HISMX6, cdc5::pCLB2-CDC5:KanMX6/cdc5::pCLB2-CDC5:KanMX6, PDS1-18MYC:LEU2/PDS1-18MYC:LEU2, ubr1::HIS3/ubr1::HIS3, REC8/REC8-3HA:URA3*
A33119	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, ama1::HISMX6/ama1::HISMX6, PDS1-18MYC:LEU2/PDS1-18MYC:LEU2, ubr1::HIS3/ubr1::HIS3, REC8/REC8-3HA:URA3*
A33292	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, cdc5::pCLB2-CDC5:KanMX6/cdc5::pCLB2-CDC5:KanMX6, PDS1-18MYC:LEU2/PDS1-18MYC:LEU2, ubr1::HIS3/ubr1::HIS3, REC8/REC8-3HA:URA3*
A33293	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, PDS1-18MYC:LEU2/PDS1-18MYC:LEU2, ubr1::HIS3/ubr1::HIS3, REC8/REC8-3HA:URA3*
A33368	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-*

	GAL4(848).ER:URA3, REC8/REC8-3V5:HISMX
A33453	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, ubr1::HIS3/ubr1::HIS3, spo11::URA3/spo11::URA3, rec8::KanMX4: Rec8S197AS386AS387AS136AT173AS199AS245AT249A-S521AS522AS314AS410AS179AS215AS465AS466AS285AS494AS421AY14AS552AT18AT19AS292AS425AS404AS125AT126AS224A-3HA/rec8::KanMX4:Rec8S197AS386AS387AS136AT173AS199AS245AT249A-S521AS522AS314AS410AS179AS215AS465AS466AS285AS494AS421AY14AS552AT18AT19AS292AS425AS404AS125AT126AS224A-3HA*
A33459	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, KanMX6:pCUP1-3HA-CDC5/KanMX6:pCUP1-3HA-CDC5, REC8/REC8-3V5, NDC10/NDC10-13MYC*
A33469	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, ubr1::HIS3/ubr1::HIS3, spo11::URA3/spo11::URA3, rec8::KanMX4:REC8-3HA/rec8::KanMX4:REC8-3HA*
A33491	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, rec8::KanMX4/rec8::KanMX4, leu2::REC8-S136DS179DS197D-3HA:LEU2/leu2::hisG, cdc20::pCLB2-CDC20:KanMX6/cdc20::pCLB2-CDC20:KanMX6, spo11::URA3/spo11::URA3, NDC10/NDC10-6HA:HIS3MX6*
A33493	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, REC8-3HA:URA3/rec8::KanMX4, cdc20::pCLB2-CDC20:KanMX6/cdc20::pCLB2-CDC20:KanMX6, spo11::URA3/spo11::URA3, NDC10/NDC10-6HA:HIS3MX6*
A33497	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, KanMX6:pCUP1-3HA-CDC5/KanMX6:pCUP1-3HA-CDC5, spo13::SPO13-13MYC:HIS3MX6/spo13::SPO13-13MYC:HIS3MX6*
A33501	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, spo13::SPO13-13MYC:HIS3MX6/spo13::SPO13-13MYC:HIS3MX6*
A33513	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-*

	GAL4(848).ER:URA3, CDC14/CDC14-3HA, cdc5-as1(cdc5L158G)/cdc5-as1(cdc5L158G)
A33765	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, REC8-3HA:URA3/REC8-3HA:URA3, ubr1::HIS3/ubr1::HIS3,ama1::HIS3MX6/ama1::HIS3MX6, spo13::hisG/spo13::hisG, KanMX6:pCLB2-CDC5/KanMX6:pCLB2-CDC5*
A33766	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, REC8-3HA:URA3/REC8-3HA:URA3, ubr1::HIS3/ubr1::HIS3,ama1::HIS3MX6/ama1::HIS3MX6, KanMX6:pCLB2-CDC5/KanMX6:pCLB2-CDC5*
A33767	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, REC8-3HA:URA3/REC8-3HA:URA3, ubr1::HIS3/ubr1::HIS3,ama1::HIS3MX6/ama1::HIS3MX6*
A33768	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, ura3, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, REC8-3HA:URA3/REC8-3HA:URA3, ubr1::HIS3/ubr1::HIS3,ama1::HIS3MX6/ama1::HIS3MX6, spo13::hisG/spo13::hisG*
A33791	*MATa/alpha, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER:URA3/ura3::pGPD1-GAL4(848).ER:URA3, REC8/REC8-3V5, NDC10/NDC10-13MYC*

CHAPTER 4:

Discussion and Future Directions

Summary of key conclusions

Research in the field of meiotic chromosome segregation has made important advances in the past few years, in large part due to research in budding yeast, where sporulation is inducible. Further, discovery of a method to synchronize the meiotic divisions in this organism has allowed us to add population-based assays to our arsenal of methods with which to investigate meiotic chromosome segregation (Carlile and Amon, 2008). Using this approach, we have uncovered previously unappreciated layers of regulation of essential cell cycle regulators. The research presented in this thesis improves our understanding of how cell cycle regulators and signaling pathways function during gametogenesis. Understanding how these cell cycle engines drive the specialized meiotic cell divisions has provided significant insight into how cell cycle regulatory pathways are modulated to accommodate different types of cell division. Since the cell-cycle regulators studied here are conserved, the research presented in this thesis may shed light on the causes of meiotic chromosome missegregation in humans. Furthermore, understanding how cell cycle regulatory pathways can be modulated under different conditions may be relevant to cancer, a disease of aberrant cell division and hyper-proliferation.

Two universal cell cycle regulatory kinases - cyclin-dependent kinase (CDK) and polo kinase - are critical for the proper execution of mitotic and meiotic divisions. CDK activity drives progression through mitosis, and CDK inactivation by the mitotic exit network (MEN) is required for mitotic exit (Jaspersen et al., 1998; Visintin et al., 1998). In Chapter 2, I demonstrate that the MEN, a pathway essential for vegetative growth, is dispensable for the meiotic divisions. Instead, MEN components function at late stages

of gametogenesis to control spore wall formation. Furthermore, during meiosis the MEN signals via a mechanism distinct from mitosis, suggesting that cell cycle regulatory pathways can evolve divergent signaling mechanisms in different types of cell divisions. Research presented in Chapter 3 addresses the role of polo kinase in meiosis. I have probed the additional roles that polo kinase takes on in meiosis and established a central role for polo kinase in meiosis I. Polo kinase coordinates several modifications necessary for homologous chromosomes to segregate in meiosis I, and establishes permissive conditions for meiosis II segregation. I will discuss several issues that arise from this research below, centering on four general questions. (1) What is the mechanism of MEN signaling in meiosis? (2) How is exit from meiosis II controlled? (3) How does Cdc5 take on additional roles in meiosis I but not in meiosis II? (4) How is the stepwise loss of cohesion achieved?

The mechanism of MEN signaling in meiosis

During mitosis, localization of MEN components to the SPB is required for MEN signaling (Rock and Amon, 2011; Valerio-Santiago and Monje-Casas, 2011). In fact, tethering Cdc15 to the SPB bypasses the requirement for *TEM1* and *CDC5* in MEN activation (Rock and Amon, 2011). Furthermore, recent data indicate that the scaffold at the SPB, Nud1, participates in activating the Dbf2-Mob1 kinase complex (J. Rock, personal communication). Therefore, one of the most surprising findings presented in this thesis is that MEN signaling, which is restricted to meiosis II, occurs independently of SPBs. Several lines of evidence support this conclusion. First, MEN components, although expressed, are not detected at SPBs during any stage of meiosis. Second, the

SPB-associated scaffold Nud1 is dispensable for MEN activity. Third, tethering the MEN component Tem1 to SPBs fails to activate MEN signaling in meiosis (See Appendix).

MEN signaling is controlled in an SPB-independent manner

One reason why the SPB is not required as a hub for MEN signaling in meiosis is the nature of the meiotic division in budding yeast. Mitosis is an asymmetric division, in which Tem1 acts as a sensor that is only activated in the bud, thus ensuring that mitotic exit is only triggered when the spindle is positioned properly (Bardin et al., 2000; Chan and Amon, 2010; Pereira et al., 2000). In meiosis, the two divisions occur within the mother cell without any obvious asymmetry. Consistently, we and others have not yet identified a role in meiosis for Tem1 or Tem1-interacting factors, which mediate the spatial control of MEN signaling (Kamieniecki et al., 2005). Since factors governing the spatial control of MEN signaling are not important for MEN signaling in the symmetric meiotic divisions, it is possible that signals mediating temporal control of MEN signaling are relevant. In mitosis, *CDC5* is one mediator of the temporal control of MEN signaling (Rock and Amon, 2011). Interestingly, in meiosis, *CDC5* has essential roles prior to anaphase I (Clyne et al., 2003; Lee and Amon, 2003), but does not activate the MEN. However, in mitosis *CDC5* activates MEN signaling by inactivating the Tem1 GAP at SPBs and by recruiting Cdc15 to SPBs (Hu et al., 2001; Rock and Amon, 2011). It is therefore likely that *CDC5* fails to activate MEN signaling in meiosis I because MEN signaling is controlled via a mechanism that does not require the SPB.

Although the meiotic divisions do not rely on the same spatial cues as mitosis, it is unknown why MEN components would not still use the SPB to assemble signaling

modules. One possibility is that the outer plaque of the SPB undergoes restructuring in meiosis II in order to act as a platform for prospore membrane formation and spore formation (Neiman, 2005). Perhaps assembly of the meiosis II outer plaque inhibits the ability of MEN components to bind to this organelle. Alternatively, it was possible that the outer plaque architecture impeded our ability to detect MEN components at SPBs. This possibility is unlikely because the MEN component Bfa1 is detected at SPBs in meiosis II.

Since MEN components do not localize to SPBs, the mechanism by which the MEN signals in meiosis must be different than in mitosis. Moreover, not only are MEN components not detected at SPBs, but MEN signaling in meiosis does not require the mitotic scaffolding protein Nud1. How do signaling modules form? It appears that the Dbf20-Mob1 interaction is the key step regulating meiotic MEN signaling, but many details remain to be discovered (Figure 1, top). The Dbf20-Mob1 interaction depends on Cdc15 kinase activity, and is likely to also depend on phosphorylation of Dbf20 and Mob1. Dbf20 exhibits a mobility shift on SDS-PAGE during meiosis II and Mob1 exhibits a mobility shift on SDS-PAGE during meiosis I and meiosis II. How Cdc15, Dbf20, and Mob1 are linked in meiosis is unclear and the identity of a candidate meiosis-specific scaffold is unknown. To address this question, it will be interesting to take a proteomic approach to identify Dbf20, Mob1, and Cdc15-interacting factors in meiosis II that may function as a meiosis-specific scaffold. A wave of transcription occurs late in meiosis, and many of these genes have roles in spore wall morphogenesis and the completion of sporulation (Chu et al., 1998). A meiosis-specific scaffold produced late in meiosis could link Cdc15 to Dbf20 and Mob1 in the cytoplasm, and promote MEN signaling during

meiosis II. Coupling MEN activation to spore wall formation would not be surprising since *CDC15* and *CDC5* are required for spore wall formation (Chapters 2 and 3; (Pablo-Hernando et al., 2007)). It will also be interesting to determine if Cdc15 kinase activity is cell cycle regulated in meiosis. At the time of these studies, a readout for Cdc15 activity was lacking. However, we now have Nud1 phospho-specific antibodies recognizing *CDC15*-dependent phosphorylation sites. Nud1 undergoes a mobility shift on SDS-PAGE in meiosis, and it will be interesting to determine how phosphorylation of these *CDC5*-dependent sites changes during meiosis.

The mechanism by which the Dbf20-Mob1 interaction and its associated kinase activity are cell cycle regulated is unknown. In meiosis II, the Dbf20-Mob1 interaction is enriched, and this interaction depends on Cdc15 kinase activity. On the other hand, in mitosis, Dbf20 and Mob1 localize to SPBs and physically interact throughout the vegetative cell cycle, but this kinase complex is inactive, at least by our *in vitro* assay. It is unclear how Dbf20-Mob1 complexes are kept inactive in meiosis I and mitosis. Again, proteomic approaches may shed light on the trans-acting factors that keep Dbf20 and Mob1 apart during meiosis I or inhibit the activation of the Dbf20-Mob1 complexes that do form during meiosis I. Comparing proteins that co-precipitate with Dbf20 and Mob1 in mitosis, meiosis I, and meiosis II will therefore be informative.

