The regulatory network controlling the transition from prophase I into metaphase I

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Dedicated to my mother

Carmen Francisca Miranda-Miranda

and my grandmother

Aida Miranda-Miranda,

whose passion for life is infinite.

Este trabajo esta dedicado a mi Madre

Carmen Francisca Miranda Miranda

y a mi abuela

Aida Miranda Miranda,

porque su alegria de vivir es infinita.

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Abstract

Living systems are frequently confronted with decisions between mutually exclusive states. For instance, a critical decision for germ cells is whether to initiate the meiotic program or to proliferate through mitotic divisions. However, the biochemical mechanisms that orchestrate these decisions are rarely understood in detail. Here, we have studied how the budding yeast *Saccharomyces cerevisiae* makes decisions during the transition from prophase into metaphase of meiosis I. In late prophase I, *S. cerevisiae* cells are presented with three options: (1) to stay in prophase I, (2) to enter metaphase I, or (3) to return to mitosis. During prophase I, M phase-promoting Cdk1 activity is low and programmed double-strand breaks (DSB) initiate homologous recombination. As long as DSB are present, the meiotic recombination checkpoint (MeiRC) blocks entry into M phase by repressing the transcription factor Ndt80. Once DSB are repaired, Ndt80 is activated, M-phase Cdk1 activity rises, and metaphase I is established. Our analysis of the main regulators of this transition led to the following conclusions: (1) Proteolysis mediated by a meiosis-specific ubiquitin-ligase, called APC/C-Ama1, is essential for suppressing mitotic cell cycle controls and for maintaining the low-kinase state characteristic of prophase I. (2) Cdc5, the yeast polo-like kinase, is a strong inhibitor of the MeiRC. (3) Once recombination is completed, Ndt80 generates inhibitors of APC/C-Ama1, such as Cdk1 bound to the Clb1 cyclin, and inactivates the MeiRC by producing Cdc5. Mathematical modeling of this regulatory network revealed bi-stability as an emergent property of the system. We confirmed experimentally that the decision to abandon the low-kinase state of prophase I and to enter the high-kinase state of metaphase I is controlled by a bi-stable switch, which explains the irreversibility of this transition. The control of APC/C-Ama1 activity is a crucial element of the system, which is also relevant for the exit from meiosis and for the return to mitotic proliferation. We concluded that a bi-stable control system determines whether to stay in prophase I, with the option of return to mitosis, or to continue into metaphase I to complete meiosis. The bi-stable switch might be a ubiquitous mechanism for cellular decision-making.

1. Introduction

One essential property of all living systems is their capacity for decision-making. Life is always faced with decisions between mutually exclusive states. Cells divide or remain quiescent, but never attempt to do both simultaneously. During our own development, stem cells give rise to very specific lineages, such as neurons or lymphocytes. The decision over the cell fate is so efficient that intermediates or mixed cells, e.g. a "neuro-lymphocyte", are never observed. When our bodies produce a limb, this decision also includes preventing that other organs, such as a heart or an eye, are created instead. Regardless of whether it is choosing which organ, which cell type, or which program of cell division to produce, all living systems are confronted with the problem of how to make decisions between mutually exclusive states.

At the cellular level, one of the most important decisions is whether to undergo mitosis or meiosis. The choice between these two types of cell division is crucial for all sexually reproducing organisms on this planet. From the unicellular yeast *Saccharomyces cerevisiae* to humans, the decision to undergo meiosis is responsible for the production of sex cells, i.e. gametes. Failure to properly carry out the meiotic program can cause sterility (Judis et al., 2004). In humans, defects in the correct execution of meiosis can lead to conditions such as Down syndrome (Sherman et al., 2006). In other cases a faulty meiosis is lethal; a significant number of miscarriages in humans are the result of unhealthy gametes (Hassold and Hunt, 2001; Hunt, 2006). For many plants and unicellular organisms, errors during meiosis also represent death (Klug and Cumings, 2003). Sexual reproduction is, nevertheless, one of the most efficient strategies to perpetuate life, generation after generation. Its success relays on a single decision: at a critical point in the life cycle, the right cells must choose meiosis over mitosis.

1.1. Meiosis or mitosis?

At first glance, mitosis and meiosis share the common purpose of segregating DNA after it has been replicated. However, the final products of these two types of cell division are very different. During mitosis, a cell grows, synthesizes a new copy of

its DNA, and then divides, giving rise to two cells with the same genetic material. These events are repeated endlessly in what is called the cell cycle (**Figure 1**). In higher eukaryotes, most cells are created through mitosis whereas meiosis occurs only in a special type of diploid cell, called germ cells (Bowles and Koopman, 2010). This process is a developmentally regulated program of cell division that leads to the production of specialized sex cells. During meiosis, one round of DNA replication is followed by two rounds of chromosome segregation (**Figure 2**). This, however, does not mean that meiosis is simply doing mitosis two times. Several general features set meiosis apart from mitosis:

(1) Whereas DNA replication and cell division strictly alternate in mitosis, during meiosis, a single round of DNA replication is followed by two rounds of DNA segregation. Furthermore, meiosis usually culminates in a differentiation program that creates specialized cells, spores in yeast and other fungi, or sperm and egg in animals.

(2) After DNA replication is completed, mitotic cells soon segregate their DNA. By contrast, meiotic cells enter a long stage, called prophase I, in which meiotic recombination occurs (Padmore et al., 1991). Recombination allows the exchange of genetic information between paternal and maternal homologous chromosomes. More importantly, it creates the physical link between homologous chromosomes that is essential for their accurate segregation.

(3) The main principle for the segregation of chromosomes during mitosis is to link them during DNA replication, so that they can be put under tension on the mitotic spindle and then segregated to opposite poles (Dewar et al., 2004). This strategy is insufficient for two rounds of chromosome segregation. In meiosis, there are two ways to link chromosomes: during meiosis I, homologous chromosomes are physically connected, usually as a result of recombination. With the help of meiosis I specific proteins, homologues can be put under tension and segregated. Meiosis II takes

advantage of a persistent link between sister chromatids to put them under tension and finally separate them.

(4) Mitosis generates two cells with an equal amount of identical genetic information. By contrast, meiosis can generate four cells that contain half of the genetic information. Due to recombination, these haploid cells can present new genetic combinations. The generation of genetic diversity is a main feature of meiosis, but it is avoided in mitosis.

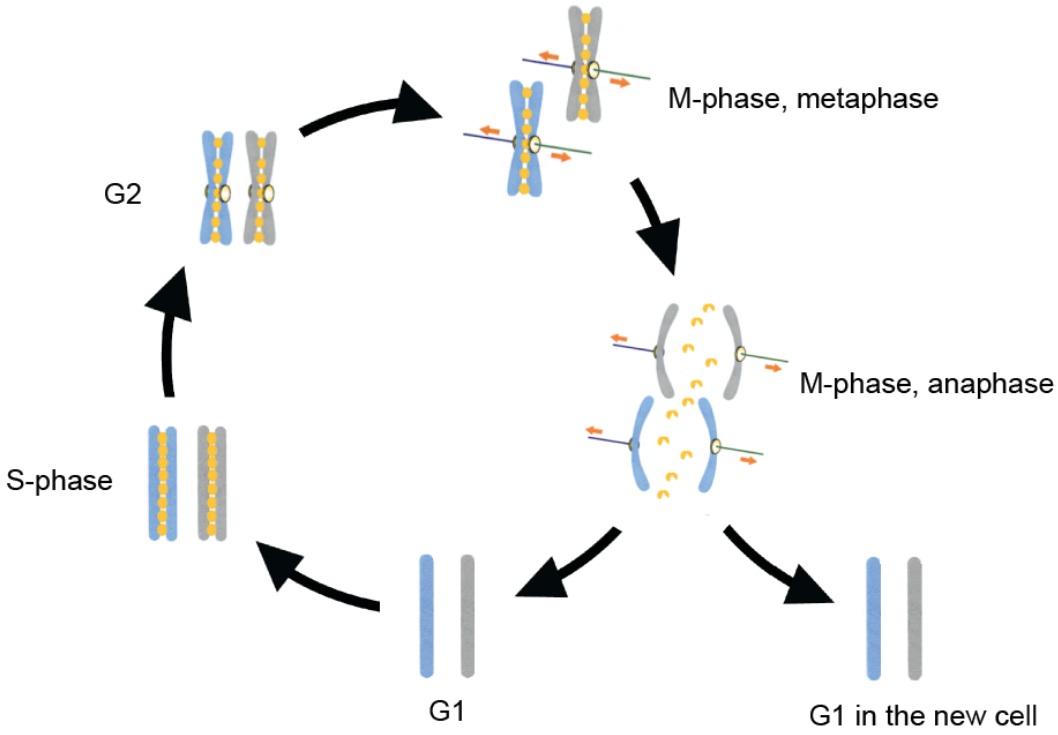

Figure 1. **The mitotic cell cycle in *S. cerevisisae*.** Proliferating cells exit from G1 into S-phase to duplicate their genomic DNA. After replication, each chromosome is composed of two sister chromatids held together by the mitotic cohesin complex (yellow balls). After the G2 stage, cells enter M-phase. During metaphase, chromosomes are put under tension in the mitotic spindle. Microtubules emanating from opposite poles of the cell attach to kinetochores assembled on the centromeric DNA of each sister chromatid. When all kinetochores are attached and under tension, cells enter anaphase, the cohesin complex subunit Scc1 is cleaved, and sister chromatids separate. The resulting two new cells have identical copies of the original genetic information, and proceed to the next cell cycle.

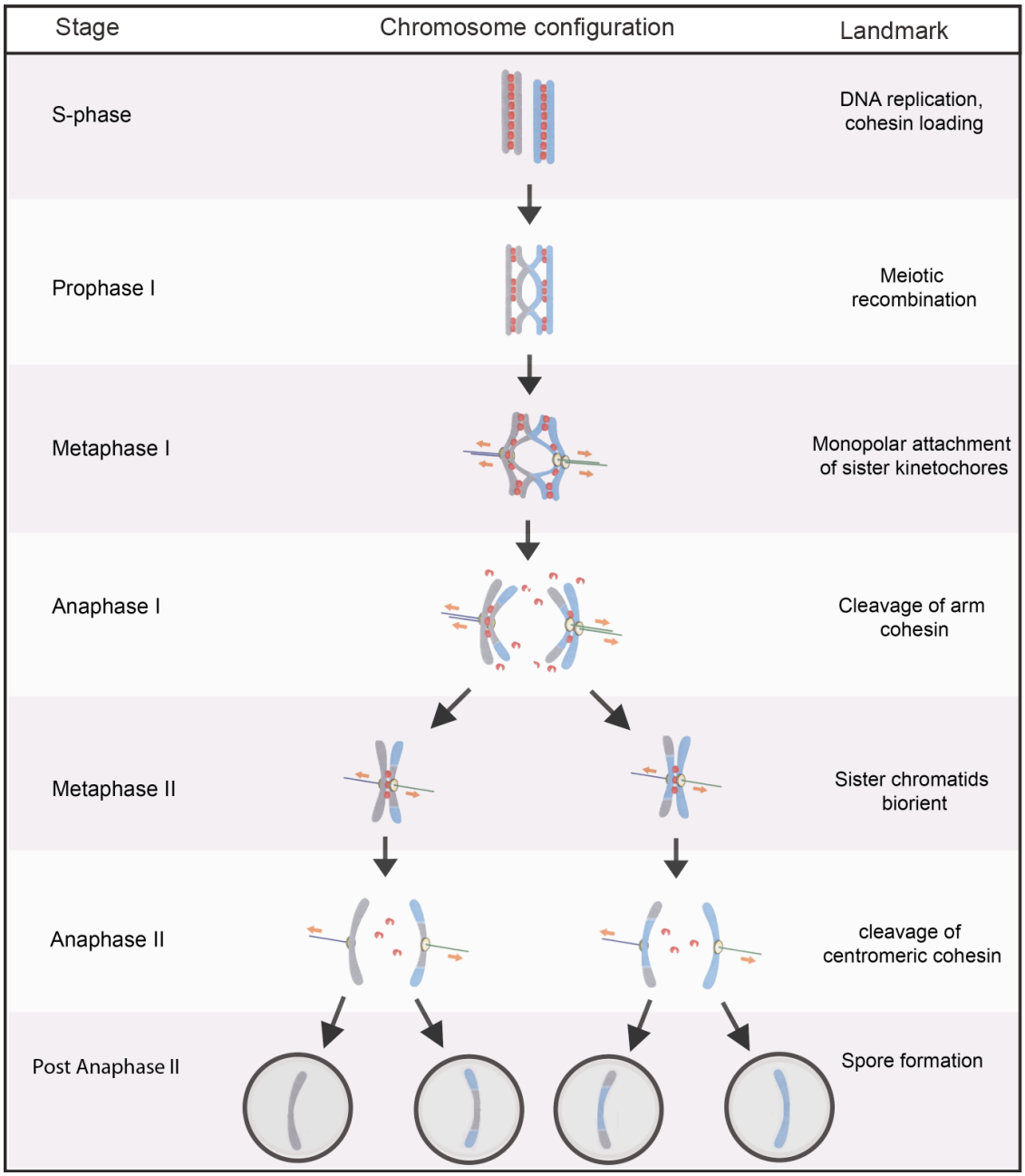

Figure 2. The meiotic program of chromosome segregation in *S. cerevisisae*. After the meiotic G1 stage (not shown), meiotic S-phase takes place, turning each homologous chromosome into a pair of sister chromatids held together by the meiotic cohesin complex (red balls). During a long prophase I, homologous chromosomes become linked by meiotic recombination. At metaphase I, sister kinetochores are forced to face the same pole of the cell, and homologous chromosomes are put under tension on the metaphase I spindle. The cohesin is cleaved along the arms, in anaphase I, allowing the separation of homologous chromosomes. Sister chromatids remain together because of persistent centromeric cohesion, which is used to put the chromatids under tension in the metaphase II spindle. At anaphase II, centromeric cohesin is finally cleaved and four haploid nuclei are produced, which can contain a different rearrangement of the original genetic information. After the second division, the cells terminally differentiate generating structures such as spores in yeast.

Mitosis and meiosis are therefore mutually exclusive fates. Once a cell has chosen meiosis, it must not attempt to divide in a mitotic way. In order to produce healthy gametes, the decision of undergoing meiotic divisions must be irreversible and carried out to completion. A clear example of making decisions between meiosis and mitosis is observed in a sporulating *S. cerevisiae* culture. Upon starvation, the cells activate the transcriptional program necessary for meiosis. If the starvation conditions persist, the cells carry on and enter the prophase I stage. Remarkably, if the cells are transferred to rich medium at this point, they will exit from meiosis and resume the mitotic cycle (Dayani et al., 2011; Zenvirth et al., 1997). However, if the cells are allowed to reach the first meiotic division, they will finish the meiotic program even if they are transferred back to rich medium (Tsuchiya et al., 2014). This behavior shows that *S. cerevisiae* posses the mechanisms to decide rapidly between meiosis and mitosis. The capacity for robust and irreversible decision-making is also found in other cellular processes, such as during cell differentiation in higher eukaryotes (Ferrell, 2012).

How are these decisions made? What kind of biochemical mechanisms determines whether a cell embarks on a mitotic or a meiotic division? To answer these questions, we have studied how *S. cerevisiae* makes the decision to execute meiotic divisions. We have focused on a key point of the meiotic program: the transition from prophase I into metaphase I. At this stage, *S. cerevisiae* cells must select between three possible options: (1) stay in prophase I, (2) continue meiosis, or (3) return to mitosis. In this work, we have identified the molecular basis of how *S. cerevisiae* cells decide which program of cell division will finally prevail. The following section describes in detail molecular principles of cell division and the meiotic program in *S. cerevisiae* with emphasis on the prophase I-to-metaphase I transition.

1.2. The logic of cell division: cyclin-dependent kinase I (Cdk1) versus the anaphase-promoting complex (APC/C)

In 1996, Kim Nasmyth proposed that the cell cycle can be understood as alternating states of high and low activity of the conserved protein kinase Cdk1, also called Cdc28 in *S. cerevisiae* (Nasmyth, 1996). On the one hand, during the low kinase state,

or interphase, growth and DNA replication occur. On the other hand, segregation of genetic material takes place in a high kinase state, called M-phase. A similar reasoning can be applied to the meiotic program. Cells start out in a state of low Cdk1 activity. Meiotic DNA replication and meiotic recombination occur during this period. After prophase I, the cells proceed to a high kinase state, which generates the conditions for nuclear divisions.

Cdk1 levels do not change during cell division and therefore several strategies have evolved to control its activity (Enserink and Kolodner, 2010; Mendenhall and Hodge, 1998). A universal mechanism is to activate the kinase only when bound to a regulatory subunit, called cyclin. When Cdk1 activity is needed, cells synthesize the required cyclins. Once Cdk1 has served its purpose, cyclins are destroyed and the kinase is inactivated. Targeting the cyclins for degradation is generally the task of the E3 ubiquitin ligase APC/C (Peters, 2006; Zachariae and Nasmyth, 1999). Cyclins are ubiquitinated and sent for proteolysis to the 26*S* proteosome. APC/C activity must also be tightly regulated. The core proteins of this gigantic 1.5-MDa complex are not a limiting factor. The accumulation of APC/C activators is the key to restrict its activity to the right periods. APC/C can be activated by WD40 proteins, such as Cdc20 or Cdh1, that dictate the substrate specificity and the timing of APC/C activity (Pesin and Orr-Weaver, 2008).

Cell division is thus characterized by waves of Cdk1 activity that are timely counteracted by APC/C activity. However, several factors determine whether a cell enters a period of low or high Cdk1 activity. During the low kinase state, APC/C is dominant, cyclins are poorly transcribed and constantly destroyed, hindering the activation of Cdk1. To maintain the low kinase activity, cells have evolved additional strategies, like inhibitory post-transcriptional modifications on Cdk1 or the synthesis of Cdk1-inhibitors, such as Sic1. Once the cells go to the high kinase state, these mechanisms are reversed: (1) Cyclins are actively synthesized and stabilized (2) Cdk1 inhibitors are destroyed and ultimately (3) APC/C is inactivated (Kapuy et al., 2009).

1.3. Meiosis starts in period of low Cdk1 activity called meiotic G1

In higher eukaryotes, a hormonal signal in the cell milieu can trigger the differentiation of germ cells into oocytes or sperm (Bowles and Koopman, 2010). In yeast, the initiation of the meiotic program is triggered by external cues. In the absence of nitrogen and fermentable carbon sources, several pathways promote a transient arrest in a G1 state. This response is a signaling cascade that requires the kinases Rim15 and Snf1, as well as the TOR and cAMP/PKA pathways (van Werven and Amon, 2011). During this state, M-phase Cdk1 activity is kept low by the APC/C-Cdh1-dependent destruction of cyclins. The starvation signals ultimately lead to the production of the meiosis-specific transcription factor Ime1 (Mitchell et al., 1990; Rubin-Bejerano et al., 2004). This triggers the expression of the early meiotic genes, which are required for replication and recombination. At this stage, the diploid cell contains one maternal and one paternal version of each chromosome, called homologues. At the end of meiotic G1 they are ready to be duplicated.

1.4. Meiotic S-phase

The initiation of DNA replication during meiosis requires the collaborative action of several kinases such as the Dbf4-Dependent Cdc7 kinase, Ime2, and Cdk1 bound to the S-phase cyclins Clb5 and Clb6 (Benjamin et al., 2003; Dirick et al., 1998; Sclafani, 2000; Stuart and Wittenberg, 1998). The kinases Ime2 and Cdk1 cause the destruction of the Cdk1-inhibitor Sic1 (Benjamin et al., 2003). This facilitates further activation of Cdk1-Clb5, which then inhibits APC/C-Cdh1. S-phase cyclins are then further stabilized and, in collaboration with Ime2, support the firing of the origins of replication. As DNA replication takes place, maternal and paternal chromosomes are duplicated. At the end of meiotic S-phase, each chromosome is composed of two sisters chromatids. A ring-shaped protein complex, called cohesin, is loaded onto the DNA during replication and ties sister chromatids together (Nasmyth and Haering, 2009). The cohesin complex is present both after mitotic and meiotic DNA replication. The meiotic form of the complex contains the subunit Rec8, instead of the mitotic subunit Scc1. Meiotic cohesin, unlike its mitotic counterpart, is essential for

two accurate rounds of chromosome segregation and also takes part in the following process of meiotic homologous recombination.

1.5. Prophase I: the formation of the synaptonemal complex

As cells enter prophase I, homologous chromosomes composed of two sister chromatids attempt to pair up. Initially, the pairing of homologues is established by a set of proteins that assemble directly along their entire length. This proteinaceous platform is called the axial elements (AE); in yeast they are composed of the proteins Red1, Hop1, and Mek1. The cohesin complex is also considered to be a structural part of the AE. Crucially, Red1 is required for the loading of all other axial element proteins.

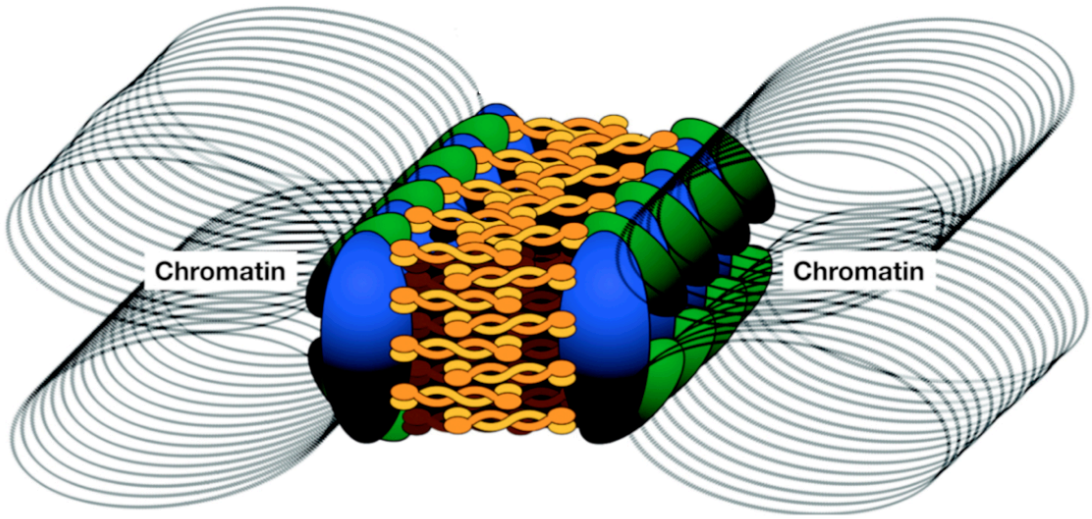

Figure 3. Model of the Synaptonemal Complex in *S. cerevisiae*. The synaptonemal complex (SC) is a railway-like structure along the homologous chromosomes (chromatin). Axial Elements (AE), the first to be assembled, include cohesin (blue ovals) and proteins such as such as Red1 and Hop1 (green ovals). The central elements are long coiled-coils (orange rods), such as the transverse filament protein Zip1, that connect both homologous chromosomes. Other factors required for SC assembly include the proteins of the synapsis-initiation complex Zip2, Zip3, and Zip4 (not shown). Modified from Page and Hawley (2004).

Once AEs are assembled onto chromatin, they are linked through the polymerization of the protein Zip1. This connects the AEs of the two homologues chromosomes and brings them into close proximity. Also the proteins Zip2, Zip3, and Zip4, or synapsis-initiation complex, are required for the proper pairing of homologues. The resulting railway-like structure that holds homologous chromosomes together is called the synaptonemal complex (SC) (Page and Hawley, 2004) (**Figure 3**).

1.6. Prophase I: meiotic homologous recombination

Meiotic homologous recombination initiates when the conserved endonuclease Spo11 introduces double-strand breaks (DSBs) in the DNA of the homologous chromosomes (Lam and Keeney, 2014) (**Figure 4**). Spo11 remains covalently bound to the 5' ends at the DSB site, because it uses a topoisomerase-like trans-esterase reaction to produce nicks in the DNA backbone. To start DSB repair, the MRX complex (Mre11, Rad50 and Xrs2) cleaves a short DNA fragment at the 5' end, which removes Spo11 from the DSB site. Then, the 5' end is further resected, leaving behind 3'-overhangs of single-stranded DNA (Mimitou and Symington, 2009). At this point, DNA repair could be accomplished either by using the sister chromatid or the homologous chromosome. However, AE promote repair by using the homologue as a template. The 3' single-stranded DNA, helped by the recombinases DMC1 and Rad51, invades and probes the homologous chromosome (Cloud et al., 2012). This search continues until the complementary sequence that can be used for repair is found (Bishop et al., 1992; Rockmill et al., 1995). If the interaction between the 3' single-stranded DNA and its homologous sequence is not stable, DNA synthesis starts but eventually the single-stranded DNA is ejected, abandoning the homologous chromosome to re-anneal with its initial partner strand. This is called a non-crossover (NCO). If the interaction between the 3'-single-stranded DNA and its homologous sequence is stable, the second overhang of 3'-single-stranded DNA can also anneal with its complementary sequence in the homologue, a process called second-end capture. Both 3'-strands are synthesized using the homologue sequence as a template, and then ligated. This produces a structure known as Double Holliday Junctions (DHJ) (Schwacha and Kleckner, 1995) that is finally resolved by exchanging the maternal

and paternal DNA strands at the site of the DSB. Since the cohesin complex still holds chromatids together, the exchange of the DNA strands means that homologues chromosomes have become physically connected, an event called crossover (CO) (Marston and Amon, 2004).

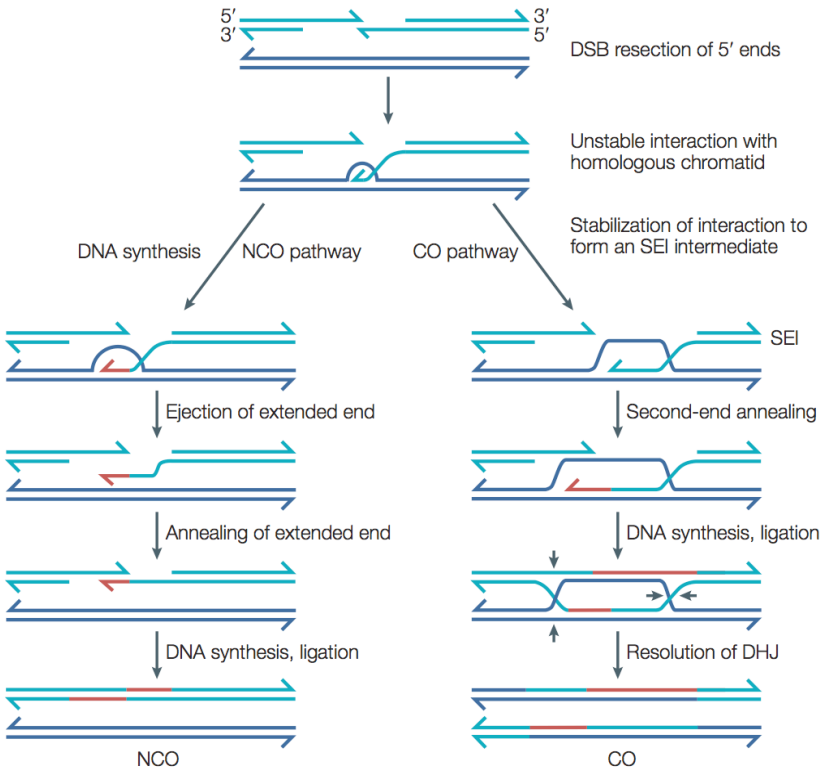

Figure 4. Model of meiotic recombination. After double-strand breaks (DSBs) are introduced by the conserved endonuclease Spo11, the DNA 5'-ends are resected by the Mre11-Rad50-Xrs2 (MRX) complex. The 3'-single-strand overhangs invade the homologous chromosome in search for its complementary sequence (single end invasion, SEI). Once the homologous sequence is found, the invading 3'-single-strand can initiate DNA synthesis (red), but still be ejected to re-anneal with its initial partner strand. This event will produce a non-crossover (NCO, left). In the crossover pathway (CO, right), the 3'-single-strand invasion is stabilized and the second 3'-single-strand will also anneal with its homologous complement (Second-end annealing or capture). Both 3'-strands are synthesized (red) and finally ligated to produce a Holliday junction (DHJ). Single-strand nicks (arrows) resolve the DHJ, producing an exchange of sequences between the homologous chromosomes. Adapted from Marston and Amon (2004). Because the cohesin complex still holds the chromatids together (not shown), both homologous chromosomes become physically linked after meiotic homologous recombination.

The formation of COs has two important consequences. (1) DNA sequences are exchanged between the homologues, leading to the production of chromosomes that are a mosaic of paternal and maternal genetic information. (2) The DNA exchange between homologous chromosomes creates a physical link, or chiasmata, that will allow their segregation during the first meiotic division. Thus, recombination not only creates genetic variation but also produces the linkage that is used to orient maternal and paternal chromosomes on the meiosis I spindle. Cells must ensure that homologues chromosomes are connected by at least one CO at the end of prophase I. Structures such as the synaptonemal complex, the ZMM proteins (Zip2, Zip3, Zip4), the helicase Mer3, and the DHJ-stabilizing Msh4/Msh5 complex, enforce the production of COs (Börner et al., 2004).

1.7. The meiotic recombination checkpoint monitors DSB repair

The meiotic recombination checkpoint (MeiRC) prevents progression out of prophase I until the last DSB has been repaired (Hochwagen and Amon, 2006). Otherwise, cells might attempt to start the first meiotic division with broken chromosomes. To detect the presence of unrepaired DSBs, the MeiRC takes advantage of sensor proteins used during the DNA damage response in mitotic cells. These include the kinases Tel1 and Mec1, as well as the sensor proteins Rad24, Rad17, and Ddc1 (Hong and Roeder, 2002; Lydall et al., 1996). The AE proteins Red1, Hop1, and Mek1 act as the platform on the chromosome that allows the checkpoint machinery to sense unrepaired DSB (Malone et al., 2004). The main target of the MeiRC is the meiosis-specific transcription factor Ndt80 (Tung et al., 2000), which is the source of the proteins required for meiotic nuclear divisions (**Figure 5**).