One of our most interesting observations is that, during meiosis, Dbf20-associated kinase activity is restricted to meiosis II. This observation led us to postulate that it is important to keep MEN signaling inactive during meiosis I. We imagined that MEN signaling during meiosis I, and thus premature CDK inactivation, would lead to cessation of meiosis after the first meiotic division, DNA re-replication, or premature

spore wall formation. To test this hypothesis, we attempted to delete negative regulators and hyperactivate positive regulators of the MEN pathway to try to activate MEN signaling during meiosis I (See Appendix). However, we have thus far been unable to hyperactivate MEN signaling during meiosis I to examine the consequences of MEN signaling in meiosis I. In the future it will be important to exploit what we learn about the Dbf20-Mob1 interaction to examine the importance of keeping the MEN inactive in meiosis I. If a Dbf20-Mob1 fusion is active as a kinase, we could address the consequences of Dbf20 kinase activation in meiosis I. If we discover a meiosis II-specific scaffold or another trans-acting activator, these could also be expressed in meiosis I. Together, these experiments will elucidate how MEN signaling is controlled in meiosis and improve our understanding of how cells achieve two consecutive rounds of chromosome segregation.

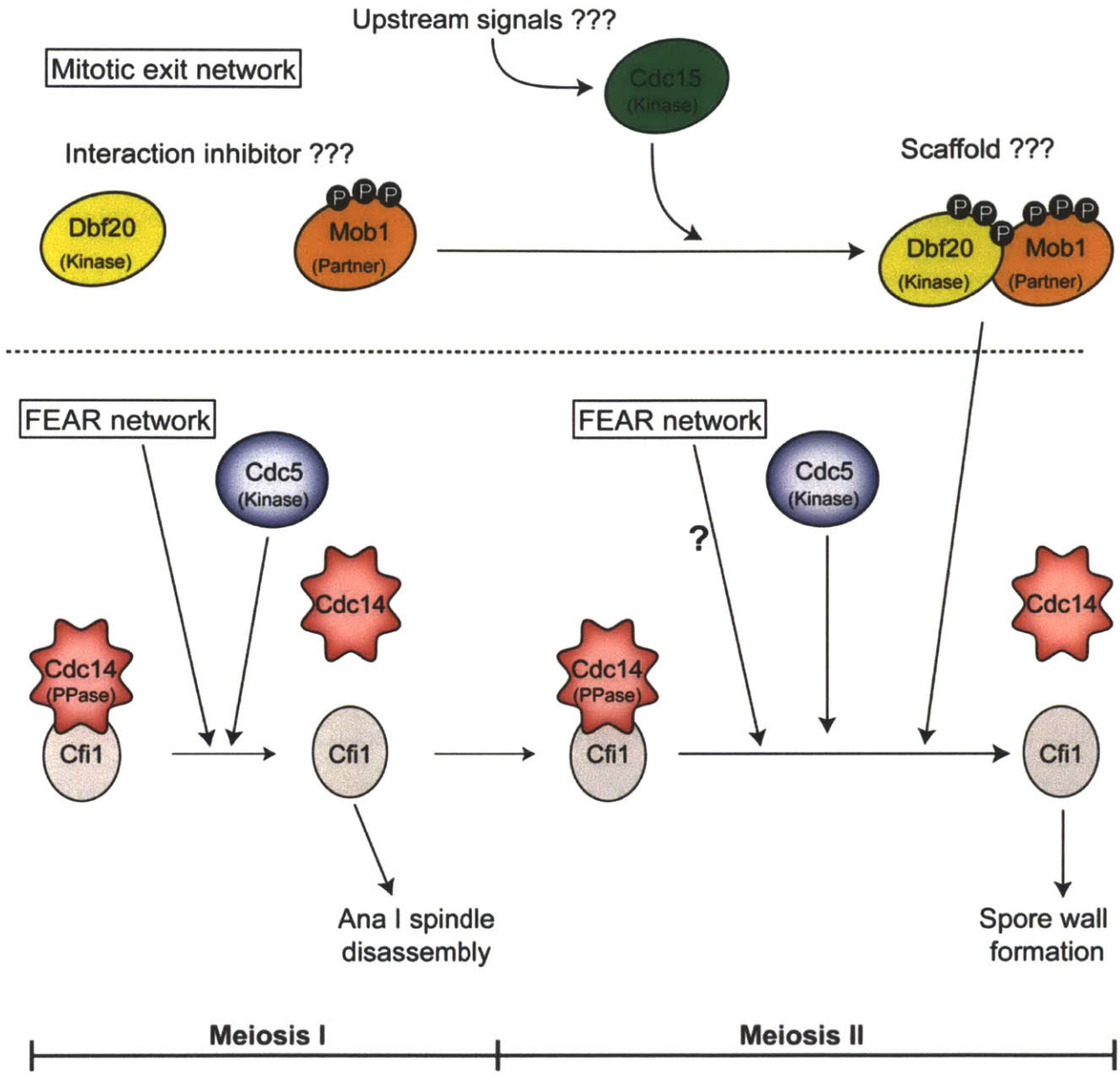

Figure 1. Control of Cdc14 release by the FEAR network and the MEN in meiosis. The MEN is wired in a different way during meiosis. Cdc15 promotes the interaction between Dbf20 and Mob1, which promotes Cdc14 release. The FEAR network provides a transient burst of Cdc14 release in meiosis I. Cdc5 kinase activity is required for Cdc14 release in both meiosis I and meiosis II. It appears that Cdc14 release is required for anaphase I spindle disassembly in meiosis I, but in meiosis II its critical function may be in spore wall formation.

Relevance to higher eukaryotes

Studying how signaling through the MEN is modulated during meiosis may be broadly relevant to higher eukaryotes. The MEN is an NDR-kinase signaling cascade and homologous pathways control cell proliferation and organ growth in vertebrates (Halder and Johnson, 2011). The Cdc15-Dbf2/20-Mob1 module is conserved as Mst1/2-Lats-Mob1A/B in human cells. A multitude of upstream signals regulate NDR kinase signaling, and these signals are poorly understood. Moreover, different signals modulate NDR kinase pathways in different cell types and tissues. Therefore, studying the MEN in meiosis may offer insights into understanding how NDR kinase pathways are controlled. Several similarities are already emerging. Just as Cdc15 promotes the Dbf20-Mob1 interaction in meiosis, Mst1/2 phosphorylate Lats and Mob1 proteins, which in turn enhance the ability of Lats and Mob1 to interact (Chan et al., 2005; Hirabayashi et al., 2008; Praskova et al., 2008). Interestingly, it is thought that WW45, a protein with several protein-protein interaction domains, links Mst1/2, Lats, and Mob1, without necessitating the localization of the signaling modules to a discrete cellular structure (Avruch et al., 2012). Much remains to be learned, but it is likely that understanding how MEN signaling modules are assembled in meiosis will inform the mammalian NDR-kinase signaling field.

The role of Cdc14 in exit from meiosis II

In order for cells to exit mitosis and rapidly reset the cell cycle to a G1 state, CDKs are inactivated. The phosphatase Cdc14 is essential for the inactivation of CDKs. Cdc14 reverses CDK-dependent phosphorylation, and one of its critical target is the

APC/C activator Cdh1 (Visintin et al., 1998). Dephosphorylation of Cdh1 leads to its association with the APC/C, and thus the degradation of cyclins. Cdc14 release is tightly controlled during the cell cycle. It is sequestered in the nucleolus for most of the cell cycle, but released into the nucleus and cytoplasm during anaphase (Shou et al., 1999; Visintin et al., 1999). Cdc14 also functions during meiosis, as cells harboring a temperature sensitive allele of *CDC14* exhibit defects in anaphase I spindle disassembly (Buonomo et al., 2000; Marston et al., 2003). Furthermore, Cdc14 is released from the nucleolus in anaphase I and anaphase II (Buonomo et al., 2000; Marston et al., 2003). It has therefore been assumed that Cdc14 release is also required in meiosis II to promote anaphase II spindle disassembly. Data presented in this thesis challenge this notion. Cells expressing an analog-sensitive allele of *CDC5* that are treated with inhibitor at the meiosis I-meiosis II transition exhibit a profound defect in Cdc14 release, yet these cells disassemble anaphase II spindles with only a subtle delay (Chapter 3). It is unclear how anaphase II spindle disassembly is achieved in the absence of Cdc14 release, but I envision two possibilities: (1) CDK inactivation does not require Cdc14 activity in meiosis II, or (2) CDK inactivation is not required for meiosis II spindle disassembly.

The regulation of CDK activity differs between mitosis and meiosis, which could change the requirement for Cdc14 activity in inactivating cyclins at exit from meiosis II. The regulation of cyclins that drive meiotic progression is complex. Clb1 kinase activity rather than protein level is regulated, and Clb3-CDK is the major CDK active until meiotic exit (Carlile and Amon, 2008). Moreover, the most potent cyclin in mitosis, Clb2, is absent during meiosis (Grandin and Reed, 1993). Because there is much less CDK

activity remaining at exit from meiosis II, the importance of Cdc14 activity in meiosis II is probably greatly diminished. It is also possible that the APC/C activity needed to promote cyclin degradation at exit from meiosis II may not require Cdc14 activity. Interestingly, another APC/C activator is present during meiosis, Ama1. *AMA1* is required for spore wall formation, suggesting that the APC/C^{Ama1} is active late in meiosis II (Cooper et al., 2000). It has been suggested that reduced CDK activity activates the APC/C^{Ama1} (Oelschlaegel et al., 2005). Therefore, cyclin degradation begun at the metaphase II-anaphase II transition by the APC/C^{Cdc20} could activate the APC/C^{Ama1} at the end of meiosis. In this manner, the remaining Clb3 could be degraded without the need for Cdc14 release. Several future experiments can shed light on the relationship between Cdc14 activity and cyclin degradation at exit from meiosis II. It will be interesting to establish if Clb3 degradation depends on Cdc14 release. Examining Clb3 degradation in cells lacking Cdc5 kinase activity in meiosis II will address this question. If cyclin degradation does not require Cdc14 activity, this would indicate that Cdc14 activity is not needed for CDK inactivation at the end of meiosis. It will also be interesting to more directly assess the requirement for *CDC14* during exit from meiosis II. We can examine the effect of inactivating *CDC14* by shifting temperature-sensitive *CDC14* mutants to the restrictive temperature during meiosis II. I predict that *CDC14* will not be required for anaphase II spindle disassembly, but may have a subtle role.

It remains possible that Cdc14 activity is not required for anaphase II spindle disassembly because the inactivation of CDKs is not required for anaphase II spindle disassembly. This model predicts that overexpressing a non-degradable cyclin in meiosis II would still be competent to disassemble anaphase II spindles. Cells

overexpressing a non-degradable version of Clb2 fail to disassemble anaphase I spindles (Marston et al., 2003), and it will be interesting to examine the effects of the same construct on anaphase II spindle disassembly. How could anaphase II spindle disassembly be triggered independently of CDK inactivation? It has been appreciated for several decades that general protein degradation is upregulated during sporulation (Zubenko and Jones, 1981). It is possible that general protein degradation contributes to meiotic exit, although the mechanism is completely unknown. Furthermore, age-induced cellular damage is eliminated late in sporulation, as the age-associated marker Hsp104 is detectable in tetranucleates but not tetrads (Unal et al., 2011). In the future, it will interesting to determine if there is a connection between meiotic exit and life-span resetting.

The control of Cdc14 release in meiosis

Cdc14 release is controlled by the non-essential FEAR network and the essential mitotic exit network (MEN) during mitosis. Despite the observation that Cdc14 release is not required for anaphase II spindle disassembly, the regulation by the FEAR network of Cdc14 release appears highly conserved between mitosis, meiosis I, and meiosis II. In these three cell cycle stages, Cdc14 release is controlled by the polo kinase Cdc5, which is a component of the FEAR network and an activator of the MEN. At least in mitosis and meiosis I, Cdc14 release is governed by FEAR network activity, thereby allowing Cdc14 release to be coupled to the metaphase-anaphase transition. In a key difference between mitosis and meiosis, I have found that the MEN is not required for Cdc14 release in anaphase I, and contributes subtly to Cdc14 release in anaphase II. It

is thought that a transient burst of Cdc14 release and sequestration brought about by the FEAR network is sufficient to promote the meiosis I-meiosis II transition without causing cells to exit the meiotic program between the two divisions (Figure 1). It will be interesting to understand in greater detail how the FEAR network controls Cdc14 release in meiosis II. It will be interesting to deplete the FEAR components *SPO12* and *SLK19* in meiosis II. I predict that Cdc14 release will be impaired in meiosis II in FEAR mutants, but that anaphase II spindles will disassemble. We can also monitor FEAR network activity by examining the phosphorylation of Spo12 (Tomson et al., 2009). In mitosis, Spo12 phosphorylation occurs in anaphase, and it will be interesting to determine if Spo12 phosphorylation peaks in anaphase I and II.

It appears then that the critical role for Cdc5 and MEN components in meiosis II is in spore wall morphogenesis, the cytokinetic event of meiosis. Both Cdc5 and Cdc15 kinase activity are required for spore wall formation. *CDC14-ts* mutants also fail to build spore walls, although this could be an effect of elevated temperature (Marston et al., 2003). A role for the MEN in spore wall formation is not surprising, as Cdc15, Dbf2, Mob1, and Cdc5 localize to the bud neck during telophase and contribute to cytokinesis during mitosis (Frenz et al., 2000; Luca et al., 2001; Song and Lee, 2001; Xu et al., 2000; Yoshida and Toh-e, 2001). This function is conserved, as the homologous pathway in fission yeast, the septation initiation network (SIN), is required for spore wall formation as well (Krapp et al., 2006).