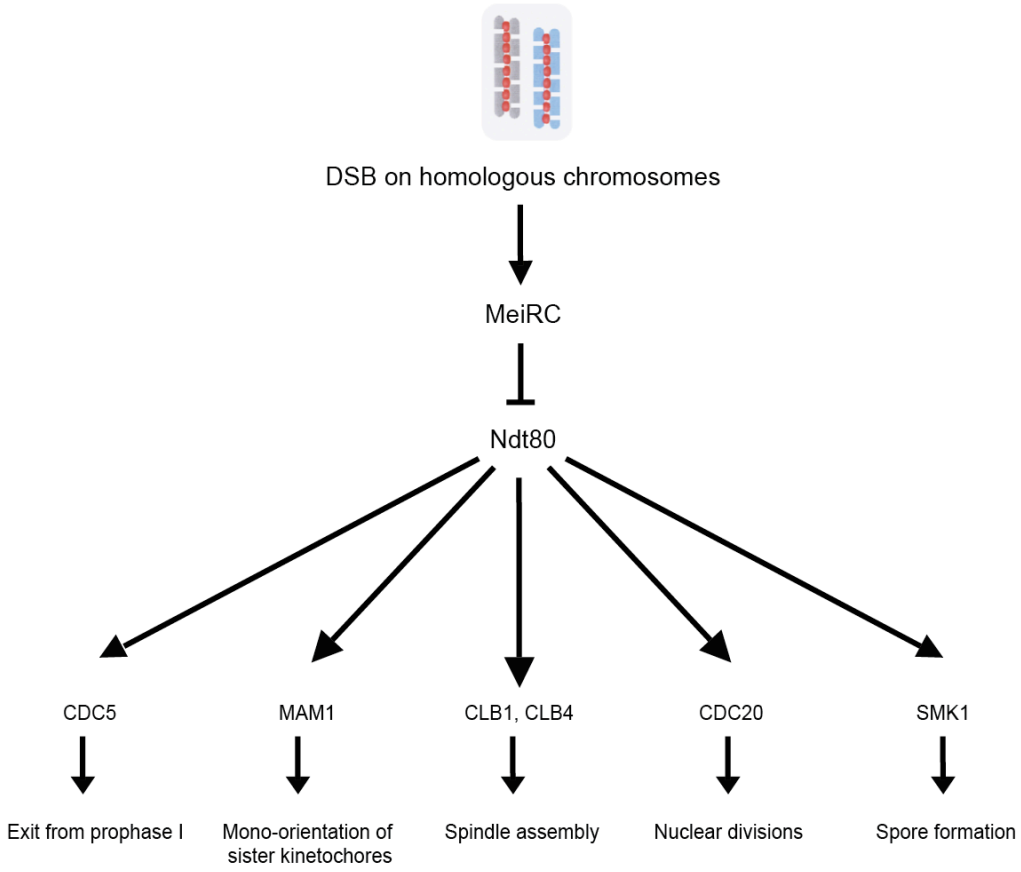

Figure 5. The meiotic recombination checkpoint (MeiRC) halts the meiotic program by inhibiting Ndt80 during prophase I. The Spo11-induced DNA double-strand breaks during homologous recombination are sensed by the MeiRC, which inhibits the activation of the transcription factor Ndt80. The entire meiotic program is stopped until DSBs are repaired and Ndt80 is allowed to trigger the accumulation of proteins required for key meiotic events. Pointed arrows, activation. Bar-headed lines, inhibition. Modified from Winter (2012).

It has been proposed that the checkpoint inactivates Ndt80 by preventing its transcription or its activity; however the mechanism remains unclear (Corbi et al., 2014; Pak and Segall, 2002b; Shubassi et al., 2003; Sopko et al., 2002). As long as DSBs are present, the MeiRC keeps the cells in prophase I by repressing Ndt80. Once DSBs have been repaired, Ndt80 accumulates and the cells undergo a very abrupt transition into metaphase I. At this point, the SC is disassembled, DHJs are resolved into COs, and the cells enter the high-kinase state.

1.8. The first meiotic division occurs in period of high M-phase Cdk1 activity

After prophase I, a long period of low M-phase Cdk1 activity, Ndt80 triggers the accumulation of cyclins and the establishment of the high-kinase state (**Figure 6**). Ndt80 products include B-type cyclins (Clb1, Clb3, Clb4, but not Clb2), the yeast homolog of the polo-like kinase, Cdc5, the APC/C activator Cdc20, and approximately 200 other proteins (Chu and Herskowitz, 1998). It is important to remark that, during mitosis, Ndt80 is blocked by the transcriptional repressor Sum1 and entry into the high-kinase state crucially depends the transcriptional activator Ndd1 (Koranda et al., 2000). Ndd1 and Ndt80 share a set of targets, such as B-type cyclins, Cdc5, and Cdc20, known as M-phase proteins. One exception is Clb2, the main mitotic cyclin, whose expression can only be triggered by Ndd1. Cdk1 enhances the activity of both transcription factors. Thus, by producing cyclins, they auto-amplify their transcriptional activity. However, while Cdk1 is directly involved in the recruitment of Ndd1 to chromatin (Reynolds et al., 2003), it might support Ndt80 indirectly by inhibiting its repressor Sum1 (Pierce et al., 2003; Reynolds et al., 2003; Shin et al., 2010). Unlike Ndd1, Ndt80 promotes its own transcription (Chu et al., 1998), which has been shown to be important for meiotic progression (Tsuchiya et al., 2014).

The Ndt80-dependent rise of Cdk1 activity triggers the formation of the metaphase I spindle. At this stage, the spindle assembly checkpoint (SAC) blocks further progression until all homologue chromosomes are under tension (Musacchio and Salmon, 2007). The target of this checkpoint is the APC/C activator Cdc20 (Hwang et al., 1998). Once the SAC is silenced, homologous chromosomes will be segregated but, importantly, sister chromatids remain together. This meiosis I-specific pattern of chromosome segregation is achieved by two processes: the monopolar attachment of sister kinetochores and the persistence of centromeric cohesin linking sister chromatids after arm cohesin has been cleaved (Marston and Amon, 2004).

During the first meiotic division, the monopolin complex forces the kinetochores on the sister chromatids to face the same spindle pole body (Toth et al., 2000). This process is called mono-orientation of sister kinetochores. The monopolin complex is assembled when the proteins Lrs4 and Csm1 are released from the nucleolus at the onset of metaphase I, upon which they join the meiosis-specific subunit Mam1 and

the conserved casein kinase I homolog of yeast, Hrr25 (Petronczki et al., 2006). This tetrameric complex acts on sister kinetochores so that tension can only be established by pulling the chiasmata-linked homologous chromosomes apart. Once tension is established on all the homologues, the SAC is silenced. APC/C-Cdc20 initiates anaphase I by targeting for destruction securin/Pds1, the inhibitor of the thiol-protease separase/Esp1 (Buonomo et al., 2003). As a consequence, separase/Esp1 cleaves the meiotic cohesin subunit Rec8, thereby opening the cohesin ring and allowing the homologous chromosomes to be segregated to opposite poles of the cell. Our lab has shown that the cleavage of the cohesin ring in meiosis I requires the phosphorylation of Rec8 by the kinases Hrr25 and Dbf4-Cdc7 (Katis et al., 2010). Sister chromatids, however, remain together during meiosis I thanks to a persistent portion of centromeric cohesion, which is protected from separase/Esp1 (Kiburz et al., 2005). The protein Sgo1 is responsible for centromeric cohesin protection during meiosis I by recruiting the phosphatase PP2A. This keeps centromeric Rec8 hypo-phosphorylated, which makes it resistant to separase/Esp1. B-type cyclins are also targeted for destruction by APC/C-Cdc20, once the SAC is silenced. The down-regulation of Cdk1 at the end of the first meiotic division is sufficient to allow the disassembly of the meiosis I spindle. However, it is thought that the kinase activity remains high enough to prevent DNA replication between meiosis I and meiosis II (Petronczki et al., 2003).

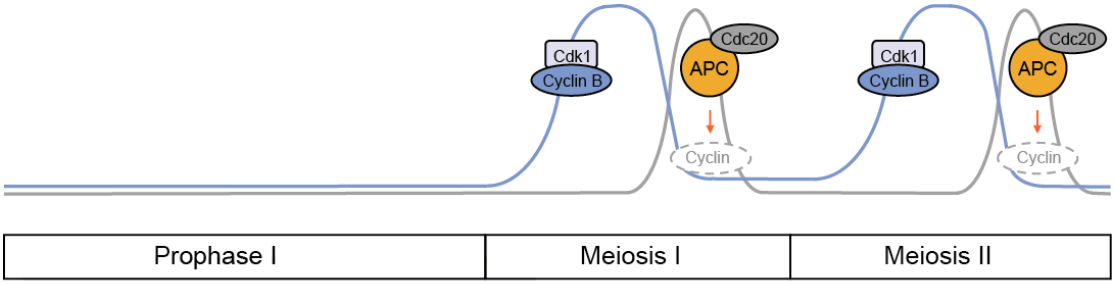

Figure 6. **M-phase Cdk1 activity in Meiosis.** After prophase I, a long period devoid of M-phase Cdk1 activity, two waves of Cdk1 activation are triggered by the accumulation of the B-type cyclins and their timely destruction mediated by APC/C-Cdc20.

1.9. The second meiotic division

Thanks to the persistence of centromeric cohesion after meiosis I, sister chromatids can be put under tension in the metaphase II spindle. Once the SAC is satisfied, APC/C-Cdc20 is activated a second time. The ensuing destruction of securin/Pds1 allows separase/Esp1 to finally cleave centromeric cohesin (Salah and Nasmyth, 2000). This time, Rec8 is not protected from separase, and chromatids are segregated. At the end of the second meiotic division, spindles disassemble and the cells enter a period of low-kinase activity because Cdk1 is fully inactivated. During meiotic exit cyclins are degraded and their synthesis is repressed due to the destruction of Ndt80. APC/C-Cdh1 is reactivated and Cdk1-inhibitors such as Sic1 re-accumulate (Benjamin et al., 2003). However, the control of chromosome segregation during the second meiotic division and the meiotic exit events are still not fully elucidated (Marston and Amon, 2004; Petronczki et al., 2003). In yeast, exit from meiosis II is coupled to the production of spores. The four resulting haploid nuclei are engulfed by a four-layered spore wall (Coluccio et al., 2004). In other eukaryotes, post-meiosis II differentiation events can lead to the development of specialized structures, such as flagella in spermatozoids.

1.10. The control of the prophase I-to-metaphase I transition is critical for meiosis

A conserved feature of meiosis is the exquisite orchestration of the prophase I-to-metaphase I transition. In most eukaryotes, prophase I is a period of low Cdk1 activity, in which homologous recombination takes place. On the contrary, metaphase I is a period of high-kinase activity, in which the recombination machinery is dismantled. The transition between the two states is marked by the silencing of the MeiRC, the rapid accumulation of B-type cyclins, the formation of the meiosis I spindle, and the destruction of the synaptonemal complex (Marston and Amon, 2004). Errors in the coordination of these events may prove disastrous for the meiotic program. Despite its complexity, it is precisely at this transition that cells decide whether to continue with meiosis or not. In extreme cases, such as the human female meiosis, a primary oocyte can be maintained in prophase I for decades (Klug and

Cumings, 2003). Remarkably, once the decision to undergo meiotic divisions is taken, the primary oocyte resumes the meiotic program making the transition to metaphase I. In a similar way, *S. cerevisiae* cells can remain in prophase I for a period equivalent to many cell cycles (Padmore et al., 1991). During this stage, cells must decide between three possible fates: (1) stay in prophase I, (2) continue meiosis, or (3) return to mitosis if nutrients are sensed. To understand how these decisions are made, we decided to analyze in detail the events of the prophase I-to-metaphase I transition in *S. cerevisiae*.

1.11. Contributions

I would like to acknowledge that the plasmids for the expression of cyclins under the *GAL1* promoter and the expression of *AMA1* from the *DMC1* promoter were created by Dr. Aliona Bogdanova. The conversion of the experimental biochemical data, obtained in this study, into the mathematical equations that compose the model of the prophase I-to-metaphase I transition, was done by Dr. Vinod Unni and Prof. Dr. Béla Novák at Oxford University. The wiring diagram in Figure 17 and the mathematical model showed in the Figures 18 and 20, as well as the potential surface representation of the model depicted in Figure 33 are the result of their work.

2. Results

2.1. APC/C-Ama1 is required for maintaining the low-kinase state of prophase I

In all sexually reproducing organisms analyzed so far, prophase of meiosis I is a long period without M-phase Cdk1 activity in which meiotic recombination takes place (Marston and Amon, 2004). This stage must be maintained until the last double-strand break (DSB) is repaired; otherwise the cells could attempt to divide with damaged chromosomes. How can the cells ensure that M-phase Cdk1 activity does not appear prematurely during prophase I? A potential mechanism is proteolysis of M-phase proteins triggered by the APC/C. In *S. cerevisiae*, only one form of APC/C has been showed to be potentially active during prophase I: the APC/C bound to its meiosis-specific activator Ama1 (Oelschlaegel et al., 2005).

To analyze the role of APC/C-Ama1 in prophase I, we performed synchronized meiotic time course experiments on cultures of wild-type and *ama1*Δ cells. Samples taken every two hours were used for immunofluorescence detection of spindles (α-tubulin) and the meiotic progression marker securin/Pds1, C-terminally tagged with 18 Myc epitopes. Samples were also taken for the preparation of TCA-protein samples, which were analyzed by SDS-PAGE followed by western blotting. We considered the appearance of the meiosis I spindle as the landmark event for the end of the low-kinase state of prophase I. In our synchronized cultures, wild-type cells started to assemble meiosis I spindles around six hours after induction of meiosis. Remarkably, in *ama1*Δ cells, spindle formation was already evident four hours after the induction of meiosis (**Figure 7**). The advanced appearance of spindles in the *ama1*Δ mutant suggested that Cdk1 had been activated prematurely. To confirm this observation, we analyzed the pattern of cyclin accumulation in wild-type and *ama1*Δ cells by western blotting. Consistent with the early appearance of spindles, the accumulation of the B-type cyclins Clb1 and Clb4, as well as the kinase Cdc5, was advanced. Early meiotic events such as the accumulation of securin/Pds1, Rec8, and Dbf4, were indistinguishable between wild-type and *ama1*Δ cells. This indicated that,

although the initial stages of meiosis occurred normally, *ama1*Δ cells exited prematurely from the low kinase state of prophase I.

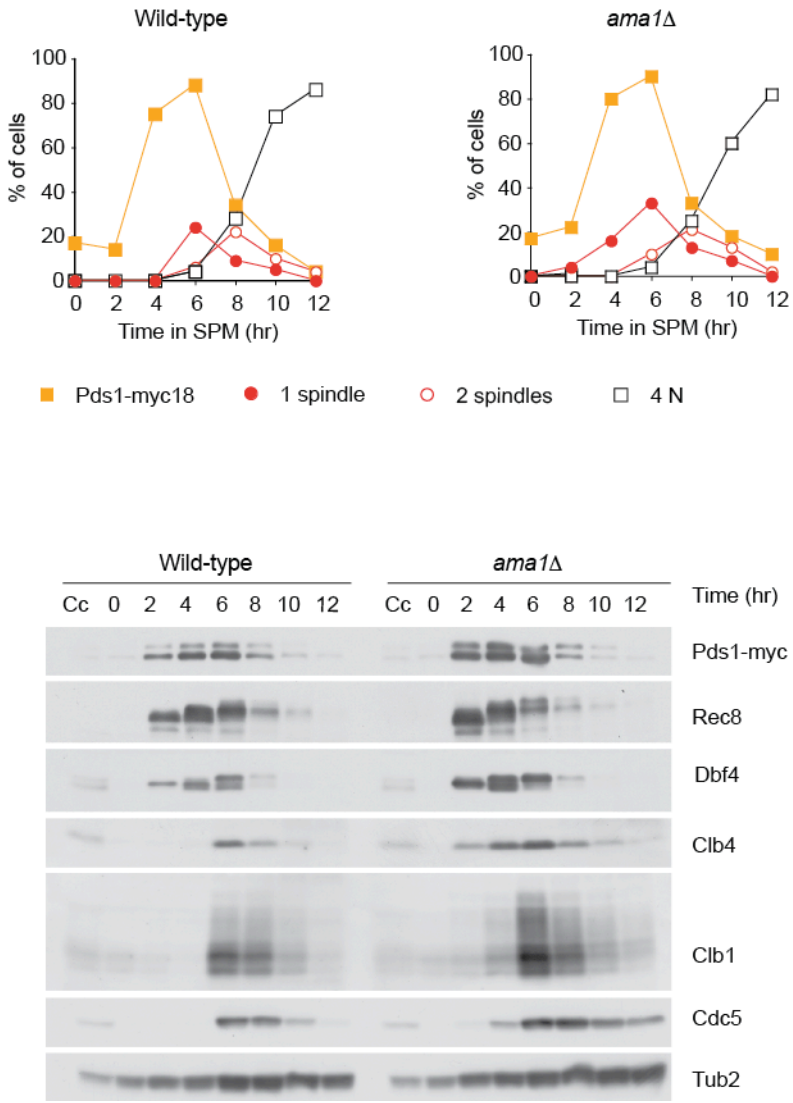

Figure 7. APC/C-Ama1 prevents premature activation of M-phase Cdk1. Meiosis was induced in synchronized cultures of wild-type (Z2828) and *ama1*Δ (Z19251) cells. After transfer to sporulation medium (SPM), samples for immunofluorescence and TCA protein extraction were collected every 2 hr. Top, quantification of meiotic progression in wild-type and *ama1*Δ cells by immunofluorescence detection of securin/Pds1-myc, meiosis I or meiosis II spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown. Bottom, immunoblot detection of protein levels during the time course. Cc means sample from proliferating cells.