The roles of polo kinase in meiosis

Polo kinases are key regulators of the cell cycle in all eukaryotes. Vertebrate polo kinases coordinate multiple processes during mitosis, whereas in budding yeast the essential function of the sole polo kinase Cdc5 is in controlling Cdc14 release and mitotic exit (Archambault and Glover, 2009; Stegmeier and Amon, 2004). Polo kinases are also important regulators of meiosis across species. In *Drosophila*, *polo* is required for nuclear envelope breakdown prior to the meiotic divisions, and Polo activation is controlled by an inhibitor called Matrimony (Xiang et al., 2007). Polo also regulates the cohesion-protective protein MEI-S332 (Clarke et al., 2005). In vertebrate meiosis, polo kinase is important for meiotic exit. In oocytes, polo kinase promotes degradation of an APC/C inhibitor in response to a calcium signal to trigger the completion of meiosis (Rauh et al., 2005). Therefore, understanding how Cdc5 coordinates meiotic events in molecular detail could provide important insights into how meiotic progression is controlled in other species.

In budding yeast, Cdc5's essential function is to promote Cdc14 release and activate the MEN. In meiosis, however, Cdc5 has gained additional functions. Cdc5 is required for the resolution of chiasmata, securin degradation, sister kinetochore co-orientation, arm cohesion removal, and Cdc14 release (Clyne et al., 2003; Lee and Amon, 2003). Since cells with *CDC5* depleted (*CDC5-mn*) in meiosis arrest in metaphase I, it has been difficult to tease apart how *CDC5* coordinates the meiotic divisions. By pairing our Ndt80 block-release synchronization protocol with an allele of *CDC5* that allows us to inhibit Cdc5 kinase activity at any point in meiosis, we determined that Cdc5 kinase activity is required during the first meiotic division, but not

during the second meiotic division. Furthermore, several Cdc5 targets are phosphorylated only in meiosis I. Figure 2 highlights the stages of meiosis in which Cdc5 functions. In the following sections, I will discuss how *CDC5* gains additional functions in meiosis I and speculate about how the phosphorylation of Cdc5 targets may be restricted to meiosis I.

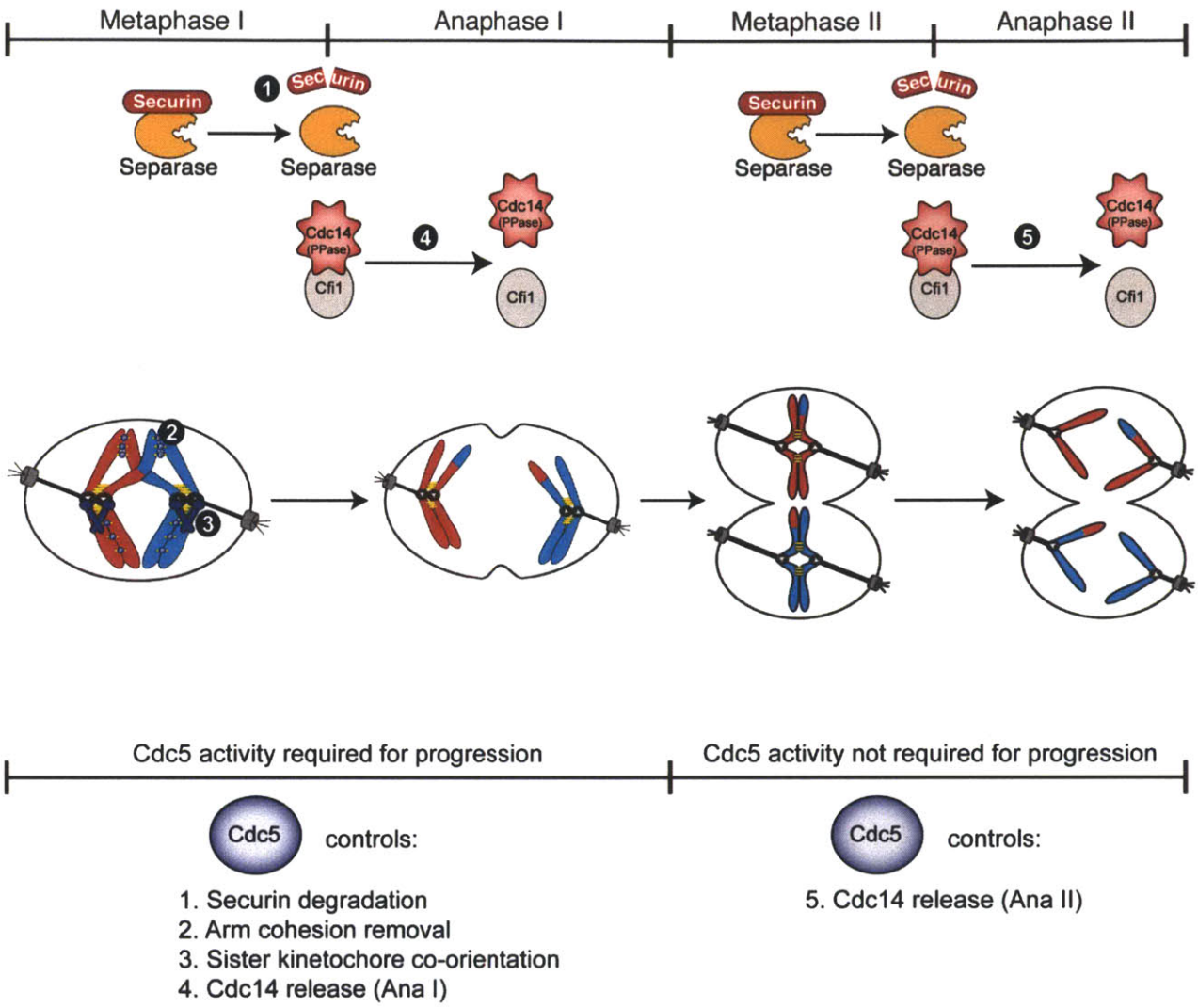

Figure 2. Cdc5 coordinates meiosis I progression.

A schematic of homologous chromosomes progressing through metaphase I, anaphase I, metaphase II, and anaphase II is shown. The events controlled by Cdc5 are numbered. *CDC5* is required for securin degradation, cohesion removal, sister kinetochore co-orientation, and Cdc14 release in meiosis I. Cdc5 kinase activity is not required during meiosis II progression, but does control Cdc14 release. The removal of centromeric cohesion does not require Cdc5 kinase activity or phosphorylation. Speculative models for how centromeric cohesion removal is achieved are discussed in the text.

Cdc5 gains additional functions in meiosis I

In order to achieve the segregation of homologous chromosomes in meiosis I, several specializations are needed. These specializations: creation of physical linkages between homologous chromosomes, regulation of the linkages between sister chromatids, and attachment of sister kinetochores to microtubules from the same pole of the meiotic spindle, establish the meiosis I chromosome segregation pattern. The additional functions of *CDC5* in meiosis I establish the meiosis I chromosome segregation pattern. Cdc5 coordinates these meiosis I specializations by regulating the meiosis-specific proteins Rec8, Spo13, and Mam1. In a key departure from mitosis, securin degradation by the APC/C^{Cdc20} depends on *CDC5* in meiosis I (Clyne et al., 2003; Lee and Amon, 2003). In the following section I will discuss how *CDC5* has evolved to regulate securin degradation and coordinate meiosis I-specific events.

CDC5 *is required for securin degradation in meiosis I*

In the future, it will be interesting to determine how securin degradation becomes dependent on *CDC5* in meiosis I. I envision that differences in CDK activity between mitosis and meiosis could be a reason why *CDC5* takes on the additional role of controlling securin degradation in meiosis I. CDK activity is required for APC/C^{Cdc20} activation towards securin degradation in mitosis, as inactivation of either Clb1/2 or Clb2/3 results in securin stabilization (Rahal and Amon, 2008). Interestingly, Clb2 is not expressed in meiosis and Clb3 activity is restricted to meiosis II (Carlile and Amon, 2008; Grandin and Reed, 1993). Therefore, to compensate for lower bulk CDK activity in meiosis, Cdc5 may have evolved the ability to activate the APC/C^{Cdc20} in metaphase

I. In fact, polo kinases have been shown to activate the APC/C^{Cdc20} *in vitro* (Kotani et al., 1998). Interestingly, deletion of the APC/C activator *AMA1* partially alleviates the requirement for *CDC5* in securin degradation (Katis et al., 2010). It is possible that a mitotic regulator that accumulates in *ama1Δ* mutants activates the APC/C^{Cdc20}. Clb2 accumulates in *ama1Δ* mutants (Okaz et al., 2012), and it is tempting to speculate that increased CDK activity in this mutant reduces the need for Cdc5 in securin degradation.

Several experiments could address these questions to help us understand the interplay between CDK activity, polo kinase activity, and APC/C activation in meiosis. If increasing CDK activity promotes activation of the APC/C^{Cdc20}, then overexpressing *CLB2* in meiosis should bypass the requirement for *CDC5* in securin degradation. Furthermore, deletion of *CLB2* or its transcriptional regulator *NDD1* should suppress the ability of *ama1Δ* mutants to degrade securin in a *CDC5*-independent manner. Alternatively, it is possible that in *ama1Δ* mutants, the APC/C is biased towards association with Cdc20, and no longer requires activation by Cdc5.

Two other observations complicate the relationship between Cdc5 and securin degradation. First, overexpression of *CDC5* stabilizes securin. Preliminary experiments suggest that this securin stabilization does not occur due to activation of the spindle assembly checkpoint, but future experiments should re-examine this possibility. Second, *CDC5* is not required for securin degradation during meiosis II. It is possible that the activation of Clb3 kinase activity and a late wave of Ime2 kinase activity (Luke Berchowitz, personal communication) may render securin degradation independent of *CDC5* at this transition. Ime2 is a meiosis-specific Cdk-like kinase that fulfills some roles played by CDKs during mitosis, and Ime2 might therefore promote securin degradation

during meiosis II. Finally, understanding how *CDC5* controls securin degradation has important consequences for our ability to study meiotic events occurring after securin destruction. Understanding the role of Cdc5 in controlling Rec8 cleavage has been obscured by the failure of *CDC5-mn* cells to degrade securin. Understanding how to easily bypass this requirement could help us look more clearly at the role of *CDC5* in cohesion loss.

CDC5 *controls sister kinetochore co-orientation*

Cdc5 has gained other meiosis I-specific functions. *CDC5* is required for sister kinetochore co-orientation. To achieve sister kinetochore co-orientation, *CDC5* controls the meiosis I-specific factors Spo13 and Mam1. Cdc5 physically interacts with Spo13, a protein required for both the protection of centromeric cohesion and sister kinetochore co-orientation (Matos et al., 2008). Cdc5 also modifies Spo13, as Spo13 protein exhibits *CDC5*-dependent slower-migrating forms on SDS-PAGE. Interestingly, either depleting *CDC5* or overexpressing *CDC5* (*CUP-CDC5*) results in sister kinetochore biorientation. In *CDC5-mn* cells, Mam1 does not localize to kinetochores and Lrs4 phosphorylation, which is thought to reflect its activity, is reduced. In cells overexpressing *CDC5*, premature Spo13 degradation leads to a failure to co-orient sister chromatids. Therefore, understanding the complex interplay between Cdc5 activity, Spo13 stability, and monopolin component localization is an important question for the future. It will be interesting to see whether Lrs4 is released and phosphorylated in *CUP-CDC5* cells. It will also be interesting to understand how overexpressing *CDC5* affects the monopolin complex independently of Spo13 degradation. Initial experiments with a non-degradable

allele of *SPO13* have been uninformative, but generating a better allele in which Spo13 is not degraded in response to *CDC5* overexpression could be explored.

Finally, *CDC5* plays a critical role in controlling the removal of arm cohesion during meiosis I. I will discuss the role of *CDC5* in the control of stepwise loss of cohesion in depth in a later section of this discussion.

CDC5 is dispensable during meiosis II

One of the most surprising findings in this thesis is that *CDC5* is not required for progression through meiosis II. Thus, the additional roles gained by *CDC5* appear to be meiosis I-specific. If Cdc5 kinase activity is not required during meiosis II progression, then *CDC5* must also be dispensable for cohesion removal in meiosis II. Indeed, we observe that *CDC5*-dependent phosphorylation sites in Rec8 are not phosphorylated in meiosis II. Consistently, we observe that Rec8 phosphorylation is not required for its removal in meiosis II (Discussed below). Next, we observed that the Cdc5 targets Lrs4 and Clb1 were phosphorylated only in meiosis I. The functional significance of restricting *CDC5*-dependent phosphorylation to meiosis I is unclear. Although the Clb1 mobility shift correlates with Clb1 kinase activity, robust Clb1 kinase activity is observed when Clb1 phosphorylation is blocked (M. Miller, personal communication). Interestingly, the restriction of *CDC5*-dependent phosphorylation to meiosis I is not simply due to the inactivation of Cdc5 kinase activity because the kinase is still required for Cdc14 release in anaphase II. How is the requirement for Cdc5 activity modulated between meiosis I and meiosis II? I envision that CDK activity, phosphatase activity,

meiosis-specific substrate targeting, or modulation of Cdc5 kinase activity could play a role.

Cdc5 is a polo kinase, a family of protein kinases characterized by a C-terminal polo box domain. The polo box binds to phosphorylated residues and therefore targets the polo kinase to substrates that have "priming" phosphorylation, which is often carried out by CDK (Elia et al., 2003). Therefore, the presence of a different suite of Clb-CDKs in meiosis II that decline over the course of meiosis II could affect the ability of Cdc5 to be targeted to its substrates. It is also possible that phosphatases antagonize *CDC5*-dependent phosphorylation at the meiosis I-meiosis II transition and in meiosis II. PP2A is a good candidate, as it can counteract Rec8 phosphorylation in meiosis I (Kitajima et al., 2006; Riedel et al., 2006).

To understand the interplay between Cdc5 and CDKs, in both meiosis I and meiosis II, it will be important to develop physiologically relevant *in vitro* kinase assays. A good assay to assess total CDK activity in meiosis would be very useful. Because different cyclins exhibit different reactivities with the substrate commonly used in the assay, histone H1, overall CDK activity in meiosis has been difficult to determine. A new set of substrates has recently been developed, and it will be interesting to reinvestigate bulk CDK activity throughout meiosis (Koivomagi et al., 2011). A robust Cdc5 kinase assay is also lacking because Cdc5 *in vitro* activity reflects Cdc5 protein levels in budding yeast. We further know that these kinase assays are not physiologically relevant because Cdc5 substrates change in a cell cycle regulated manner. In mitosis, Cdc5 phosphorylates the cohesin subunit Scc1 in metaphase, but does not phosphorylate the MEN component Bfa1 until anaphase. It would be fascinating to know

the changes in phosphorylation of all Cdc5 targets between meiosis I and meiosis II. This might be possible using a labeled bulky ATP analog that can be utilized only by Cdc5-as1 protein, paired with quantitative mass spectrometry (Carlson and White, 2012). This experiment could address the question of whether Cdc5 phosphorylates vastly fewer substrates, or whether phosphorylation of its substrates simply is not essential for meiosis II progression. It is likely that in meiosis II, like in mitosis, the essential role of *CDC5* is Cdc14 release, but that it has several nonessential roles that help sharpen cell cycle progression.

Regulation of the stepwise loss of cohesion

CDC5 *regulates the stepwise loss of cohesion*

In meiosis I, homologous chromosomes are linked by cohesion between sister chromatids distal to chiasmata. When cohesion is removed from chromosome arms at the metaphase I-anaphase I transition, the connection between homologs is removed, and homologous chromosomes segregate. Importantly, cohesion is maintained at centromeric regions, which links sister chromatids until the second meiotic division. The removal of centromeric cohesion at the metaphase II-anaphase II transition triggers sister chromatid separation. The regulation of the Rec8 subunit of the meiotic cohesin complex is at the center of the mechanism governing the stepwise loss of cohesion. In budding yeast, the cleavage of Rec8 by separase triggers the loss of arm cohesion in meiosis I (Buonomo et al., 2000). Rec8 phosphorylation regulates its removal during meiosis I. Indeed, mutants containing at least 17 phosphorylation sites mutated to alanine exhibit a striking metaphase I delay and Rec8 cleavage delay (Brar et al., 2006;

Katis et al., 2010). Phosphorylated Rec8 is thought to be a better substrate for separase, and thus dephosphorylated Rec8 at centromeric regions is protected until meiosis II. The cohesion-protective protein Sgo1 recruits the phosphatase PP2A to centromeric regions during meiosis I to maintain Rec8 in a dephosphorylated state, and thus refractory to cleavage by separase (Kitajima et al., 2006; Riedel et al., 2006). The kinases that phosphorylate Rec8 to promote its cleavage in meiosis I are a subject of controversy, and this thesis demonstrates that Cdc5 is one of the kinases responsible for Rec8 phosphorylation and cleavage.

A previous study identified DDK and casein kinase as the critical kinases for Rec8 cleavage, and concluded that polo kinase had no role in cohesion removal (Katis et al., 2010), despite the observation that a majority of Rec8 phosphorylation sites identified by mass spectrometry were *CDC5*-dependent (Brar et al., 2006). Consistent with the observations made by Katis, *et al* (2010), casein kinase but not polo kinase appears to be critical for arm cohesion removal in fission yeast (Ishiguro et al., 2010). We also note that an allele of *REC8* containing all of the identified *CDC5*-dependent sites mutated to alanine does not exhibit a defect in Rec8 cleavage (Brar et al., 2006). Furthermore, Rec8 still exhibits a mobility shift in prophase arrested cells, a stage of meiosis in which *CDC5* is not expressed (Oelschlaegel et al., 2005; Okaz et al., 2012). However, data presented in Chapter 3 show that Rec8 cleavage is *CDC5*-dependent. We performed Rec8 cleavage assays and determined that Rec8 removal does depend on *CDC5* in budding yeast. Therefore, we conclude that several kinases, one of which is the polo kinase Cdc5, collaborate to phosphorylate Rec8 on chromosome arms in

meiosis I. Future experiments aimed at understanding the interplay and hierarchy between these kinases will be interesting.

We predicted that if *CDC5* promotes Rec8 phosphorylation and cohesion loss, then overexpressing *CDC5* might compete with the cohesion protective machinery to phosphorylate Rec8, ultimately leading to the premature loss of centromeric cohesion. This occurred, although many meiotic processes were disrupted in this mutant. The premature destruction of Spo13 in *CUP-CDC5* cells suggests that overexpressing *CDC5* leads to the premature loss of centromeric cohesion through *SPO13*. However, cells lacking *CDC5* and *SPO13* fail to cleave Rec8, suggesting that even when centromeric cohesin is de-protected due to the lack of *SPO13*, *CDC5* is still needed to bring about Rec8 cleavage. It will be interesting to understand in greater detail how overexpressing *CDC5* leads to the premature loss of cohesion. Determining the localization of cohesion protective factors Sgo1 and $PP2A^{Rts1}$ in *CUP-CDC5* cells may be informative. I would expect the localization of these factors to mirror the localization observed in a *spo13Δ* mutant. It will also be interesting to use a chromatin-IP approach to determine if *CDC5*-dependent Rec8 phosphorylation sites are phosphorylated at centromeric regions in *CUP-CDC5* cells.

Towards a model for the stepwise loss of cohesion in meiosis

The balance of Rec8 phosphorylation and dephosphorylation governs the stepwise loss of cohesion in our current model. Although the balance of Rec8 phosphorylation and dephosphorylation clearly controls the loss of cohesion during meiosis I, less is known about how cohesion is lost in meiosis II. It has been assumed

that Rec8 is cleaved by separase in meiosis II based on several results: securin re-accumulates in meiosis II, securin is degraded at the metaphase II-anaphase II transition, and a non-degradable version of securin expressed in meiosis II delays cells in metaphase II (Kiburz et al., 2005; Salah and Nasmyth, 2000). If the phosphorylation of Rec8 is required for cleavage by separase, then Rec8 should be phosphorylated at centromeric regions prior to its removal at the metaphase II-anaphase II transition. However, an initial observation made by Brar et al., 2006 suggested the intriguing possibility that Rec8 phosphorylation was not required for meiosis II cohesin removal. In cells lacking *SPO11*, recombination does not occur, and homologous chromosomes are not linked. Unlinked homologs segregate randomly during meiosis I without the need for securin destruction. All of the remaining cohesin is then lost simultaneously, suggesting that the bulk of cohesin removal occurs during metaphase II in *spo11Δ* cells. In *spo11Δ rec8-17A* mutants, Rec8 cleavage is restored, despite the substantial metaphase I delay observed in *rec8-17A* mutants alone. This result suggested that Rec8 phosphorylation may not be required for cohesion loss during meiosis II (Figure 2).

Data presented in Chapter 3 further substantiate this conclusion. Using a protocol to produce highly synchronous meiotic divisions, we looked directly at populations of cells in metaphase I and metaphase II. We found that metaphase I was delayed in cells with 29 Rec8 phosphorylation sites mutated to alanine (*rec8-29A*), but metaphase II progression was not delayed. Furthermore, in *spo11Δ rec8-29A* double mutants, metaphase II progression was not delayed, even though arm and centromeric cohesion needed to be removed at once. It is unclear why phosphorylation of Rec8 is required for its removal in meiosis I, but not in meiosis II. I envision that the regulation of

separase or differences between arm and centromeric cohesin contribute to the stepwise loss of cohesion.

In the simplest model, Rec8 phosphorylation is not needed for its removal in meiosis II because there is only a small amount of cohesin remaining at metaphase II. During meiosis I, phosphorylated Rec8 is preferentially cleaved by separase, leading to the segregation of homologous chromosomes before centromeric Rec8 can be cleaved. In meiosis II, separase is able to remove the small pool of centromeric cohesion remaining regardless of its Rec8-phosphorylation status. Several lines of evidence, described below, suggest that the regulation of the stepwise loss of cohesion is more complex.

One possibility is that separase activity is regulated. Precedence for this model exists in experiments from Xenopus extracts. Separase activation depends not only on Securin destruction, but also on de-phosphorylation of Separase (Stemmann et al., 2001). Perhaps in budding yeast meiosis, Separase requires levels of regulation beyond Securin degradation. If separase activity were low in meiosis I and high in meiosis II, this could account for differences in the requirement for Rec8 phosphorylation between the two divisions. In this model, separase with a low activity will only cleave phosphorylated Rec8 in meiosis I, but separase with a high activity can cleave any Rec8 protein in meiosis II.

Several lines of evidence suggest that the meiosis I-specific factors Spo13 and Sgo1 may regulate separase activation. First, overexpression of *SPO13* in mitosis induces a metaphase arrest, and cohesin is not cleaved (Lee et al., 2002; Lee et al., 2004). This result suggests that Spo13 can inhibit cohesin removal through a

mechanism that is independent of the identity of the α-kleisin subunit of cohesin (Scc1 in mitosis versus Rec8 in meiosis). Another way separase activity could be regulated is through an *SGO1*-dependent mechanism. *rec8-17A* cells lacking *SGO1* cleave cohesin readily and do not exhibit a prolonged metaphase I arrest, suggesting that Rec8 phosphorylation is no longer required for its removal in meiosis I (Brar et al., 2006). This surprising result implied that *SGO1* has roles in addition to preventing Rec8 phosphorylation at centromeric regions. It is possible, although highly speculative, that cells lacking *SGO1* have an increased ability to fully activate separase, rendering Rec8 phosphorylation less important for its removal in meiosis I. However, it is possible that Rec8 was phosphorylated by other kinases in the absence of *SGO1*. Consistent with this possibility, Katis et al. 2010 concluded that depletion of *SGO1* does not rescue Rec8 removal in a *rec8-24A* mutant. Although Rec8 persists in the nucleus, Rec8 cleavage assays were not performed in this study. *SGO1* has also been implicated in the regulation of separase in mitotic cells. In mitosis, overexpression of *SGO1* inhibits cohesin cleavage in a securin-independent manner and interferes with separase function in the FEAR network (Clift et al., 2009).

An interplay between Spo13, Sgo1, and separase could exist since *SPO13* is required for full localization of Sgo1 to centromeric regions (Kiburz et al., 2005). Several experiments could further address the interplay between Spo13, Sgo1, and separase. Determining the physical interactions between Spo13, Sgo1, and separase will be interesting. Finally, a separase biosensor was recently developed, allowing the determination of the kinetics of cohesin cleavage *in vivo*. The biosensor uses a similar principle to GFP dots (see Chapter 3), with Scc1 or Rec8 cleavage sites integrated

between the Tet repressor and GFP. Upon cleavage at the Scc1 or Rec8 sites, the GFP dot disappears, providing a read-out for cohesin cleavage, and thus separase activity, in single cells. It will be interesting to apply this approach to meiosis to determine differences in timing of cohesin cleavage in meiosis I, meiosis II, and in different genetic backgrounds (Yaakov et al., 2012).