How can cyclins accumulate prematurely in the *ama1*Δ cells? We first hypothesized that the advanced spindle formation in the *ama1*Δ mutant results from premature accumulation of Ndt80. In wild-type cells, the meiosis specific transcription factor Ndt80 is responsible for the activation of M-phase Cdk1 activity at the end of prophase I (Chu and Herskowitz, 1998; Xu et al., 1995). To increase the temporal resolution of our observations, we decided to track the accumulation of Ndt80 using life-cell imaging. Dr. Aliona Bogdanova carried out this experiment. We observed the kinetics of Ndt80 accumulation in wild-type and *ama1*Δ cells. Surprisingly, Ndt80 was not significantly advanced in the *ama1*Δ mutant (Data not shown). This suggested that M-phase Cdk1 became active before Ndt80 appeared. In budding yeast, there is only one other transcription factor known to induce M-phase Cdk1 activity: Ndd1. This however, was a radical possibility since Ndd1 was best known for its prominent role during mitosis (Koranda et al., 2000) and no meiotic function had been reported.

We nevertheless reasoned that if Ndd1 was active during meiosis in *ama1*Δ cells, this should lead to the accumulation of cyclins and spindle assembly in the absence of Ndt80. To test this hypothesis, we perform synchronized time courses in *ndt80*Δ and *ama1*Δ *ndt80*Δ cells (**Figure 8**). These strains contained their endogenous Ndd1 protein tagged with three HA epitopes at its C-terminus. Samples for TCA protein extraction and immunofluorescence were collected as before. As expected, we observed that *ndt80*Δ cells remained in the low-kinase state of prophase I, as judged by the absence of spindles and B-type cyclins. Strikingly, *ama1*Δ *ndt80*Δ cells accumulated not only Ndd1, but also several of its products, like the mitotic cyclin Clb2, Clb1, Cdc5, the APC/C activator Cdc20, and the transcription factor Swi5. The cyclin Clb4 accumulated ahead of the other M-phase proteins, probably because its expression does not depend on Ndd1 (Spellman et al., 1998). Consistent with the presence of cyclins, *ama1*Δ *ndt80*Δ cells exited the low-kinase state and assembled spindles. Interestingly, these cells remained arrested in a metaphase I-like state. These observations indicated that APC/C-Ama1 was required for maintaining the low-kinase state of prophase I.

Elwy Okaz *et al.* (2012) showed that APC/C-Ama1-mediated proteolysis is the key process that prevents the accumulation mitotic M-phase promoting factors during prophase I. Clb1, Clb2, Clb4, Cdc5, and Ndd1 itself were found to be physiological

substrates of APC/C-Ama1 during prophase I. Their stability was shown to depend on their destruction box motifs and the presence of Ama1. Thus, in *ama1*Δ cells, Cdk1 is activated prematurely because B-type cyclins and the mitotic transcription factor Ndd1 are stabilized during prophase I. We concluded that APC/C-Ama1-mediated proteolysis maintains the low-kinase state of prophase I.

The mechanism of action of APC/C-Ama1 during prophase I can be described as the network motif called coherent feed-forward loop. In biochemistry, a network motif is a simple pattern of activation or inhibition, among a small number of interacting molecular species, which serves an information-processing function in the cell (Tyson and Novak, 2010). In a feed-forward loop (FFL), an element of the system affects another one in two different ways, directly and indirectly. For instance, APC/C-Ama1 uses a FFL because it inhibits the accumulation of Clb1 at two levels (1) by promoting directly the degradation of the Clb1 protein (2) by repressing *CLB1* transcription, through the destruction of the transcription factor Ndd1. Since both the direct and indirect effect on Clb1 have the common aim of inhibiting the accumulation of the protein, this set of interactions is called a coherent feed-forward loop (CFF). By destroying not only Ndd1 but also its products, APC/C-Ama1-driven coherent feed-forward loops provide a robust mechanism to prevent the activation of Cdk1 during prophase I.

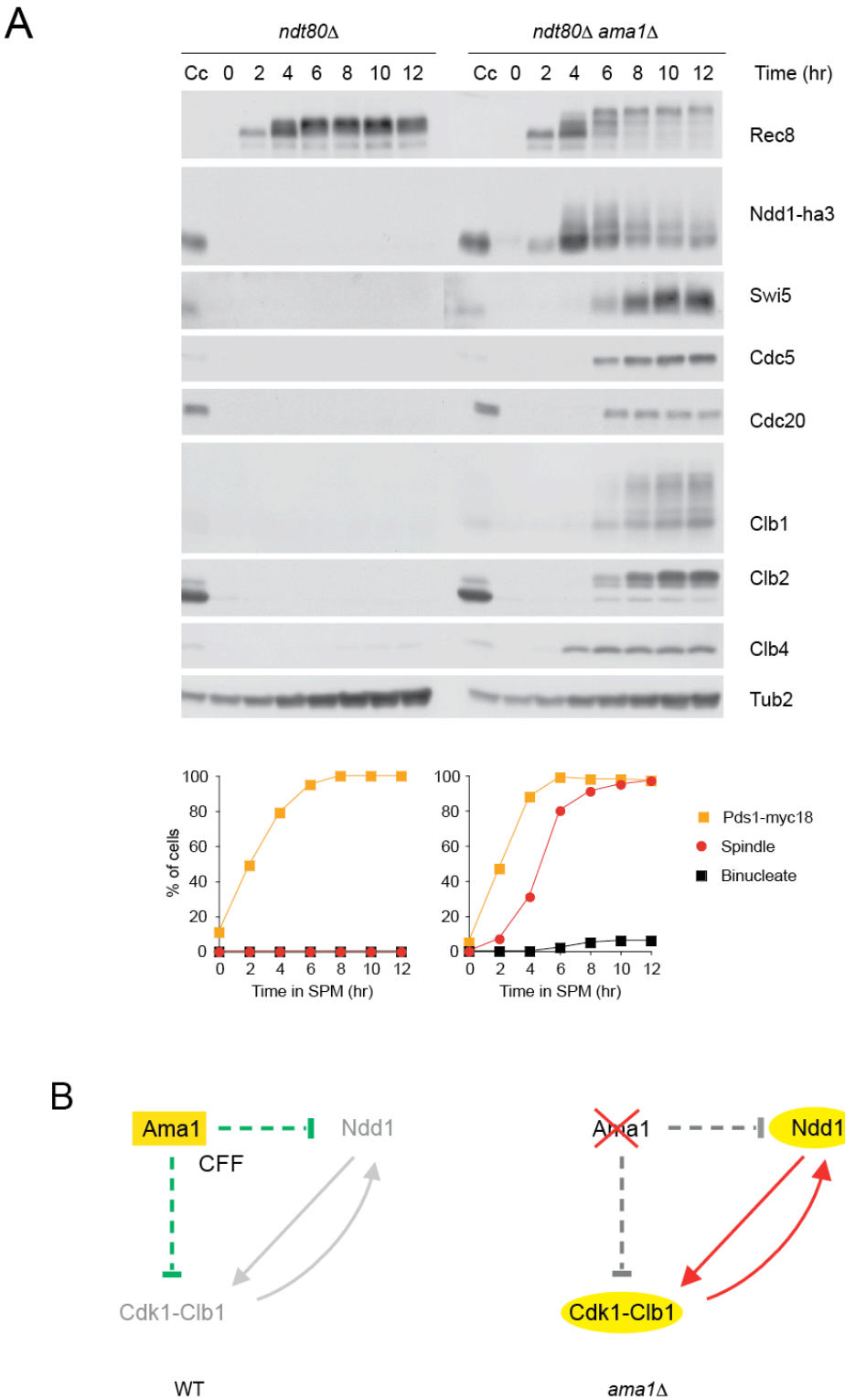

Figure 8. APC/C-Ama1 is required for maintaining the low Cdk1 kinase state of prophase I. (A) Synchronous meiotic time course of the strains *ndt80*Δ (Z17225) and *ndt80*Δ *ama1*Δ (Z17226). Top, immunoblot detection of protein levels during the time course. Cc means sample from proliferating cells. Bottom, immunofluorescence detection of securin/Pds1-myc, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. **(B)** Cdk1-counteracting coherent feed-forward loop in prophase I. Left, in wild-type cells, Ama1-driven proteolysis (green doted lines) constitutes a coherent feed-forward loop (CFF) that blocks M-phase Cdk1 activation during prophase I. Right, in *ama1*Δ cells, Ndd1 and cyclins are stabilized and activate each other (solid red lines). Modified from Okaz *et al*. (2012).

2.2. APC/C-Ama1 is inactivated during the transition from prophase I into metaphase I by Ndt80-dependent inhibitors, such as Clb1-Cdk1

We have shown that in *S. cerevisiae*, APC/C-Ama1 prevents the activation of M-phase Cdk1 during prophase I (Okaz et al., 2012). The APC/C-Ama1 mediated destruction of M-phase proteins, such a B-type cyclins, prevents premature spindle assembly. However, in wild-type cells, metaphase I is rapidly established once recombination has been completed. How do M-phase proteins, which are actively destroyed during prophase I, accumulate so abruptly at the onset of metaphase I? One possibility is that APC/C-Ama1-mediated proteolysis is inhibited as cells exit prophase I. To test whether APC/C-Ama1 was inhibited at the prophase I-to-metaphase-I transition, we measured the stability of M-phase proteins in metaphase I-arrested cells **(Figure 9**).

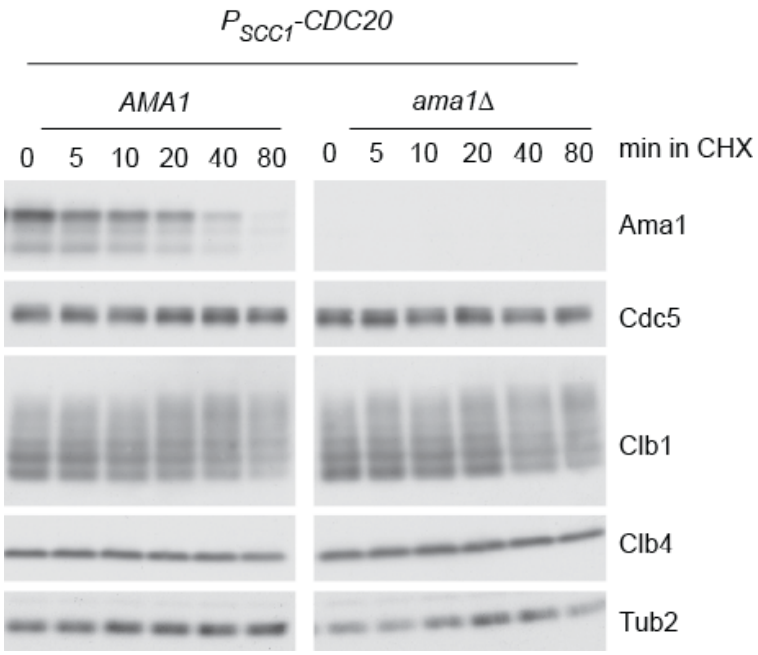

Figure 9. APC/C-Ama1 is inactive at metaphase I. Stability of M-phase proteins in wild-type (Z18334) and *ama1*Δ (Z18333) cells arrested in metaphase I by Cdc20-depletion. *CDC20* was placed under the control of the mitosis-specific promoter of *SCC1* (P_{SCC1}-*CDC20*), which depletes the protein during meiosis and causes arrest at metaphase I due to failure to activate APC/C-Cdc20. At 8 hr into meiosis, cells were treated with cycloheximide (CHX) (t = 0), and protein levels were analyzed by immunoblotting.

A stable metaphase I arrest is achieved by placing the APC/C activator Cdc20 under the control of the mitosis-specific *SCC1* promoter. During meiosis, the *SCC1* promoter is repressed, which leads to the depletion of the Cdc20 protein. P_{SCC1}-*CDC20* cells are unable to activate APC/C-Cdc20 in metaphase I, arresting with constant levels of M-phase proteins. We performed synchronized meiotic time course experiments of P_{SCC1}-*CDC20* cells, allowing the cultures to arrest at metaphase I. In order to assess protein stability, we used cycloheximide (CHX), an inhibitor of the elongation step during eukaryotic ribosomal protein synthesis (Schneider-Poetsch et al., 2010). Upon addition of cycloheximide, we observed that in metaphase I-arrested cells, M-phase proteins were very stable. Indeed, their stability was similar in the presence and absence of Ama1 **(Figure 9)**. In sharp contrast, the stability of M-phase proteins during prophase I depends on Ama1(Okaz et al., 2012). This showed that, contrary to prophase I, APC/C-Ama1 is inactive at metaphase I.

Inactivation of APC/C during transitions in the cell cycle often requires the production of APC/C-specific inhibitors. During the transition from G1 into S phase, for instance, APC/C-Cdh1 is inhibited by Clb5-Cdk1 activity (Zachariae et al., 1998). Thus, we considered that APC/C-Ama1-specific inhibitors could be produced at the onset of metaphase I. In wild-type cells, the prophase I-to-metaphase I transition is marked by an abrupt increase in M-phase Cdk1 activity. To investigate whether Cdk1 could inhibit APC/C-Ama1, we decided to express meiotic B-type cyclins in *ndt80*Δ cells. In the previous section we showed that APC/C-Ama1 activity is required for preventing the accumulation of M-phase proteins during the prophase I arrest of *ndt80*Δ cells. Therefore, we reasoned that the expression of an APC/C-Ama1 inhibitor in *ndt80*Δ cells should induce the accumulation of M-phase proteins. To produce cyclins to roughly metaphase I levels in *ndt80*Δ cells, we used an estradiol-inducible expression system (Benjamin et al., 2003). The strains bearing this expression system produce, from the *GPD1* promoter, a fusion of the Gal4 transcription factor and the hormone-binding domain of the human estrogen receptor (ER). In the presence of estradiol, the Gal4.ER protein is translocated to the nucleus and activates the transcription of genes controlled by the *GAL1* promoter. We placed each meiotic cyclin under the control of the *GAL1* promoter, or its shortened version, the *GALL* promoter, in *ndt80*Δ cells bearing the estradiol-inducible expression system. After expressing the meiotic cyclins to roughly metaphase I levels, we found that only Clb1

induced rapid accumulation of M-phase proteins (**Figure 10**). Upon Clb1 expression, the cells not only accumulated Cdc5, Cdc20, and Clb2, but also assembled spindles. In contrast, no inhibitory effect was observed upon expression of other cyclins. This result suggested that Clb1-Cdk1 is an inhibitor of APC/C-Ama1 at the transition from prophase I to metaphase I. An unexpected effect of the Clb1 expression in *ndt80*Δ cells was the destruction of securin/Pds1. This observation implied that Clb1 is able to inhibit APC/C-Ama1 and simultaneously activate APC/C-Cdc20, once Cdc20 has accumulated.