However, since separase does cleave Rec8 on chromosome arms and acts within the FEAR network in meiosis I, it is possible that its activity is not regulated. Indeed, alternative possibilities exist to explain the differential requirement for Rec8 phosphorylation in meiosis I versus meiosis II. One model that could explain why Rec8 phosphorylation is needed for its removal in meiosis I, but not in meiosis II, is the existence of two pools of cohesin complexes. Two pools of cohesins could be generated by DSBs. Indeed, during mitosis, DSBs induce genome-wide sister chromatid cohesion (Unal et al., 2007). It will be interesting to determine whether this phenomenon also occurs when DSBs are produced during meiotic prophase, and whether cohesin that is recruited to chromatin during prophase requires phosphorylation for its removal. This model could explain why cohesion loss in *spo11Δ* cells, which do not undergo homologous recombination, no longer requires Rec8 phosphorylation. In meiosis, Rec8 links homologous recombination and sister chromatid cohesion, since many prophase I events depend on *REC8*. Rec8 plays a key role in recombination and prophase I progression (Brar et al., 2009; Klein et al., 1999), and may therefore interact differently with chromatin at chromosome arms and at centromeric regions. Double-strand break (DSB) formation and crossover formation are likely to alter the physical environment for cohesin complexes, perhaps requiring that Rec8 must be phosphorylated to be

efficiently cleaved. It is possible that these two pools of cohesin, arm and centromeric, require different modifications to allow cleavage. Furthermore, *CDC5* may link prophase I and sister chromatid cohesion; *CDC5* is required for both the resolution of chiasmata and the phosphorylation and cleavage of Rec8. In the future, it will be interesting to probe the relationship between recombination and sister chromatid cohesion further. Initial experiments could examine if Rec8 localization to chromosomes changes during prophase I and metaphase I.

Concluding remarks

Many of the same regulatory concepts are preserved between meiosis and mitosis. Delicate regulation of the APC/C, polo kinase, and cyclin dependent kinase activity is required to establish both division types. In this thesis, I have contributed to our understanding of how polo kinase controls the meiotic divisions. I have also showed that a cell cycle regulatory pathway essential for vegetative growth is dispensable for the meiotic divisions. Together, the data in this thesis demonstrate how cell cycle regulatory pathways are modulated to bring about different types of cell division. Much remains to be learned, especially in understanding how the levels of CDK activity change throughout meiosis, and how CDK activity impacts APC/C and polo kinase activity. It will be important to learn more molecular details about how polo kinase coordinates meiosis I and how the loss of cohesion is achieved, both in meiosis I and meiosis II. Since the cell cycle regulatory pathways discussed here are conserved in humans, it is my hope that ongoing research in the field of meiotic chromosome

segregation will yield important insights into the molecular basis of aneuploidy, miscarriage, and congenital birth defects.

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Appendix:

The Function of the Mitotic Exit Network (MEN) During Meiosis

INTRODUCTION

After chromosomes segregate in anaphase, cells must exit from mitosis. During this cell cycle transition, the spindle is disassembled, chromosomes decondense, and cytokinesis occurs. In order to achieve mitotic exit, mitotic CDK (Clb-CDK) activity, which is high during mitosis, is inactivated (Stegmeier and Amon, 2004). By abruptly inactivating Clb-CDKs, the cell quickly resets to a state of low CDK activity permissive for exit from mitosis. Mitotic exit is controlled by the essential phosphatase Cdc14, which promotes the degradation of the Clb cyclins. Among the targets of Cdc14 are a Clb-CDK inhibitor and an activator of an E3 ubiquitin ligase called the APC/C (anaphase promoting complex/cyclosome). Upon dephosphorylation by Cdc14, this APC/C activator, known as Cdh1, associates with the APC/C, thus triggering the degradation of cyclins (Visintin et al., 1998).

Cdc14 activity is tightly controlled. It remains bound to its inhibitor Cfi1/Net1 in the nucleolus for most of the cell cycle (Shou et al., 1999; Visintin et al., 1999). During anaphase two signaling pathways govern the release of Cdc14 from the nucleolus: the FEAR network and the mitotic exit network (MEN). The FEAR network is a nonessential pathway that induces a transient burst of Cdc14 release in early anaphase (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). The MEN is an essential GTPase signaling cascade required for sustained Cdc14 release and mitotic exit (Stegmeier and Amon, 2004). The small GTPase, Tem1, localizes to spindle pole bodies (SPBs) during metaphase, but is kept inactive by a two-component GAP, Bub2-Bfa1 (Bardin et al., 2000; Geymonat et al., 2002; Pereira et al., 2000). In response to spatial cues and anaphase onset, Tem1 is activated, and it is thought that Tem1-GTP

propagates a signal to the kinase Cdc15 (Asakawa et al., 2001). Cdc15 then recruits the Dbf2-Mob1 complex to SPBs (Visintin and Amon, 2001). Dbf2 is a member of the NDR-kinase family and requires the Mob1 subunit for activation. The MEN forms signaling modules at the SPB, and SPB localization is required for MEN activation in mitosis. The core MEN components are anchored at the SPB by Nud1, an SPB protein. Nud1 is required for MEN component localization and MEN activity (Adams and Kilmartin, 1999; Gruneberg et al., 2000; Valerio-Santiago and Monje-Casas, 2011; Visintin and Amon, 2001). Furthermore, Nud1 participates in MEN signaling. Phosphorylation of Nud1 by Cdc15 creates a novel binding site for the Dbf2-Mob1 kinase complex (J. Rock, personal communication).

How the final cell cycle transition is achieved in other cell division types is less clear. In meiosis, a specialized cell division, two consecutive rounds of chromosome segregation follow one round of DNA replication (Marston and Amon, 2004). As in mitosis, CDK activity drives progression through the meiotic divisions. However, Clb-CDK activity is regulated quite differently. First, the only Clb able to drive mitosis on its own, Clb2, is absent during meiosis (Fitch et al., 1992; Grandin and Reed, 1993). Second, Clb1-CDK is regulated at the level of kinase activity (Carlile and Amon, 2008). Third, Clb3 is translationally controlled, and Clb3-CDK activity is only detected in meiosis II (Carlile and Amon, 2008). Therefore, cyclin regulation is complex and relies on layers of regulation in addition to degradation.

It is thought that CDK activity is lowered to an intermediate level between the two divisions to ensure that the meiosis I spindle can be disassembled without re-licensing replication origins or completely exiting the meiotic program (Iwabuchi et al., 2000).

Cdc14 is released from the nucleolus in both anaphase I and anaphase II, and Cdc14 release is predominantly controlled by the FEAR network. In temperature-sensitive *CDC14* mutants and FEAR network mutants, cells fail to disassemble anaphase I spindles and form dyads (Buonomo et al., 2003; Marston et al., 2003). Furthermore, the meiosis I-meiosis II transition requires cyclin degradation. Cells overexpressing a non-degradable form of the cyclin Clb2 also fail to disassemble anaphase I spindles (Marston and Amon, 2004). Intriguingly, loss-of-function MEN mutants exhibit no delay in progression through the meiosis I-meiosis II transition and exhibit only a subtle delay in exit from meiosis II. Consistent with a role only in meiosis II, the MEN is inactive during meiosis I, but is activated specifically in anaphase II (Attner and Amon, 2012; Kamieniecki et al., 2005; Pablo-Hernando et al., 2007). Furthermore, the MEN does not assemble signaling modules at SPBs in meiosis, as cells containing a temperature-sensitive mutation in *NUD1* exhibit MEN activity that is indistinguishable from wild-type (Attner and Amon, 2012). Finally, the MEN in meiosis uses a different NDR-kinase, Dbf20. Dbf2 and Dbf20 are 87% similar. Why Dbf20 is upregulated in meiosis is unclear.

Together, these data suggest that the FEAR network rather than the MEN controls Cdc14 release in meiosis, possibly to ensure that only a transient burst of Cdc14 activity will occur at the meiosis I-meiosis II transition. Here we examine the function of MEN signaling in meiosis and examine the importance of keeping the MEN inactive at the meiosis I-meiosis II transition. We find that the MEN does not regulate cyclin degradation at exit from meiosis. Furthermore, we were unable to hyperactivate MEN signaling during meiosis I by several methods. Our data show that the cell

employs multiple layers of regulation to ensure that the MEN is inactive during the meiosis I-meiosis II transition.

RESULTS

The MEN is not required for cyclin degradation at exit from meiosis II

The MEN is a pathway absolutely essential for vegetative growth. Cells lacking MEN function arrest in late anaphase with high levels of Clb2 (Jaspersen et al., 1998; Visintin et al., 1998). Our previous characterization of MEN signaling during meiosis indicated that the MEN is less important for meiotic progression. Our analysis of loss-of-function MEN mutants demonstrated that the MEN has a subtle role in controlling anaphase II spindle disassembly and Cdc14 release (Attner and Amon, 2012). We wished to understand how the MEN controls exit from meiosis II, and therefore chose to determine if the MEN was required, even only subtly, for cyclin degradation in meiosis.

To produce synchronous cultures, cells were reversibly arrested in late prophase I. Briefly, the transcription factor Ndt80 is required for entry into the meiotic divisions. *NDT80* was placed under the control of the *GAL1-10* promoter and cells also contained a *GAL4*-estrogen receptor fusion. Cells were induced to sporulate and arrested at the *NDT80* block. Upon addition of β-estradiol to the medium, cells progress synchronously through the meiotic divisions (Carlile and Amon, 2008). To analyze a loss-of-function MEN mutant, we used an allele of the essential kinase *CDC15* (*cdc15-as1*) that can be specifically inhibited by addition of an ATP analogue, 1-NA-PP1, while leaving other cellular kinase activities intact (Bishop et al., 2000). Vegetative cells containing the *cdc15-as1* allele treated with inhibitor arrest in anaphase (see Chapter 2).

We chose to examine protein levels of the cyclin Clb3, the predominant cyclin remaining at the end of meiosis II (Carlile and Amon, 2008). In contrast, Clb1 kinase activity, Clb4 protein, and Clb5 protein are all greatly reduced by anaphase II (Carlile and Amon, 2008). In cells progressing synchronously through meiosis, we found that Clb3 was degraded when both wild-type and *cdc15-as1* cells disassembled their anaphase II spindles (Figure 1A-B). Clb3 was degraded with an approximately 15 minute delay in *cdc15-as1* cells, which correlates with the anaphase II spindle disassembly delay observed in these cells. We conclude that *CDC15* promotes the timely disassembly of anaphase II spindles and degradation of Clb3, but that *CDC15* is not required for Clb3 degradation.

Figure 1

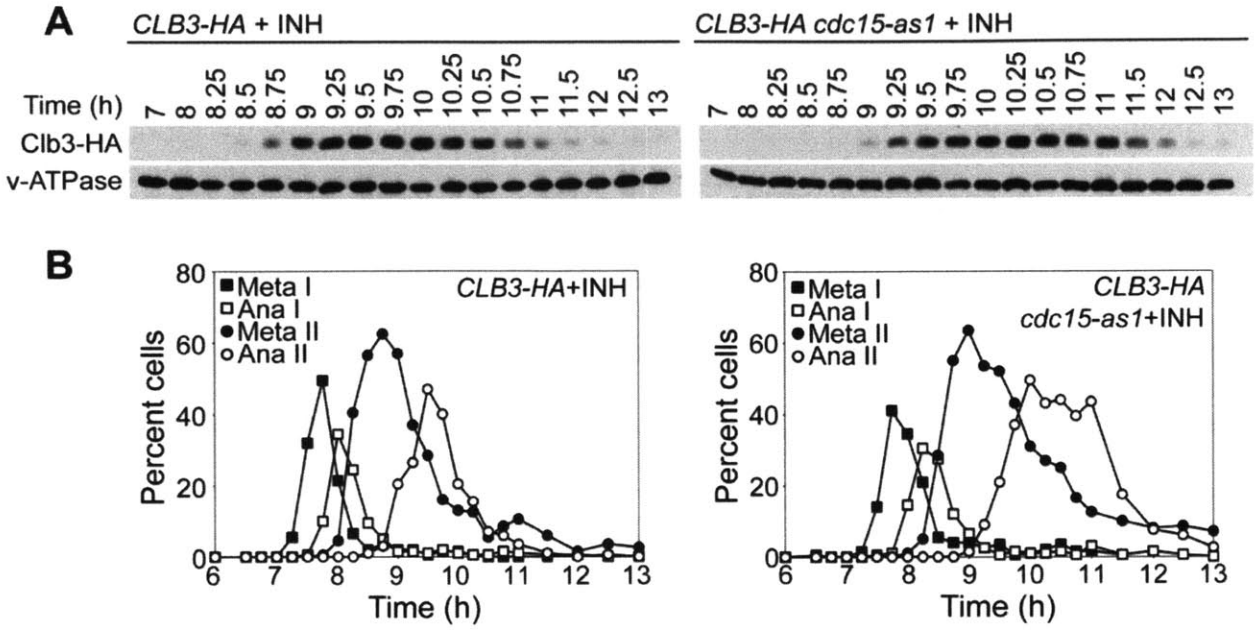

Figure 1. Clb3 degradation does not required Cdc15 kinase activity.

(A-B) Wild-type (A15802) or *cdc15-as1* (A22755) cells containing *CLB3-HA* and *GAL4-ER* fusions, and 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. (A) Samples were taken at the indicated times to determine Clb3-HA protein levels by Western blotting. V-ATPase is used as a loading control. (B) 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. At least 100 cells were analyzed at each time point.

Overexpressing the cyclin Clb2 does not enhance the requirement for the MEN in meiosis

During mitosis in budding yeast, the bulk of CDK activity is inactivated at exit from mitosis. In contrast, during meiosis it is thought that CDKs are partially inactivated at the meiosis I-meiosis II transition in order to exit the first meiotic division without exiting the meiotic program. Furthermore, most meiotic cyclins are inactivated prior to anaphase II (Carlile and Amon, 2008). It is possible that, at exit from meiosis I and II, FEAR network activity is sufficient to bring about the small amount of cyclin degradation necessary for exit from each meiotic division. To this end, we tested whether the amount of CDK activity in meiosis altered the requirement for MEN signaling in meiosis. We hypothesized that overexpressing Clb2, which is normally not present during meiosis, would enhance the requirement for the MEN in meiosis, possibly because more CDKs would need to be inactivated at the meiosis I-meiosis II transition. However, in wild-type and *cdc15-as1* mutants progressing synchronously through meiosis, Clb2 protein was degraded concomitantly with anaphase II spindle disassembly (Figure 2A). Interestingly, neither wild-type nor *cdc15-as1* cells exhibited defects in progression through the meiosis I-meiosis II transition (Figure 2B-E). This result suggests that the FEAR network can handle the burden of extra CDK activity at the meiosis I-meiosis II transition, as Clb2-CDKs are active when Clb2 is overexpressed in meiosis (M. Miller, personal communication). Taken together, we conclude that the MEN has a subtle, if any, role in triggering cyclin degradation at the end of meiosis.

Figure 2

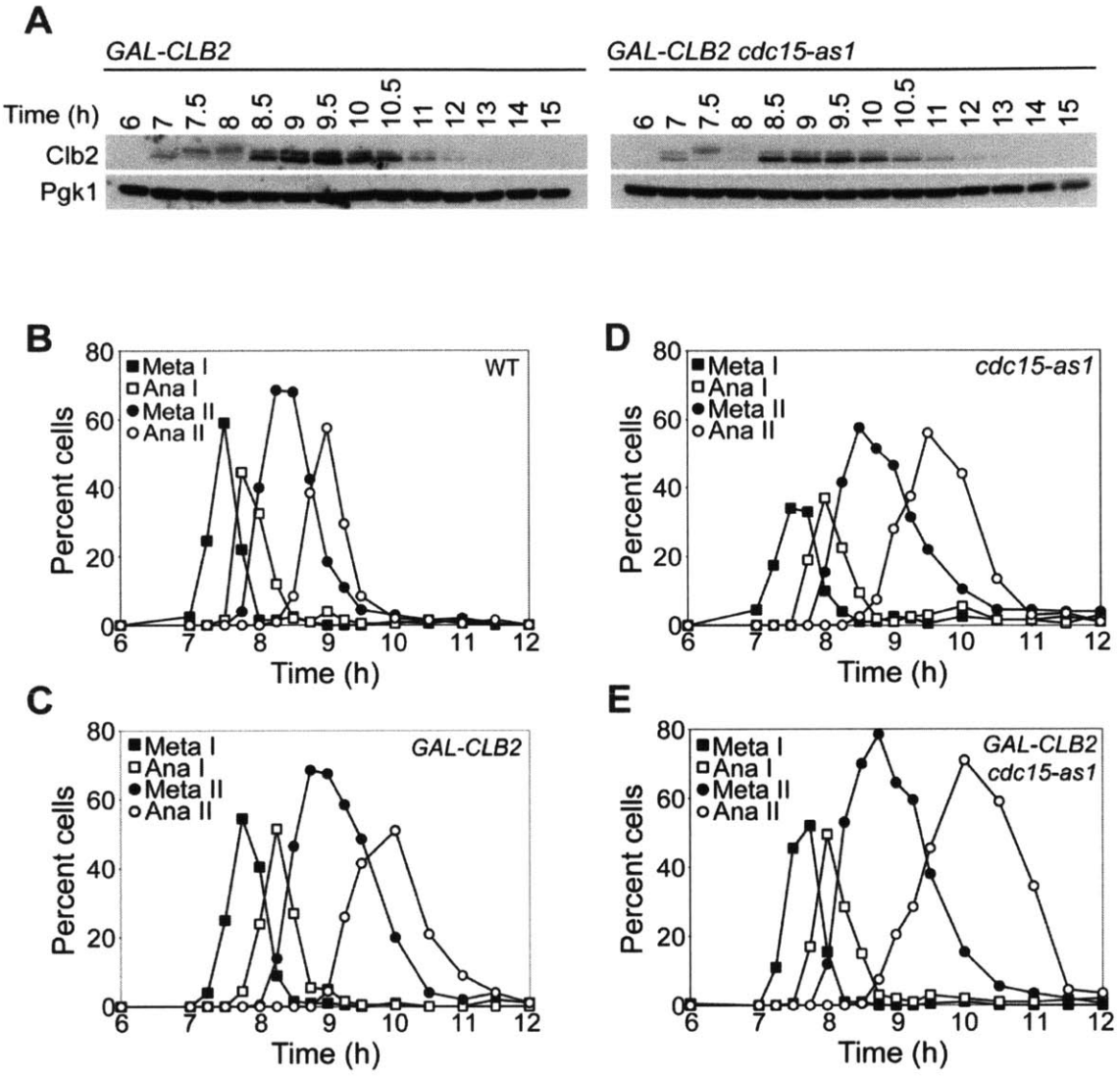

Figure 2. *CLB2* overexpression does not enhance the requirement for the MEN in meiosis I or meiosis II.

(A-E) Wild-type (A14201), *cdc15-as1 (A19440)*, *GAL-CLB2 (A20600)*, and *GAL-CLB2 cdc15-as1* (A24250) mutants containing *GAL-NDT80* and *GAL4-ER* fusions were sporulated as in Figure 1. Expression of *CLB2* and *NDT80* both occur upon β-estradiol addition to the medium. (A) For *GAL-CLB2* and *GAL-CLB2 cdc15-as1* cultures, samples

were taken at the indicated times to determine Clb2 protein levels by Western blotting. Pgk1 is a loading control. (B-E) The percentage of wild-type (B), *GAL-CLB2* (C), *cdc15-as1* (D), and *GAL-CLB2 cdc15-as1* (E) cells with metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), anaphase II (open circles) was determined at the indicated times. At least 100 cells were analyzed at each time point.

Deletion of *BUB2* does not hyperactivate MEN signaling in meiosis

Having established that the MEN is not required for exit from meiosis II, we wished to determine the function of MEN signaling during meiosis. Intriguingly, despite the observation that the MEN is not required for meiotic exit or cyclin degradation, we previously found that MEN activity during meiosis is tightly controlled (Attner and Amon, 2012). The MEN becomes active specifically during meioisis II, which raises the interesting possibility that it is important to keep the MEN inactive during meiosis I. We hypothesized that if the MEN were hyperactivated during meiosis I, all Clb-CDKs would be inactivated, leading to re-replication, cessation of meiosis after the first meiotic division, and premature initiation of spore formation. Alternatively, if activation of the MEN led to Cdc14 release prior to anaphase I, progression through meiosis I could be greatly hampered. Overexpression of *CDC14* in mitosis, and premature release of Cdc14 during meiosis I both have disastrous consequences for mitotic and meiotic progression, respectively (Bizzari and Marston, 2011; Visintin et al., 1998).

MEN activity in mitosis is assessed by *in vitro* Dbf2-associated kinase activity, and there are several established methods for hyperactivating Dbf2 kinase activity during mitosis. The Bub2-Bfa1 GAP complex keeps SPB-localized Tem1 inactive

(Geymonat et al., 2002). Upon anaphase onset, Bub2-Bfa1 is inactivated, thus promoting Tem1 activity and MEN signaling (Hu et al., 2001). Therefore, one way to hyperactivate Dbf2 kinase activity during mitosis is to delete *BUB2*. When *BUB2* is deleted, Tem1, presumably in its GTP-bound form, activates MEN signaling prematurely (Fesquet et al., 1999; Visintin and Amon, 2001). To assess the effect of deleting *BUB2* on MEN signaling in meiosis, we monitored Dbf20-associated kinase activity. Dbf20 is highly upregulated in the meiotic divisions, and is the predominant effector kinase in meiotic MEN signaling (Attner and Amon, 2012). We observed that the MEN is activated normally in anaphase II in *bub2Δ* cells progressing synchronously through meiosis (Figure 3A-D). Therefore, we conclude that deletion of *BUB2* does not hyperactivate Dbf20 kinase activity in meiosis I. The failure of *bub2Δ* mutants to activate MEN signaling could stem from the observation that MEN components do not localize to SPBs during meiosis.

Figure 3

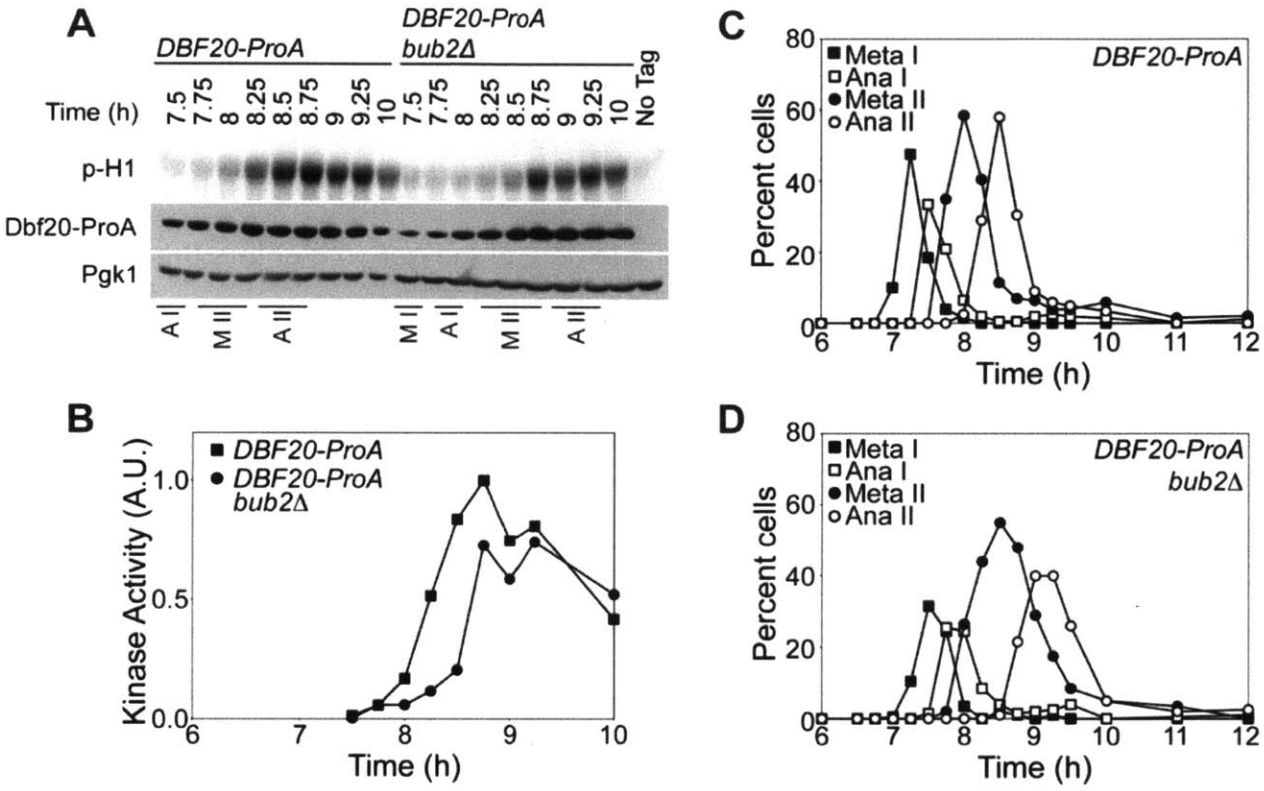

Figure 3. Deletion of *BUB2* does not activate MEN signaling in meiosis I.

(A) Cells containing *GAL-NDT80*, *GAL4-ER*, and *DBF20-ProA* fusions and *BUB2* (A23162) or *bub2Δ* (A23428) were sporulated as described in Figure 1, except that 1-NA-PP1 was not added. Samples were taken at the indicated timepoints to determine Dbf20- associated kinase activity (assessed using phosphorylation of the substrate H1; p-H1), and Dbf20 protein levels. 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 for *DBF20-ProA* (closed squares) and *DBF20-ProA bub2Δ* (closed circles). Kinase activity was normalized to the peak kinase activity observed. (C-D) The percentage of *DBF20-ProA* (C) and *DBF20-ProA bub2Δ* (D) cells in metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), anaphase II (open circles) was determined at the indicated times. At least 100 cells were analyzed at each time point.