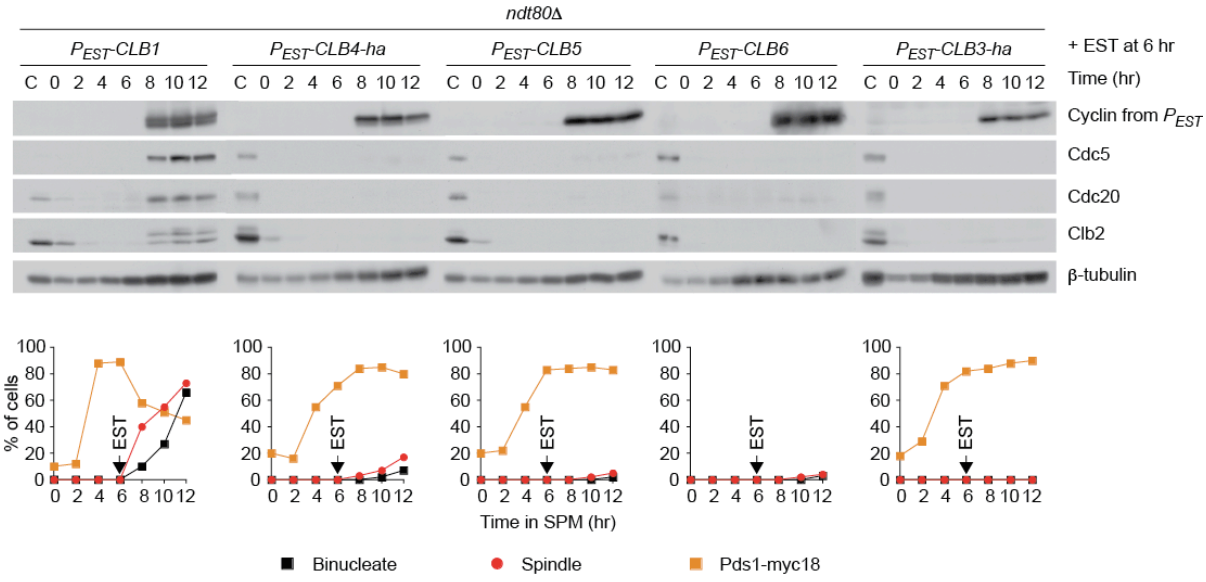

Figure 10. Clb1-Cdk1 is an inhibitor of APC/C-Ama1. Synchronous meiotic time course of *ndt80*Δ strains bearing the cyclins *CLB1* (Z19409), *CLB4* (Z18669), *CLB5* (Z18883), *CLB6* (Z19211), and *CLB3* (Z19478), under the control of the estradiol inducible promoter (P_{EST}-*Cyclin*). Samples for whole cell protein extracts and immunofluorescence were taken every 2 hr. At 6 hr into meiosis, cells were treated with estradiol to induce the corresponding cyclin. Top, immunofluorescence detection of securin/Pds1-myc, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Bottom, immunoblot analysis of protein levels. Cc means sample from proliferating cells. Modified from Okaz *et al*. (2012).

To confirm that the kinase activity of Clb1-Cdk1 was required for inhibition of APC-Ama1, we expressed Clb1 in *ndt80*Δ P_{SCC1}-*CDC20* cells bearing a conditional allele of the yeast homolog of the Cdk1 kinase, *cdc28-as1* (Bishop et al., 2000). We performed this experiment in P_{SCC1}-*CDC20* background to avoid to effects of the potential activation of APC/C-Cdc20 by Clb1. In the *cdc28-as1* mutant kinase, the ATP-binding pocket has been enlarged by replacing the bulky gatekeeper residue phenylalanine at position 88 for a smaller aminoacid, glycine. This single amino acid substitution allows cell-permeable ATP-analogs, such as 1NM-PP1, to specifically bind the enlarged ATP-binding pocket of *cdc28-as1*, resulting in kinase inhibition. Furthermore, in the absence of the inhibitor, *cdc28-as1* displays only a small reduction in activity when compared to the wild-type kinase.

We performed synchronized time courses with *ndt80*Δ P_{SCC1}-*CDC20* and *ndt80*Δ P_{SCC1}-*CDC20 cdc28-as1* cells. After 6 hours from the transfer to SPM, 1NM-PP1 was added to a final concentration of 5 μM. Consistent with our previous experiments, the induction of Clb1 in the presence of active Cdk1 triggered the accumulation of M-phase proteins and spindle assembly. Due to the depletion of Cdc20, the degradation of Pds1 was prevented and the cells were arrested in a metaphase I-like state. By contrast, no effect was observed when Clb1 was expressed after the inactivation of Cdk1 (**Figure 11**). We concluded that Clb1-Cdk1 kinase activity was sufficient to generate the exit from the low-kinase of prophase I by inhibiting APC/C-Ama1.

The robust inhibition of APC/C-Ama1 observed, upon activation of Clb1-Cdk1, in *ndt80*Δ cells made us ask whether Clb1 would be essential for timely entry into metaphase I. If Clb1-Cdk1 were the only APC/C-Ama1 inhibitor produced by Ndt80 at the onset of metaphase I, then *clb1*Δ cells should not exit from prophase I due to a failure to inactivate proteolysis of M-phase proteins. However, our analysis of synchronized meiotic time courses showed that the kinetics of accumulation of M-phase proteins and meiosis I spindles were similar between wild-type and *clb1*Δ cells (**Figure 12**).

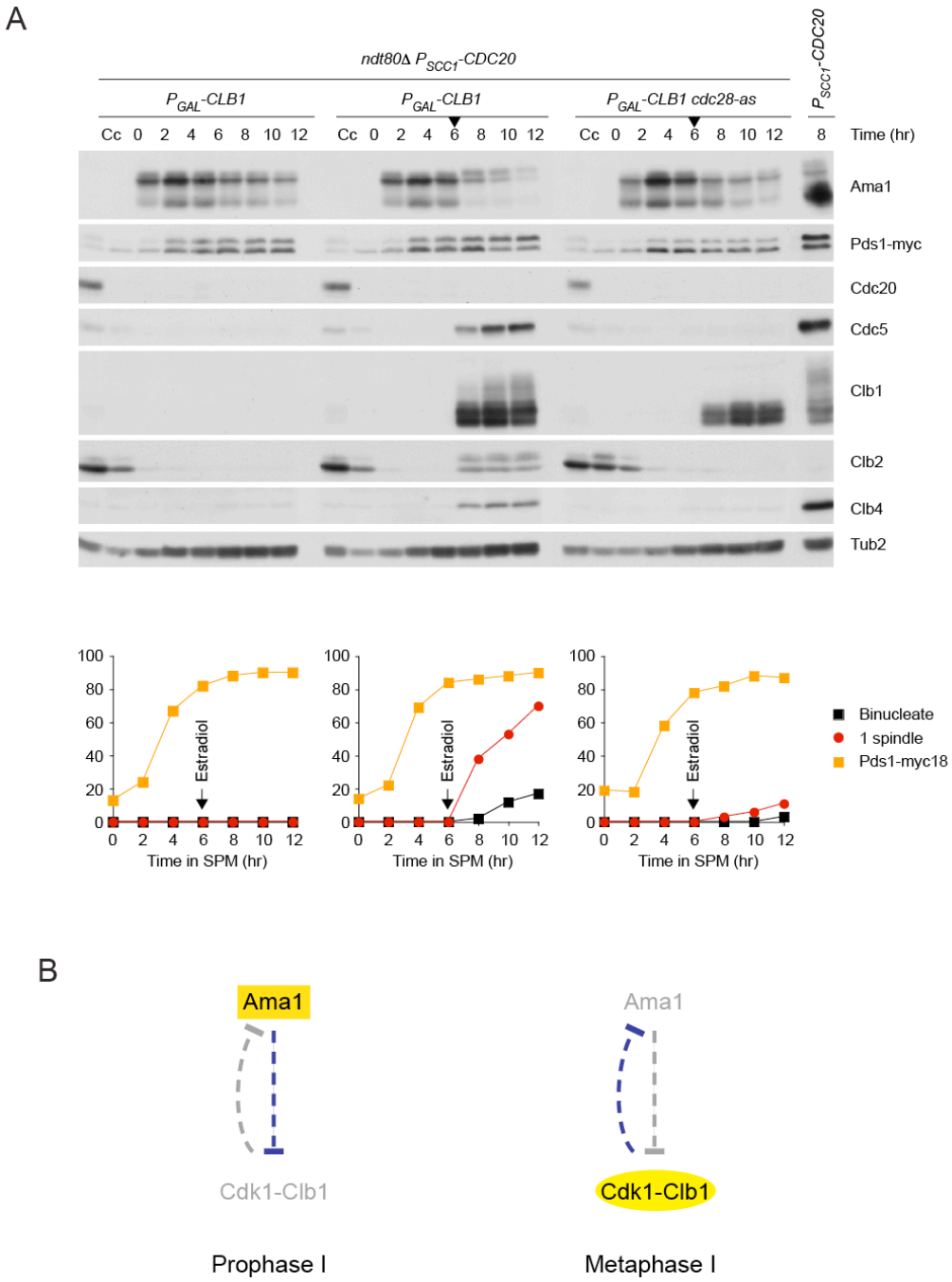

Figure 11. Cdk1 activity is required for Clb1-Cdk1 inhibition of APC/C-Ama1. (A) Synchronous meiotic time course of the strains *ndt80Δ* P_{SCC1}*-CDC20* P_{EST}*-CLB1* (Z19409) and *cdc28-as1 ndt80Δ* P_{SCC1}*-CDC20* P_{EST}*-CLB1* (Z19408). At 6 hr into meiosis, cells were treated with DMSO (left panel) or 5 µM estradiol and 5 µM 1NM-PP1 (black triangles, center and right panel) to induce Clb1 and inhibit Cdc28, respectively. Top, immunoblot analysis of protein levels. Cc means sample from proliferating cells. An extra sample collected from cells arrested at metaphase I by Cdc20-depletion shows that the levels of expressed Clb1 closely match physiological metaphase I-levels. Bottom, immunofluorescence detection of securin/Pds1-myc, MI spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. **(B)** The mutual inhibition (dashed lines) between APC/C-Ama1 and Cdk1-Clb1 creates a double-negative feedback loop. In prophase I, APC/C-Ama1 destroys Clb1 (right) whereas at metaphase I, Cdk1-Clb1 inhibits Ama1 (left). Modified from Okaz *et al*. (2012).

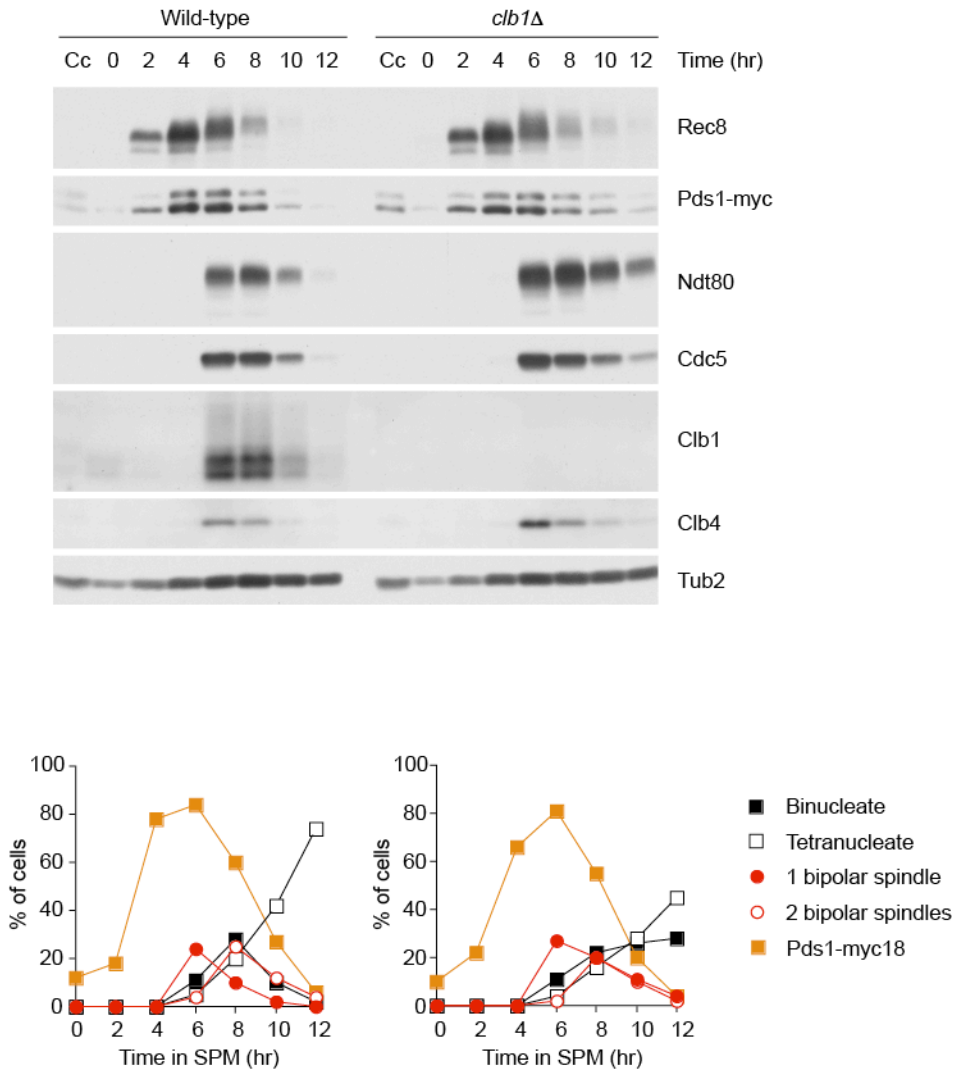

Figure 12. Clb1-Cdk1 is not essential for the exit from prophase I. Synchronous meiotic time course of wild-type (Z2828) and *clb1*Δ (Z19376) cells. Samples for whole cell protein extracts and immunofluorescence were taken every 2 hr. Top, immunoblot detection of protein levels during the time course. Cc means sample from proliferating cells. Bottom, immunofluorescence detection of securin/Pds1-myc, meiosis I or meiosis II spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown. Modified from Okaz *et al*. (2012).

Thus, Clb1-Cdk1 activity was not essential for the prophase I-to-metaphase I transition. *clb1*Δ cells, however, experienced an anomalous progression through meiotic divisions, probably due to the role of Clb1 as an APC/C regulator.

Two scenarios could explain why the prophase I-to-metaphase I transition occurs with wild-type kinetics in *clb1*Δ cells. First, Cdk1 activity is not essential for the inhibition

of APC/C-Ama1 and additional inhibitors produced by Ndt80 inactivate proteolysis at the onset of metaphase I. Second, in the absence of Clb1-Cdk1 activity, M-phase proteins are unstable, but accumulate nevertheless, because Ndt80-driven transcription overwhelms APC/C-Ama1 mediated proteolysis. To distinguish between these possibilities, we analyzed the stability of M-phase proteins in cells lacking Cdk1 activity. For this purpose, we performed synchronized meiotic time courses with *cdc28-as1* P_{SCC1}*-CDC20* cells and inhibited Cdk1 before the onset of metaphase I. Consistent with our previous results, M-phase proteins in P_{SCC1}*-CDC20* cells were stable upon addition of cycloheximide. Strikingly, in the absence of Cdk1 activity, M-phase proteins were equally stable (**Figure 13**).

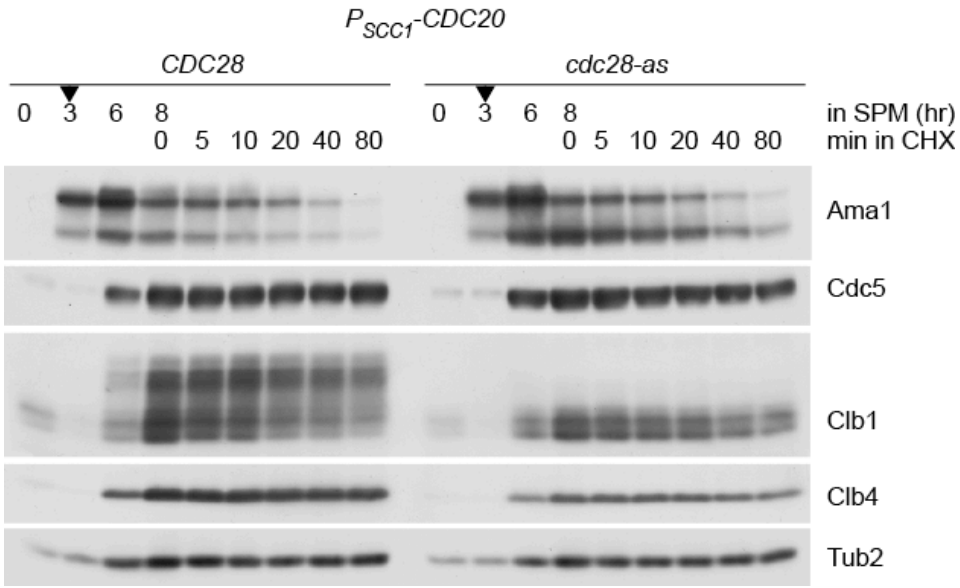

Figure 13. Cdk1 activity is not essential for the stabilization of M-phase proteins at the prophase I-to-metaphase I transition. Stability of M-phase proteins in P_{SCC1}*-CDC20* (Z18334) and *cdc28-as1* P_{SCC1}*-CDC20* (Z17972). At 3 hr into meiosis, the cells were treated with 5 μM 1NM-PP1 to inhibit Cdk1 (black triangle). At 8 hr, cells were treated with cycloheximide (CHX, t = 0) and protein levels were analyzed by immunoblotting. Modified from Okaz *et al*. (2012).

This result showed that Cdk1 activity was not essential for the accumulation of M-phase proteins as cell exit from prophase I. Therefore, Ndt80 must produce factors in addition to Clb1-Cdk1 to ensure that APC/C-Ama1 is inhibited during the prophase I-to-metaphase I transition.

Our results show that during prophase-I, APC/C-Ama1 is active and targets for degradation its own inhibitor Clb1. Inversely, at metaphase I, APC/C-Ama1 is inactivated by the rapid accumulation of Clb1-Cdk1 and other Ndt80-dependent inhibitors. The mutual inhibition between APC/C and Ndt80-dependent inhibitors, such as Clb1-Cdk1, creates a double-negative feedback loop (Tyson and Novak, 2010). This network motif occurs when two components of a system inactivate each other. In biological systems, double negative-feedback loops often lead to the generation of two mutually exclusive states where one component is ON while the other is OFF. Intermediate states in which the components are partially active or partially inhibited are not stable over time and tend to toggle automatically to the one or the other state. In our case, these two states are represented by (1) prophase I, in which APC/C-Ama1 prevails and Cdk1 activity is low, and (2) metaphase I, in which Cdk1 dominates and APC/C-Ama1 is inactive **(Figure 11.B)**.

2.3. Cdc5, the polo-like kinase of S. cerevisiae, is an inhibitor of the MeiRC

The polo like kinase of *S. cerevisiae*, Cdc5, is another important Ndt80 product that is controlled by APC/C-Ama1-dependent proteolysis (Okaz et al., 2012). It has been shown that Cdc5 regulates landmark events of the prophase I-to-metaphase I transition, such as the dissociation of the synaptonemal complex from chromatin (Clyne et al., 2003; Sourirajan and Lichten, 2008). This prompted us to analyze the consequences of the accumulation of Cdc5 in prophase I.

We expressed Cdc5 from an estradiol-inducible promoter in *ndt80*Δ cells (**Figure 14.A**). In contrast to Clb1, Cdc5 expression did not cause accumulation of M-phase proteins, suggesting that it cannot inhibit APC/C-Ama1 at the prophase I-to-metaphase I transition. Interestingly, we observed that the axial element (AE) protein Red1 and the lateral element Zip1 disappeared rapidly upon Cdc5 expression. However, other AE proteins such as Hop1 or the cohesin subunit Rec8 remained stable.

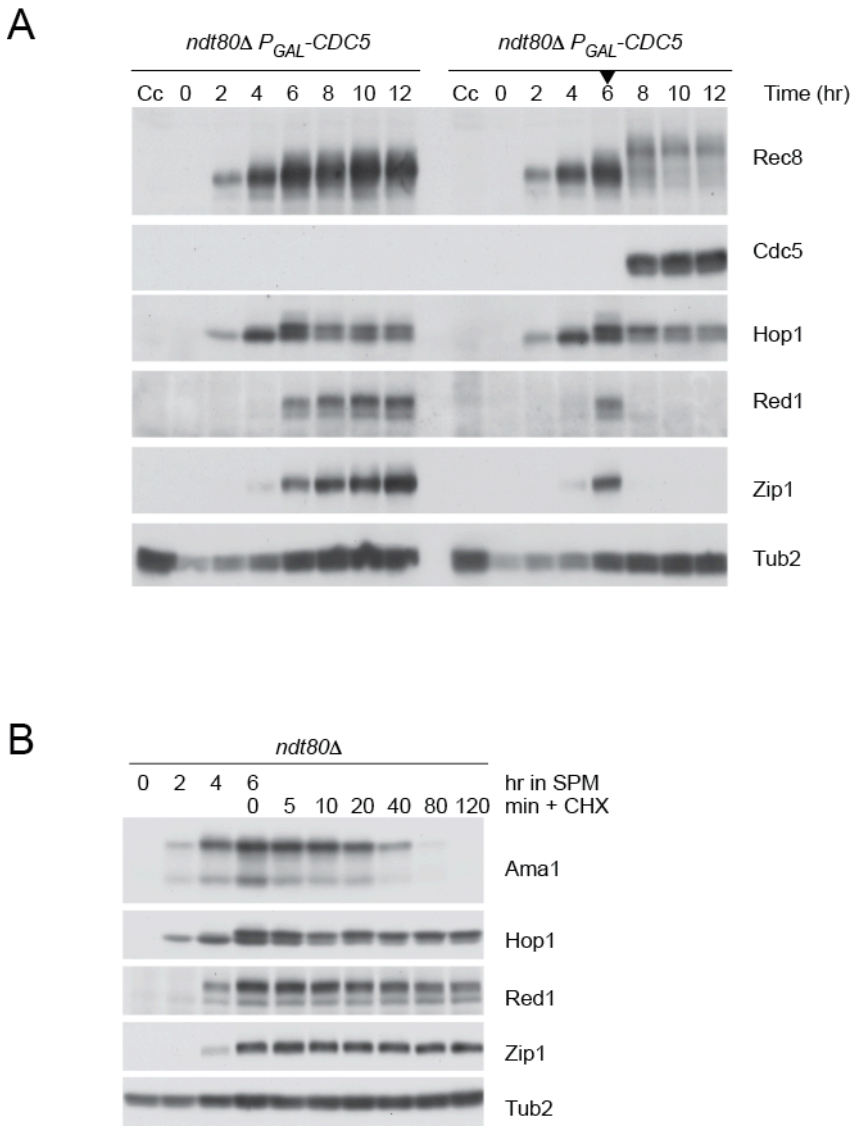

Figure 14. Cdc5 triggers the destruction of the SC proteins Red1 and Zip1. (A) Synchronous meiotic time course of the *ndt80*Δ P_{EST}-*CDC5* (Z18948). At 6 hr into meiosis, the cultures were treated with DMSO or 5 μM estradiol to induce *CDC5*. Immunoblot analysis of protein levels upon *CDC5* expression is shown. Cc means sample from proliferating cells. **(B)** Stability of Hop1, Red1, and Zip1 during prophase I. After 6 hr into meiosis, *ndt80*Δ cells were treated with cycloheximide (CHX, t = 0) and protein levels were analyzed by immunoblotting. Modified from Okaz *et al*. (2012).

Two scenarios could explain the decrease in the levels of Red1 and Zip1. First, these SC proteins could be constitutively unstable and expression of Cdc5 blocks their synthesis. Second, Red1 and Zip1 could be very stable and their dramatic decrease upon Cdc5 induction requires proteolysis. To distinguish between these possibilities,

we analyzed the stability of SC proteins in *ndt80*Δ cells. Red1 and Zip1 accumulated during the prophase I-arrest and their stability was determined upon addition of cycloheximide. Strikingly, SC proteins were extremely stable (**Figure 14.B**). We concluded that the abrupt disappearance of Red1 and Zip1 required proteolysis triggered by Cdc5. To confirm that the kinase activity of Cdc5 was required for the destruction of Zip1 and Red1, we analyzed synchronized meiotic time courses of *ndt80*Δ *ama1*Δ cells bearing a conditional allele of Cdc5, *cdc5-as* (Snead et al., 2007). Due to the L158G mutation in the ATP-binding pocket of Cdc5, the kinase can be irreversibly inactivated by pyrrolopyrimidine chloromethylketone (CMK). Inhibition occurs when CMK binds covalently to the cysteine at position 96 in the active site. After two hours into the time course, CMK was added to a final concentration of 20 μM and the levels of several SC proteins were detected (**Figure 15**).

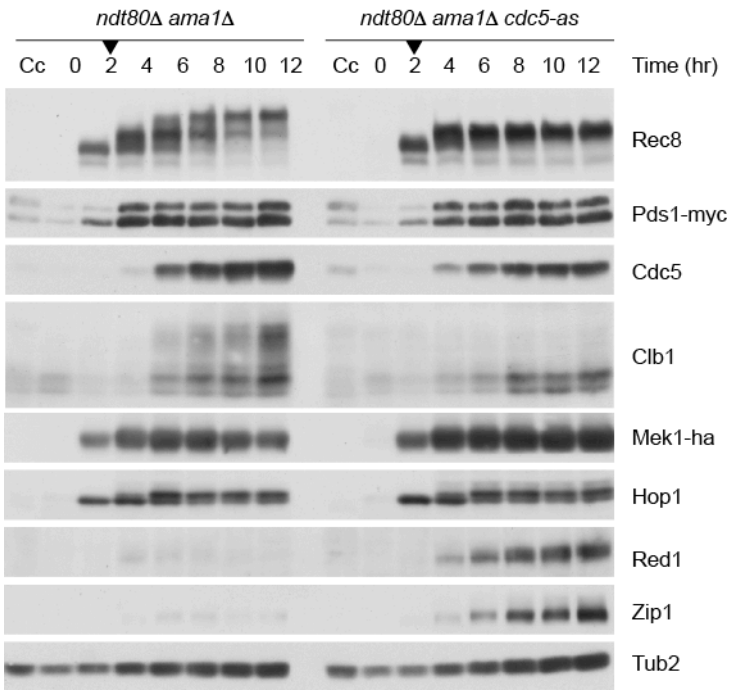

Figure 15. Cdc5 activity is required for the degradation of Zip1 and Red1. Synchronous meiotic time course of the strains *ndt80*Δ *ama1*Δ (Z19050) and *cdc5-as ndt80*Δ *ama1*Δ (Z19092). At 2 hr into meiosis, cells were treated with 20 μM CMK (black triangles) to inhibit Cdc5. Immunoblot analysis of protein levels is shown. Cc means sample from proliferating cells.

Consistent with our previous results, *ndt80*Δ *ama1*Δ cells produced Cdc5 and were unable to accumulate Zip1 or Red1. *ndt80*Δ *ama1*Δ *cdc5-as1* cells also produced Cdc5 with comparable kinetics. However, in the absence of the kinase activity, Zip1 and Red1 accumulated to very high levels. The inhibition of Cdc5 did not affect the levels of other AE proteins, such as Hop1, Mek1 or the cohesin subunit Rec8. Our data suggested that during the prophase I-to-metaphase I transition, Cdc5 induces the disassembly of SC by triggering specifically the destruction of Zip1 and Red1.

Axial Element (AE) proteins, such as Red1, perform several functions in the process of meiotic recombination: (1) SC assembly, (2) efficient DSB formation, (3) MeiRC signaling, and (4) DSB repair from the homologous chromosome and not from the sister chromatid (Page and Hawley, 2004). Among these functions, the role of Red1 as a platform for the MeiRC machinery is key for a functional checkpoint. All proteins involved in MeiRC signaling require Red1 for localization to chromatin. In wild-type meiosis, the repair of DSBs is essential for the silencing of the MeiRC. Our results, however, suggested that the Cdc5-dependent destruction of Red1 could be an alternative mechanism to inactivate the checkpoint and trigger the accumulation of Ndt80, independently of the repair DSBs. In this scenario, the accumulation of Cdc5 leads to the silencing of the MeiRC and the activation of Ndt80, which in turn produces more Cdc5. This set of interactions can be described as a positive feedback loop, because Cdc5 and Ndt80 mutually amplify their own activation. Since this mechanism would rapidly promote the silencing of the MeiRC and the activation of Ndt80, it predicts that even small amounts of prematurely accumulated Cdc5 could force the cells to exit from prophase I, despite the presence of DSBs.

To test this idea, we decided to express Cdc5, tagged with three HA epitopes, in cells that arrest in prophase I due to persistent DSBs. For this purpose we used *dmc1*Δ *rad51*Δ cells that lack the recombinases required for DSB repair (**Figure 16**). During synchronized meiotic time courses, *dmc1*Δ *rad51*Δ cells arrested at prophase I with constant levels of SC proteins and phosphorylated Hop1, which is indicative of MeiRC activity (Carballo et al., 2008). Remarkably, expression of small amounts of HA-tagged Cdc5 in *dmc1*Δ *rad51*Δ cells set in motion the events expected from a Cdc5-Ndt80 positive feedback loop: HA-tagged Cdc5 triggered the destruction of Red1 and the accumulation of Ndt80, which indicates inactivation of the checkpoint. Crucially, this led to the production of endogenous Cdc5, which then enforced the

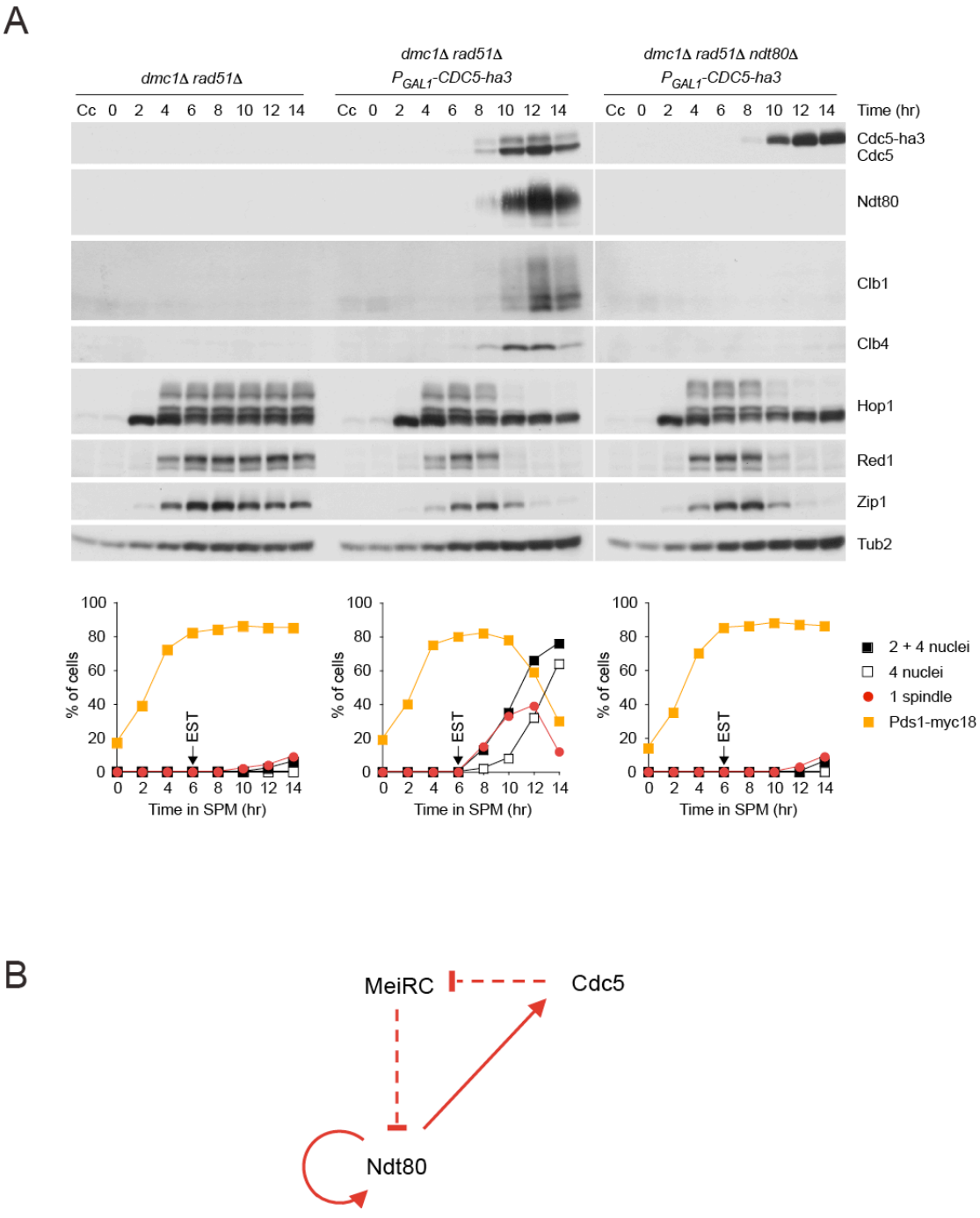

Figure 16. Cdc5 silences the MeiRC independently of DSB repair. **(A)** Analysis of synchronous meiotic time courses of *dmc1*Δ *rad51*Δ (Z19716) and *dmc1*Δ *rad51*Δ P_{EST}-*CDC5* (Z19731) and *dmc1*Δ *rad51*Δ *ndt80*Δ P_{EST}-*CDC5* (Z19732). At 6 hr into meiosis, cells were treated with estradiol to induce Cdc5. Top, immunoblot analysis of protein levels. Cc means sample from proliferating cells. Bottom, immunofluorescence detection of securin/Pds1-myc, MI spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. **(B)** Cdc5-Ndt80-positive feedback loop. By inhibiting the meiotic recombination checkpoint (MeiRC), Cdc5 triggers a positive feedback loop that enforces its own production through the activation of Ndt80. Dashed lines, inhibition. Solid lines, synthesis.

activation of Ndt80 and the production of more Cdc5. The Cdc5-Ndt80-positive feedback loop forced *dmc1*Δ *rad51*Δ cells, not only to exit from prophase I upon HA-tagged Cdc5 induction, but also to progress through both meiotic divisions producing uneven nuclei. Consistently, when HA-tagged Cdc5 was induced in the absence of Ndt80, the MeiRC was silenced, but the cells were unable to exit from the prophase I state. This showed that the induction of small amounts of the HA-Cdc5 in *dmc1*Δ *rad51*Δ cells, led to the firing of an M-phase promoting positive feedback loop between the endogenous Cdc5 and Ndt80 (**Figure 16.B**).