Overexpression of a *CDC15* truncation in meiosis does not hyperactivate Dbf20 kinase.

During meiosis, the two divisions occur symmetrically within the confines of the mother cell. In contrast, mitosis is an asymmetric division. Mitotic exit is tightly coupled with spindle position to ensure that one complement of the genome enters the bud (Stegmeier and Amon, 2004). Our analysis of MEN signaling in meiosis led to the conclusion that the MEN is controlled in a very different manner during meiosis. A role for Tem1 and Tem1-regulatory factors have not been identified, and MEN components do not localize to SPBs in either meiosis I or meiosis II (Attner and Amon, 2012). We therefore chose to analyze the effects of MEN genes downstream of *TEM1*.

We first examined the consequences of expressing a hyperactive allele of *CDC15* on Dbf20 kinase activity. It was previously shown that an allele of *CDC15* lacking the C-terminal 224 amino acids and tagged with GFP (henceforth *CDC15[1-750]*) hyperactivates MEN signaling in mitosis. The *CDC15[1-750]* mutant massively hyperactivates Dbf2 kinase activity and drives release of Cdc14 from the nucleolus in metaphase (Bardin et al., 2003). Before testing if *CDC15[1-750]* could hyperactivate MEN signaling in meiosis, we tested if this allele would hyperactive Dbf2 kinase activity in the SK1 strain background in mitosis. We expressed the truncation from the copper inducible promoter, a promoter suitable for overexpression in meiosis. To analyze cells progressing through the mitotic cell cycle in a synchronous manner, we arrested wild-type and *CDC15[1-750]* cells in G1 with pheromone. Upon release from the G1 arrest, cells progressed synchronously through mitosis. As expected, Dbf2 kinase activity was

approximately eight-fold higher and prematurely activated in cells overexpressing *CDC15[1-750]* (Figure 4A-D).

Figure 4

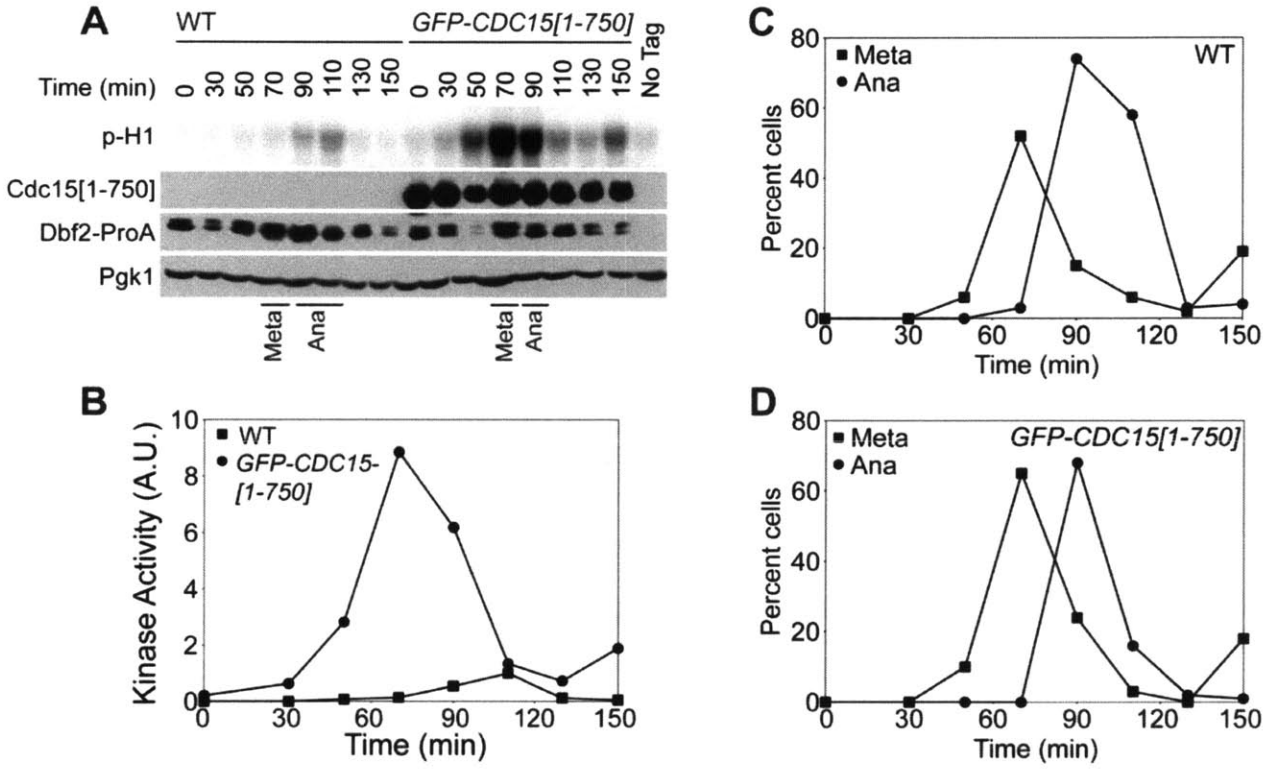

Figure 4. Overexpressing a *CDC15* truncation is sufficient to hyperactivate MEN signaling in mitosis.

(A-D) Cells containing a *DBF2-ProA* fusion and *CDC15* (A24347) or *pCUP1-GFP-CDC15[1-750]* (A25757) 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 $CuSO_4$. (A) Dbf2-associated kinase activity, GFP-Cdc15[1-750] protein, Dbf2-ProA protein, and a Pgk1 loading control were analyzed at the indicated time points. The peaks of metaphase and anaphase are indicated below the

blot. (B) Quantification of Dbf2-associated kinase activity for *DBF2-ProA* (closed squares) and *DBF2-ProA GFP-CDC15[1-750]* (closed circles). Kinase activity was normalized to the peak kinase activity observed in the *DBF2-ProA* strain.
(C-D) The percentage of wild-type (C) and *GFP-CDC15[1-750]* (D) cells in metaphase (closed squares) and anaphase (closed circles) was determined at the indicated times. At least 100 cells were analyzed at each time point.

Having established the *CDC15[1-750]* allele in the SK1 strain background, we asked whether MEN activity in meiosis was hyperactive by assessing Dbf20-associated kinase activity in cells undergoing a synchronous meiosis. In meiosis II, Dbf20-associated kinase activity was elevated approximately two-fold in *CDC15[1-750]* cells, a modest difference compared with the effects of *CDC15[1-750]* on Dbf2-associated kinase activity in mitosis. For reasons that are unclear, Cdc15[1-750] protein levels are low in meiosis II. However, overexpression of the *CDC15* truncation did not activate Dbf20 kinase activity in meiosis I, despite ample amounts of Cdc15[1-750] produced (Figure 5A-D). We investigated the possibility that *CDC15[1-750]* could hyperactivate Dbf2 kinase but not Dbf20 kinase activity. Despite overexpression of both *CDC15[1-750]* and *DBF2* in meiosis, cells still formed tetrads, and were not analyzed further (data not shown). We conclude that additional mechanisms must exist in meiosis to restrict MEN signaling to meiosis II.

Figure 5

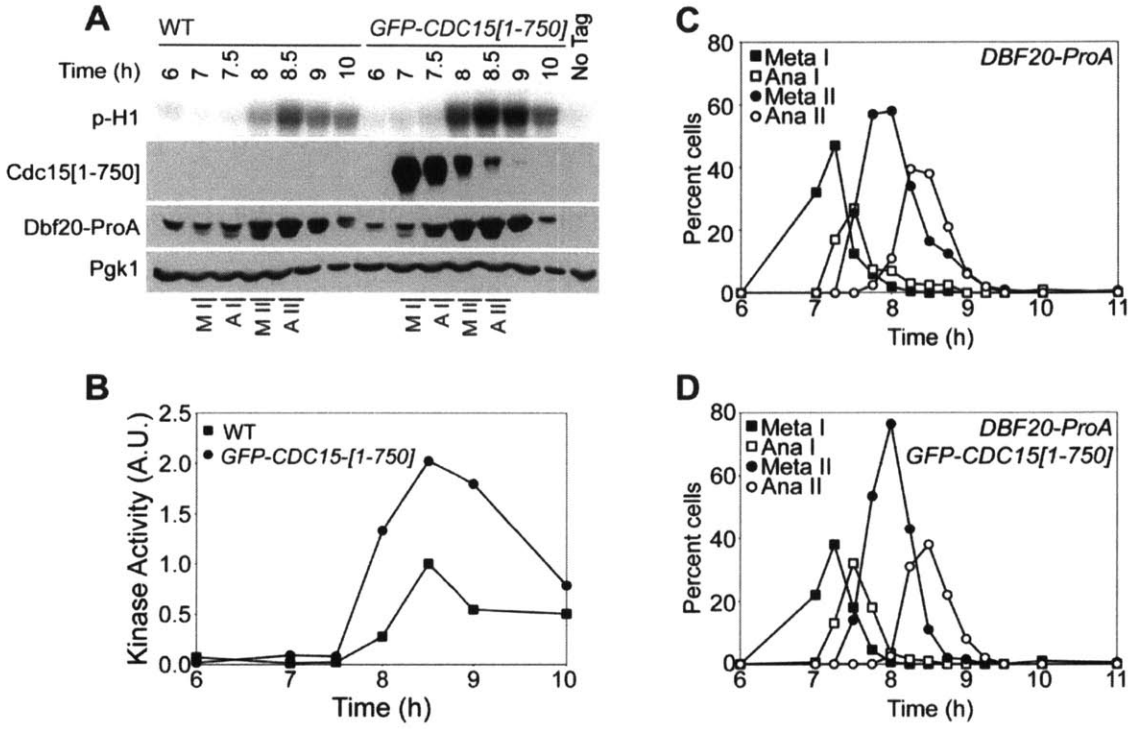

Figure 5. Overexpressing a *CDC15* truncation does not activate Dbf20 kinase in meiosis I.

(A-D) Cells containing *GAL-NDT80*, *GAL4-ER*, and *DBF20-ProA* fusions and *CDC15* (A23162) or *pCUP1-GFP-CDC15[1-750]* (A25817) were induced to sporulate. β-estradiol, 1 μM, and CuSO4, 50 μM, were added to cultures 6 h after transfer into sporulation medium. (A) Samples were taken at the indicated timepoints to determine Dbf20-associated kinase activity (assessed using phosphorylation of the substrate H1; p-H1), and Dbf20 protein levels. 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 for *DBF20-ProA* (closed squares) and *DBF20-ProA GFP-CDC15[1-750]* (closed circles). Kinase activity was normalized to the peak kinase activity observed in the *DBF20-ProA* strain. (C-D) The percentage of *DBF20-ProA* (C) and *DBF20-ProA GFP-CDC15[1-750]* (D) cells in metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), was determined at the indicated times. At least 100 cells were analyzed at each time point.

Tethering Tem1 to the SPB does not promote MEN signaling in meiosis

During mitosis, it is essential for MEN components to localize to SPBs, where they are anchored by Nud1. Furthermore, cells expressing a Tem1-SPB fusion recruit Cdc15 to SPBs prematurely (Valerio-Santiago and Monje-Casas, 2011). During meiosis, in contrast, MEN components are not seen on SPBs, and it appears that meiosis has evolved a different mechanism to control MEN signaling (Attner and Amon, 2012). We wondered if we could hyperactivate MEN signaling by forcing MEN components to assemble signaling modules at the SPB. We again reasoned that if active MEN signaling modules were assembled in meiosis I, cells would fail to traverse the meiosis I-meiosis II transition. To test this hypothesis, *TEM1* was fused to the SPB component *CNM67* and eGFP. Since *TEM1* is expressed at relatively low levels in meiosis, we drove expression of the *TEM1-SPB* fusion from the *CLB1* promoter. Tem1-SPB cells exhibited no defects in meiotic kinetics, and Dbf20 kinase was activated only in anaphase II in these cells (Figure 6A-D). We addressed the possibility that the Tem1-SPB fusion was not properly localized to SPBs, but we saw Tem1-SPB localized at foci (most likely at the SPB) at all stages of meiosis examined (Figure 6E-F). It is unknown if Cdc15 was recruited to SPBs in Tem1-SPB cells. However, we conclude that fusing Tem1 to the SPB does not result in hyperactivation of Dbf20 kinase activity during meiosis. Taken together, our data support the conclusion that the MEN is wired in a fundamentally different way in meiosis, preventing us from hyperactivating MEN signaling during meiosis I.

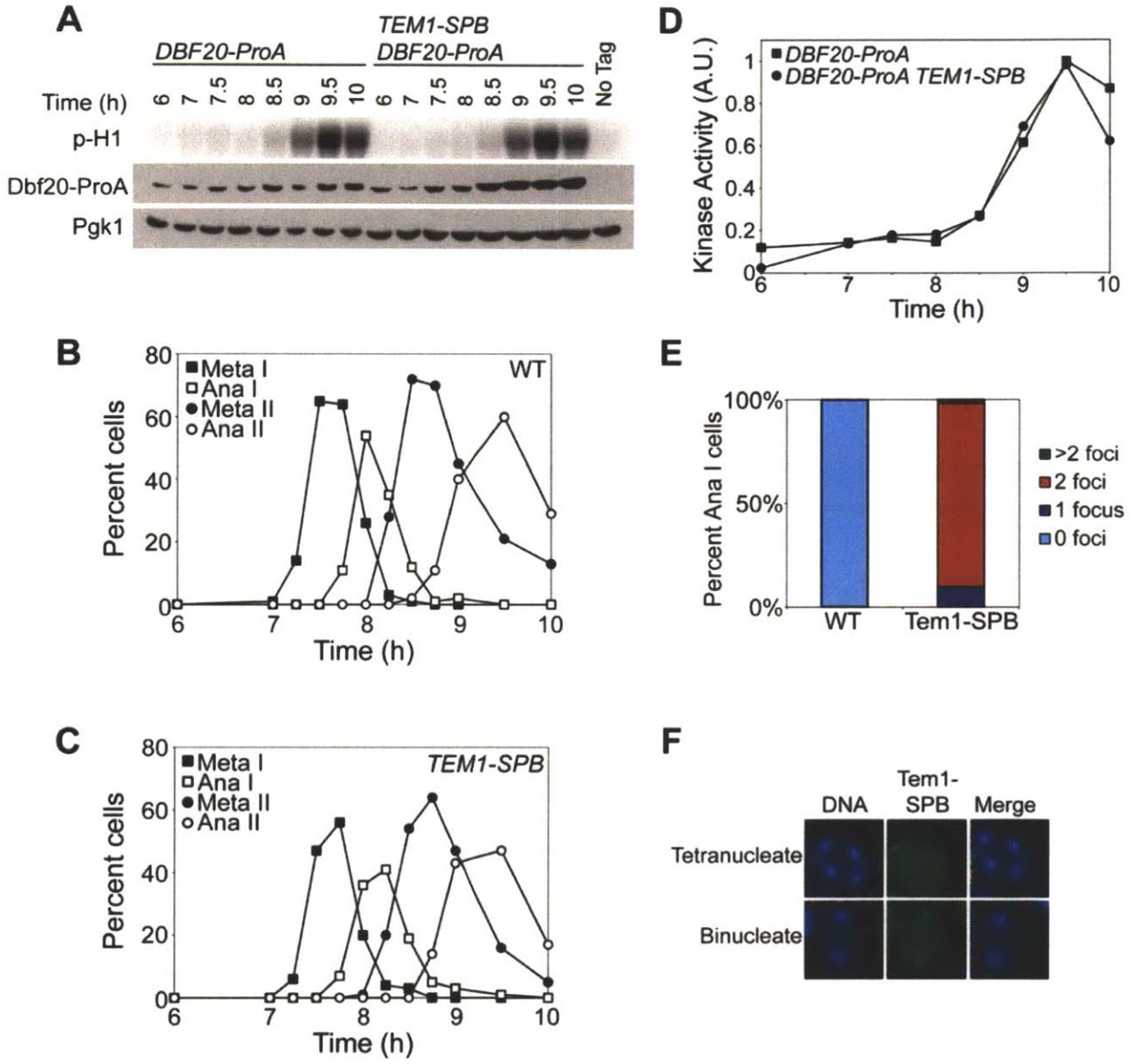

Figure 6. Fusing Tem1 to the spindle pole body does not activate Dbf20-kinase in meiosis I.

(A-D) Wild-type (A23162) or *pCLB1-eGFP-TEM1-CNM67* (A27983; *TEM1-SPB*) cells containing *GAL-NDT80*, *GAL4-ER*, and *DBF20-ProA* fusions were sporulated as described in Figure 3. Samples were taken at the indicated timepoints to determine Dbf20- associated kinase activity (assessed using phosphorylation of the substrate H1; p-H1), and Dbf20 protein levels. Pgk1 was used as a loading control. (B-C) The

percentage of wild-type (C) and *TEM1-SPB* (D) cells in metaphase I (closed squares), anaphase I (open squares), metaphase II (closed circles), anaphase II (open circles) was determined at the indicated times. At least 100 cells were analyzed at each time point. (D) Quantification of Dbf20-associated kinase activity for *DBF20-ProA* (closed squares) and *DBF20-ProA TEM1-SPB* (closed circles). Kinase activity was normalized to the peak kinase activity observed. (E) Quantification of Tem1-SPB localization. Tem1-SPB localization in binucleates was counted at the 8 hour timepoint, when binucleates were in anaphase I. A Tem1-SPB focus within a nucleus was scored. Cells with 0 foci are shown in light blue, one focus in dark blue, two foci (each in a different nucleus) in red, and greater than 2 foci in green. At least 50 cells were counted for each strain. (F) Examples of Tem1-SPB localization in a tetranucleate cell (top) and a binucleate cell (bottom). DNA is shown blue and Tem1-SPB in green.

DISCUSSION

In this appendix, we have shown that the MEN is not required for cyclin degradation in meiosis. We previously observed that MEN activity, as assayed by Dbf20 kinase activity, was restricted to meiosis II, and reasoned that it is important to keep the MEN inactive in meiosis I. Therefore, we searched for insights into the function of the MEN by investigating the importance of keeping the MEN inactive during meiosis I. Deleting a negative regulator of the MEN, hyperactivating a positive regulator of the MEN, and forcing a MEN component to localize to the SPB all failed to hyperactive Dbf20 kinase activity in meiosis I. We conclude that the MEN is regulated in a fundamentally different way in meiosis, precluding our ability to investigate the importance of keeping the MEN inactive during meiosis I.

The function of the MEN in meiosis II

We show here and in Chapter 2 that the MEN has a subtle role in controlling exit from meiosis II, in contrast to its essential role in mitosis. One reason for this may be that there are simply fewer CDKs that need to be inactivated, and the MEN is not needed to trigger their destruction. The situation at exit from meiosis II may more closely resemble mitosis in higher eukaryotes, in which most CDK inactivation is triggered at the metaphase-anaphase transition (Peters, 2002). Instead, it appears that the critical role for the MEN, or at least some MEN components, is in spore wall morphogenesis (Kamieniecki et al., 2005; Pablo-Hernando et al., 2007). In this sense, the MEN in meiosis may function more like its homologous pathway in fission yeast, the septation initiation network (SIN). The SIN is required for cytokinesis in vegetative cells and spore wall formation in meiosis (Krapp et al., 2006; Krapp et al., 2004).

The MEN cannot be hyperactivated in meiosis I

We have been unable to hyperactivate MEN signaling in meiosis I in order to better understand the importance of keeping the MEN inactive in meiosis I. We speculate that it is important for the MEN to be kept inactive in meiosis I in order to prevent the cessation of meiosis after the first meiotic division. One method for hyperactivation of MEN signaling still remains to be tested thoroughly. Fusing *CDC15* to a component of the SPB (*CDC15-SPB*) results in dramatic hyperactivation of Dbf2 kinase activity in mitosis (Rock and Amon, 2011). We have been unable to test this construct in meiosis because cells carrying the *CDC15-SPB* allele do not enter gametogenesis efficiently (unpublished observations). It will be interesting to optimize

the expression levels of this construct in order to monitor the effect of the Cdc15-SPB fusion on progression through the meiotic divisions.

It is possible that we were unable to hyperactivate MEN signaling in meiosis because the MEN is wired in a fundamentally different way. In meiosis, it is thought that one critical point of MEN regulation is at the level of the Dbf20-Mob1 interaction. The physical interaction between Dbf20 and Mob1 is cell cycle regulated in meiosis and depends on Cdc15 kinase activity (Attner and Amon, 2012). Furthermore, it was previously discovered that mobility shifts on SDS-PAGE change throughout meiosis for Dbf20 and Mob1 (Attner and Amon, 2012). It is possible that a meiosis-specific gene expressed in meiosis II is required for Dbf20-Mob1 activation. In a non-mutually-exclusive possibility, Cdc15 kinase could be activated at the meiosis I-meiosis II transition through an unknown mechanism. Both possibilities could preclude our ability to hyperactivate MEN signaling in meiosis I. Given the importance of the Dbf20-Mob1 interaction in meiosis, it will be interesting to determine the factors that restrict Dbf20-Mob1 binding to meiosis II and to examine the consequences of expressing a Dbf20-Mob1 fusion.

Another reason that we were unable to hyperactivate MEN signaling in meiosis could be due to differences between the MEN effector kinases Dbf2 and Dbf20. Although Dbf20 seems to replace Dbf2 in meiosis, the two proteins are not functionally equivalent. When *DBF2* and *DBF20* are expressed from the same promoter in mitosis, Dbf2 kinase activity peaks in anaphase, but Dbf20 kinase activity remains low throughout the cell cycle (Attner and Amon, 2012). When *DBF2* is expressed from an inducible promoter in meiosis, the protein was active as a kinase in meiosis II. In the

future, it will be important to overexpress *DBF2* in meiosis, and test whether we can hyperactivate Dbf2 kinase activity in meiosis.

Taken together, our data raise the interesting possibility that is important to keep the MEN inactive during meiosis I. The consequences of MEN signaling in meiosis I, however, remain unclear. It will be interesting to determine whether hyperactivating the MEN in meiosis I would lead to premature spore wall formation or exit between the meiotic divisions. Finally, understanding in greater detail how the MEN signals in different cell division types could provide insights into how NDR-kinase signaling pathways function in different organisms and tissues.

Methods and Materials

Yeast strains

All yeast strains are derivatives of SK1 and are listed in Table 1. *CLB3-3HA*, *GAL-CLB2*, *DBF20-ProA*, *DBF2-ProA*, *bub2*Δ, and *pCUP1-GFP-CDC15[1-750]* were constructed using a PCR-based method (Longtine et al., 1998). The *eGFP-TEM1-CNM67* construct was a gift from Fernando Monje-Casas. The *CLB1* was promoter was cloned into the vector, and the construct was integrated at the *URA3* locus.

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. 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). For eGFP-Tem1-SPB imaging, cells were fixed for 5-15 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).

Immunoblot analysis

Immunoblot analysis was performed as previously described (Attner and Amon, 2012). Conditions for Dbf2-ProA and Dbf20-ProA detection are previously described. Clb3-HA was detected with anti-HA (HA.11; Covance) antibodies at a dilution of 1:1000. Clb2 protein was detecting using anti-Clb2 antibodies at a dilution of 1:2000. The Clb2 antibody was a gift from Fred Cross. eGFP-Cdc15[1-750] was detected using anti-GFP antibodies (JL8 epitope; Clontech) at a dilution of 1:1000. Pgk1 was detected with an anti-Pgk1 antibody (Invitrogen, Carlsbad, CA) using a 1:5000 dilution.

Dbf2 and Dbf20 kinase assays

Kinase assays were performed as previously described (Attner and Amon, 2012). Quantification was performed using ImageQuant software.

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Table 1. All strains are in the SK1 background.

Strain	Genotype
A14201	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3*
A15802	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3, CLB3/CLB3-3HA:KANMX6*
A19440	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3, cdc15-as1:URA3/cdc15-as1:URA3*
A20600	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3, CLB2/clb2::GAL-CLB2:TRP1 (2 copies)*
A22755	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3, CLB3/CLB3-3HA:KANMX6, cdc15-as1:URA3/cdc15-as1:URA3*
A23162	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3, DBF20-TEV-ProA-7HIS:HIS3MX6/DBF20-TEV-ProA-7HIS:HIS3MX6*
A23428	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3, DBF20-TEV-ProA-7HIS:HIS3MX6/DBF20-TEV-ProA-7HIS:HIS3MX6, bub2Δ::URA3/bub2Δ::URA3*
A24250	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3, CLB2/clb2::GAL-CLB2:TRP1 (2 copies), cdc15-as1:URA3/cdc15-as1:URA3*
A24347	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG, DBF2-TEV-ProA-7HIS:HIS3MX6*
A25757	*MATa, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG, DBF2-TEV-ProA-7HIS:HIS3MX6, leu2::CDC15:LEU2, KANMX6:pCUP1-GFP-CDC15[1-750]:HIS3*
A25817	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3, DBF20-TEV-ProA-7HIS:HIS3MX6/DBF20-TEV-ProA-7HIS:HIS3MX6, leu2::CDC15:LEU2/leu2::CDC15:LEU2, KANMX6:pCUP1-GFP-CDC15[1-750]:HIS3/KANMX6:pCUP1-GFP-CDC15[1-750]*
A27983	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, leu2::hisG/leu2::hisG, his3::hisG/his3::hisG, trp1::hisG/trp1::hisG, GAL-NDT80:TRP1/GAL-NDT80:TRP1, ura3::pCLB1-eGFP-CNM67-TEM1/ura3::pGPD1-GAL4(848).ER::URA3, DBF20-TEV-ProA-7HIS:HIS3MX6/DBF20-TEV-ProA-7HIS:HIS3MX6*