In wild-type cells, the Cdc5-Ndt80-positive feedback loop can ensure the rapid inactivation of the MeiRC at the onset of metaphase I. For this same reason, during prophase I, Cdc5 accumulation must be prevented until the last DSB has been repaired. Even a small amount of prematurely accumulated Cdc5 could result in MeiRC inactivation, forcing the cells into the high-kinase state with damaged chromosomes. We concluded that Cdc5 is a strong inhibitor of the MeiRC. By targeting Cdc5 for degradation during prophase I, APC/C-Ama1 prevents the unscheduled firing of a positive feedback loop, capable of dismantling the MeiRC before recombination has been completed.

2.4. The prophase I-to-metaphase I transition is controlled by a bi-stable switch

Our results showed that the MeiRC and APC/C-Ama1 are required for maintaining the prophase I state. In the presence of DSBs, cells are maintained in prophase I because (1) the MeiRC blocks the expression of the transcription factor Ndt80 and (2) APC/C-Ama1-mediated proteolysis prevents premature stabilization of M-phase proteins. Once DSBs are repaired, Ndt80 inactivates the MeiRC by producing Cdc5 and stops APC/C-Ama1 with inhibitors, such as Clb1-Cdk1. Thus, in prophase I, Ndt80 is repressed while the MeiRC and APC/C-Ama1 are active. At the onset of metaphase I, the opposite holds true.

The MeiRC and APC/C-Ama1 are embedded in a highly interconnected protein network that controls the prophase I-to-metaphase I transition. We decided to investigate the properties of this system by developing a mathematical model in collaboration with Dr. Vinod Unni and Prof. Dr. Béla Novák at Oxford University,

UK. The model integrated our results on the regulation of APC/C-Ama1 and the MeiRC, with previously published work, for instance, on the control of Ndt80 transcription by Sum1 (Pak and Segall, 2002a; Shin et al., 2010). The conversion of our experimental biochemical data into the mathematical equations that compose the model of the prophase I-to-metaphase I transition was done by Dr. Vinod Unni and Prof. Dr. Béla Novák. The resulting regulatory network represented as a wiring diagram is shown in (**Figure 17)**.

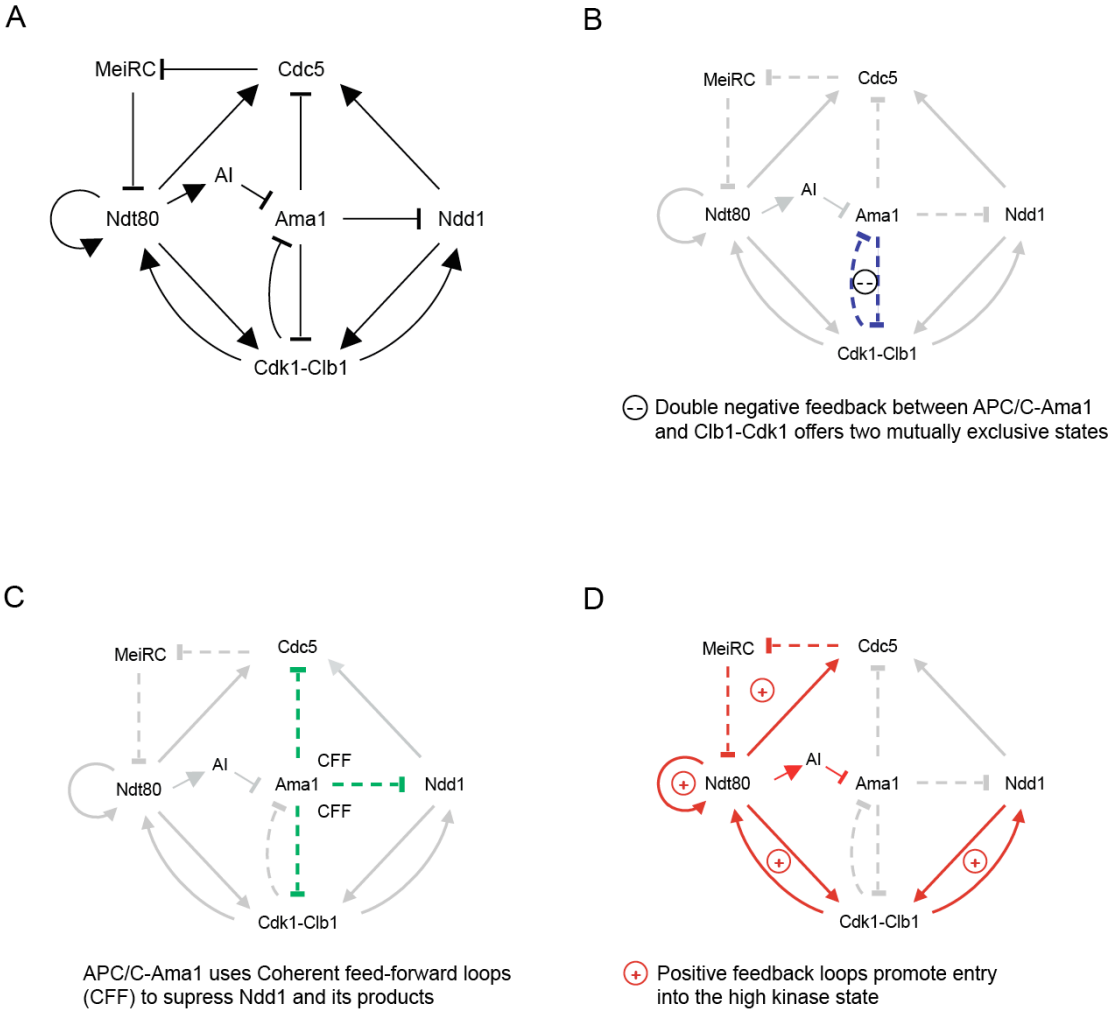

Figure 17. Wiring diagram of the regulatory network of the prophase I-to-metaphase I transition. Work by Dr. Vinod Unni and Prof. Dr. Béla Novák. **(A)** Wiring diagram. Arrows mean synthesis or activation; bar-headed lines represent degradation or inhibition. MeiRC = Recombination Checkpoint. AI = Additional Ndt80-dependent Inhibitor of APC/C-Ama1. Main features of the network are shown. **(B)** Double-negative feedback loop between APC/C-Ama1 and Clb1-Cdk1 creates two mutually exclusive states. **(C)** Coherent feed-forward loops driven by APC/C-Ama1 suppress M-phase promoting loops. **(D)** Several positive-feedback loops promote rapid entry into M-phase. Modified from Okaz *et al*. (2012).

At the heart of this system lays the double-negative feedback loop between Ndt80-dependent inhibitors, such as Clb1-Cdk1, and APC/C-Ama1, which creates two mutually exclusive states. Several positive-feedback loops enforce the entry into metaphase I: (1) Cdk1-Clb1 inhibits APC/C-Ama1 leading to the stabilization of more Clb1. (2) Ndt80 and Ndd1 enhance their own activity by the production of cyclins. (3) Ndt80 induces its own transcription. (4) Cdc5 inactivates the MeiRC triggering the production of Ndt80, which leads to more Cdc5.

To maintain the low kinase state of prophase I in the presence of DSBs, all these positive feedback loops must be counteracted by the MeiRC-mediated repression on Ndt80 and the coherent feed-forward loops used by APC/C-Ama1 to promote proteolysis of M-phase proteins (Clb1, Cdc5, Ndd1). Each of these interactions was translated in to a set of ordinary differential equations that describe the synthesis, degradation, activation, and inhibition of the components of the network. A formal description of the resulting mathematical model can be found in Okaz *et al*., (2012).

Mathematical simulations revealed that the network contains two stable steady states, (1) a low-kinase state, or prophase I, in which M-phase proteins are unstable and poorly synthesized and (2) a high-kinase state, or metaphase I, where the situation is reversed. In a steady state, all the dynamical variables of the system remain constant over time. In the model of the prophase I-to-metaphase I transition, enzyme activities or regulated protein levels are examples of dynamical variables. Steady states can be stable or unstable depending on how they react towards perturbations. On the one hand, an unstable steady state is very hard to maintain over time, because any perturbation forces the system to abandon such a state. A soccer ball resting precisely at the tip of a sharp mountain ridge would be an example of a system in an unstable steady state; whereas the ball can theoretically rest on the ridge, the slightest perturbation, such as gust of wind, causes it to roll down on either side of the mountain. In other words, a perturbation causes the ball moves away from the unstable steady state. On the other hand, experiencing small perturbations is not a threat for a stable steady state, because the system always returns to the previous conditions, after being disturbed. Our soccer ball could find a stable steady state by settling at the bottom of a valley, one could kick the ball uphill many times, but it will always roll back to the bottom of the valley. Thus, by identifying prophase I and metaphase I as stable steady states, the model shows that both stages can be kept for

long periods of time because they can recover from perturbations. However, the stability of a steady state depends on the values of the parameters of the model. Dramatic changes in the synthesis of a protein like Ndt80, or the sudden inactivation of a major regulator of proteolysis such as APC/C-Ama1, can transform previously stable steady states into unstable ones. The critical point in which a steady state experiences an abrupt change in its stability is called bifurcation. Such transitions can be plotted as one-parameter bifurcation diagrams. In this graphical depiction of the behavior of the steady states, all variables are kept constant except one, known as the bifurcation parameter, which is independent of the system. This representation is analogous to a signal-response curve, since it shows how the system responds to changes in the bifurcation parameter (signal). The concentration of Ama1 is an example of a bifurcation parameter, because, unlike its activity, it is independent of the system. Ama1 levels remain constant during the prophase I-to-metaphase I transition, and, importantly, the concentration of Ama1 could be changed experimentally. Any dynamic variable of the system can be plotted against the concentration of Ama1. We chose Cdc5 since it is an intuitive indicator of the kinase state. Low Cdc5 levels correspond to the low-kinase state of prophase I; conversely, high Cdc5 levels correspond to metaphase I.

The resulting bifurcation diagram shows the behavior of the system when DSBs are present (**Figure 18)**. The solid lines correspond to the stable steady states, which are separated by unstable steady states (dashed line). The curve displays some features intuitively expected from a signal-response curve between Ama1 and Cdc5. For example, very high values of Ama1 correspond to very low values of Cdc5, and vice versa. However, the predominant feature of the system is the overlap of the two stable steady states over a physiological range of Ama1 levels. This emergent property, called bi-stability, proposes that for any value of Ama1 within the bi-stable region, the system has two responses, one in the low-kinase state and other in the high-kinase state. For meiotic cells, this prediction means that both prophase I and metaphase I could be reached with the same levels of Ama1.

To test this prediction experimentally, we constructed a *dmc1*Δ *rad51*Δ *ama1*Δ P_{SCC1}-*CDC20* strain in which the estradiol-inducible promoter controls the expression of Ama1 (P_{EST}-*AMA1*). Due to the absence of the recombinases Dmc1 and Rad51, these

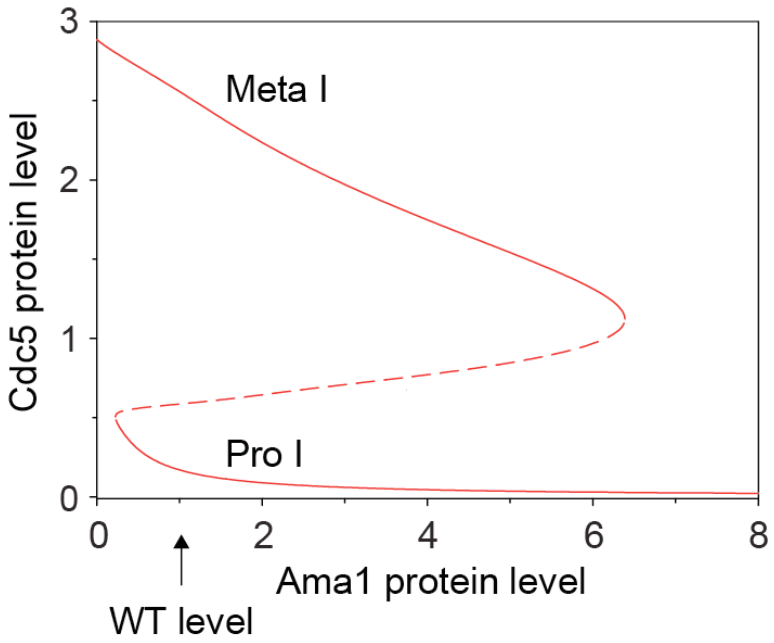

Figure 18. Bi-stability of the prophase I-to-metaphase I transition. Work by Dr. Vinod Unni and Prof. Dr. Béla Novák. One-parameter bifurcation diagram, or signal-response curve, showing the effect of Ama1 protein level (the signal) on Cdc5 steady-state concentration (the response of the system) in the presence of DSBs. Cdc5 steady-state concentration was chosen because it is an indicator of the kinase state: low Cdc5 values correspond to prophase I, while high values represent metaphase I. The stable steady states (solid lines) at low (prophase I) and high (metaphase I) Cdc5 levels are separated by unstable steady states (dashed line). Arrow, cellular Ama1 level at prophase I/metaphase I in the wild-type. Notice that the two stable steady states overlap over a physiological range of Ama1 protein levels. Within this region, the system is bi-stable, that is, both the low- and the high-kinase states can be reached with the same level of Ama1. Modified from Okaz *et al*. (2012).

cells cannot repair DSBs. Consistent with our previous results, when Ama1 was not expressed, *dmc1*Δ *rad51*Δ *ama1*Δ P_{SCC1}-*CDC20* P_{EST}-*AMA1* cells could not maintain the low-kinase state of prophase I because of the premature accumulation of Cdc5. As a consequence, the MeiRC was inactivated and the cells were forced to arrest in the high-kinase state of metaphase I, due to Cdc20 depletion (**Figure 19)**. We then asked which stable steady state would prevail if (1) Ama1 was induced at an early time point, ahead of Ndt80, when the low-kinase state is still available, or (2) the same levels of Ama1 were induced after the appearance of Ndt80, once the cells have reach the high-kinase state. When Ama1 was expressed ahead of Ndt80, the *dmc1*Δ

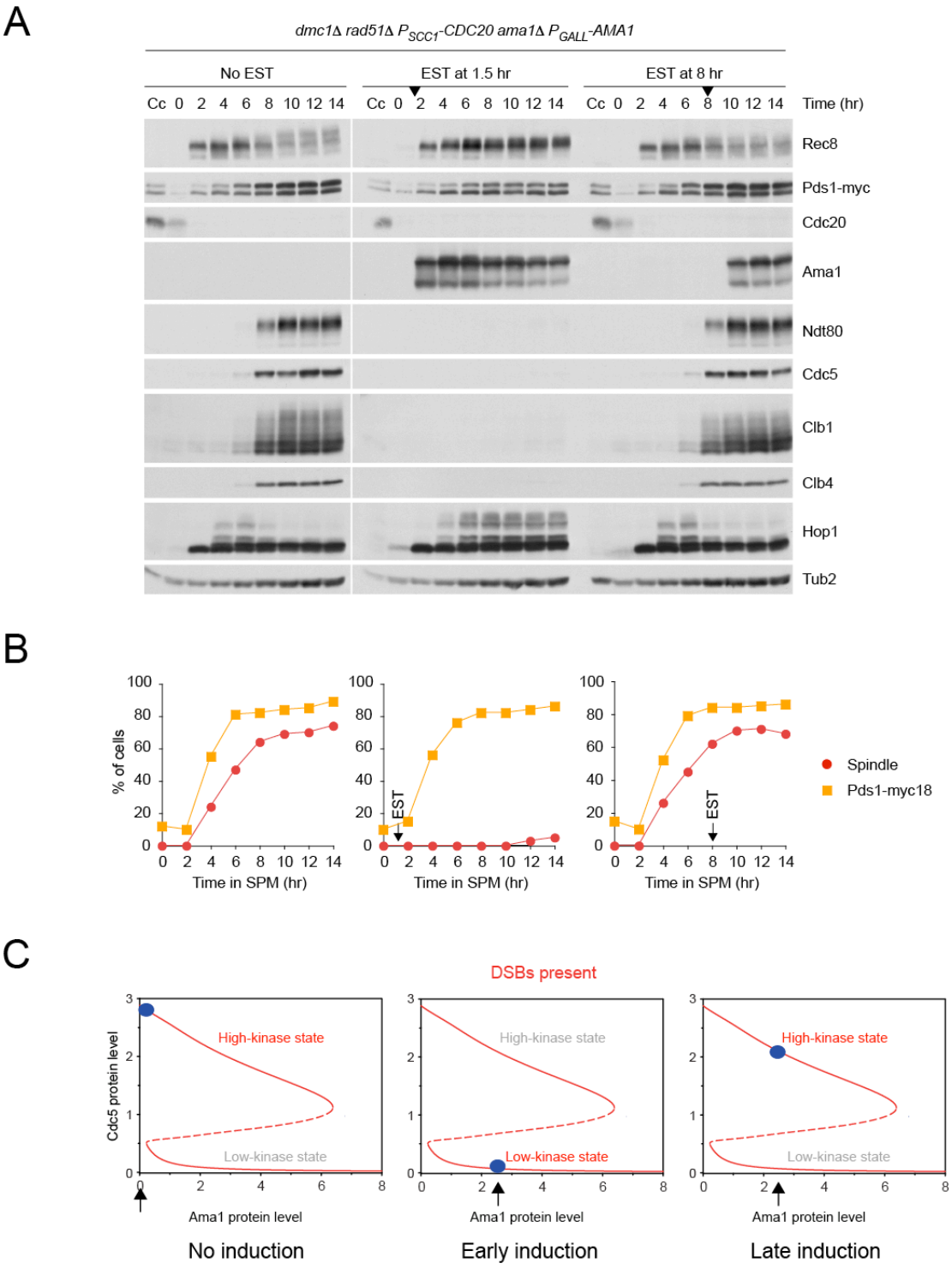

Figure 19. Bi-stability is an emergent property of the prophase I-to-metaphase I transition. Synchronous meiotic time course of *dmc1Δ rad51Δ ama1Δ* P_{SCC1}*-CDC20* P_{EST}*-AMA1* (Z19785). Cells were left untreated or treated with estradiol at 1 hr or 8 hr into meiosis. **(A)** Immunoblot analysis of protein levels. Cc means sample from proliferating cells. **(B)** Immunofluorescence detection of securin/Pds1-myc and MI spindles (α-tubulin) in fixed cells. **(C)** Graphical interpretation of the experiment according to the quantitative model of the prophase I-to-metaphase I transition.

rad51Δ ama1Δ P_{SCC1}*-CDC20* P_{EST}*-AMA1* cells remained arrested in the low-kinase state of prophase I. The MeiRC was active, as judged by Hop1 phosphorylation, and M-phase proteins did not accumulate. Under these conditions, APC/C-Ama1 prevented premature accumulation of Cdc5 and B-type cyclins, allowing the MeiRC to block Ndt80 and to maintain the cells stably in the low-kinase state of prophase I. Remarkably, when the same levels of Ama1 were expressed after the appearance of Ndt80, the cells maintained the high-kinase state of the metaphase I-arrest. The late induction of Ama1 did not trigger the degradation of cyclins or the return to the prophase I-conditions. In agreement with the model, prophase I and metaphase I were stable steady states that could be reached with the same levels of Ama1.

This result showed that the prophase I-to-metaphase I transition is controlled by a bi-stable switch. In wild-type cells, the low-kinase state of prophase I is maintained stably as long as DSBs persist, because the MeiRC and APC/C-Ama1 counteract M-phase promoting loops. However, once DSBs are repaired, Ndt80 fires several positive feedback loops that force the cells into the high-kinase state. The switch can be visualized by comparing the behavior of the system before (red curve) and after (blue curve) DSB repair (**Figure 20**).

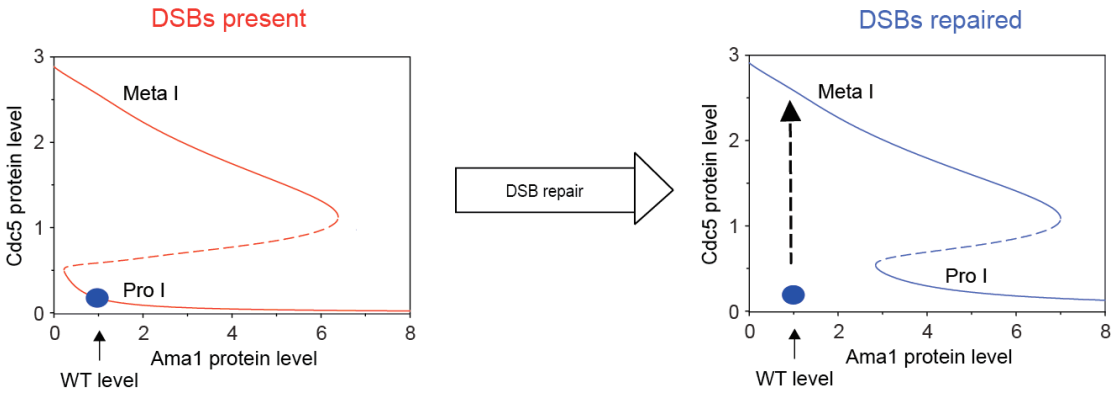

Figure 20. The transition from prophase I-to-metaphase I as a bistable switch. Work by Dr. Vinod Unni and Prof. Dr. Béla Novák. One-parameter bifurcation diagrams, or signal-response curve, showing the effect of Ama1 protein level (the signal) on Cdc5 steady-state concentration (the response). Left, as long as DSBs are present, the low-kinase state of prophase I is a stable steady state at wild-type levels of Ama1. Right, once DSBs are repaired, the low kinase state moves towards higher values of Ama1. Since the levels of Ama1 remain constant, the only stable steady state left is the high kinase state, which forces the cells to enter metaphase I. Modified from Okaz *et al.* (2012).

In the presence of DSBs, both the low- and the high-kinase state can be reached with prophase I-levels of Ama1 (red curve). Wild-type cells rest in the stable steady low-kinase state of prophase I first, because they activate the MeiRC and APC/C-Ama1 before Ndt80 appears. However, once DSBs are repaired, Ndt80 is activated, producing inhibitors of the MeiRC and APC/C-Ama1. This change in the system makes the low-kinase state available only at higher values of Ama1 (blue curve). Since Ama1 levels remain constant during the transition, the cells have no option but to go towards the only stable steady state left, metaphase I**.** A bi-stable control system can explain why the decision to exit prophase I is an abrupt, all-or-nothing, irreversible, and therefore, unidirectional process.

The model of the prophase I-to-metaphase I transition offered two other testable predictions (**Figure 21)**. (1) At Ama1 levels close to zero, only the high-kinase state is available. The low-kinase state does not extent to very small values of Ama1. This predicts that cells will be forced to the high-kinase state if the Ama1 levels drop abruptly during prophase I. (2) At very high levels of Ama1, only the low-kinase state exists. Interestingly, this prediction implies that an increase in the Ama1 levels could be a mechanism to exit from the high-kinase state.

2.5. Return-to-growth involves the destruction of Ama1 and the accumulation of Ndd1

In the model of the prophase I-to-metaphase I transition, the low-kinase state does not extent to values of Ama1 approaching zero. This feature offers the possibility to establish the high-kinase state if Ama1 would be suddenly destroyed. Although this could be seen as a theoretical consideration, there is indeed a physiological situation in which such mechanism could be used. During return to growth, yeast cells transit from the low-kinase state of prophase I into the high-kinase state of mitotic M-phase (Dayani et al., 2011). The model envisions that, during this transition, Ama1 must be down-regulated to allow the accumulation of Ndd1, the M-phase proteins and the establishment of the mitotic high-kinase state.

To test the behavior of Ama1 during return to growth, we induced *rad50S* cells to synchronously enter meiosis (**Figure 22)**. The *rad50S* strain is unable to repair DSBs

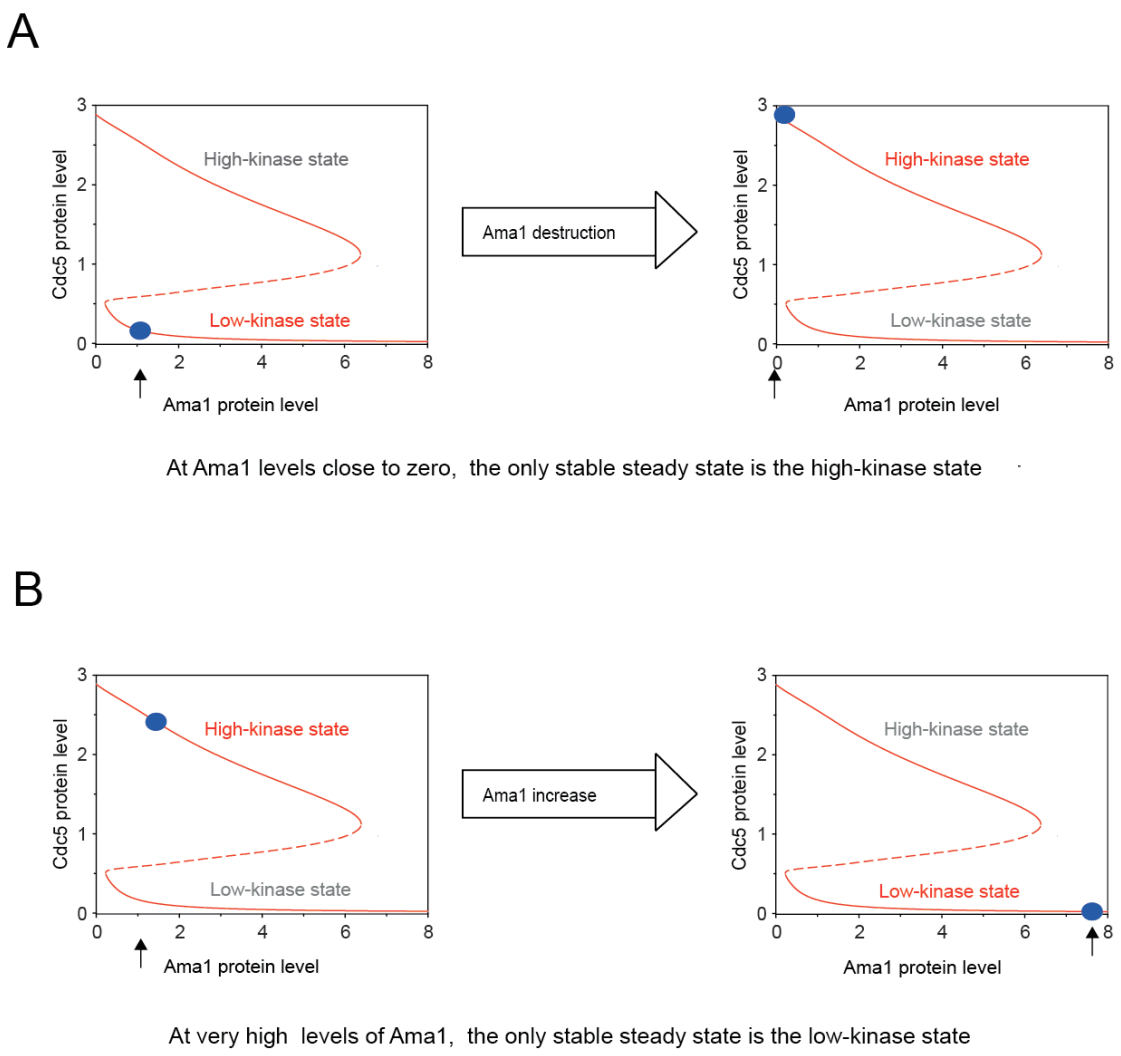

Figure 21. Ama1 levels are a critical to establish the high- or low-kinase state. Graphical interpretation of the two predictions of the mathematical model of the prophase I-to-metaphase I transition. **(A)** cells in the low-kinase state could reach the high-kinase state by destroying Ama1 (Ama1 = 0). **(B)** Cells could exit the high-kinase state by increasing Ama1 to very high levels.

due to a defect in the removal of Spo11 from the 5' end at the DSB site (Keeney et al., 1997).

In response to the unrepaired DSBs, *rad50S* cells are maintained in the low-kinase state of prophase I with an active checkpoint. After six hours into the time course, one half of the culture was resuspended in rich medium (YPD), the other half in sporulation media (SPM). Samples for immunofluorescence and TCA protein extracts were taken every hour after the transfer to rich media. Under these conditions, *rad50S* cells kept in SPM remained arrested in prophase I. By contrast, upon transfer to rich

medium, *rad50S* cells budded, and entered the high-kinase state of mitotic metaphase. Interestingly, the cells arrested in M-phase with high levels of slow migrating forms of securin/Pds1, Dbf4 and Rec8, which could be due to the unrepaired DSBs sensed by the DNA damage checkpoint (**Figure 22)**. Analysis of protein extracts by western blotting showed that M-phase proteins, such as Ndd1, Clb4, Clb2, and Cdc5, accumulated upon transfer to rich medium. Remarkably, in agreement with the prediction from the model, Ama1 was rapidly and specifically destroyed during the transition from prophase I into mitotic M-phase. By contrast, other meiotic-specific proteins, such as Red1 and Zip1, persisted until the onset of mitotic metaphase. We concluded that the establishment of the mitotic high-kinase state during return-to-growth involved the inactivation of APC/C-Ama1, by the abrupt down-regulation of the levels of Ama1.

During return to growth in *rad50S* cells, both Ndt80 and Ndd1 could potentially trigger entry into the high-kinase state, once Cdc5 is accumulated. However, we observed that *rad50S* cells selectively accumulated Ndd1, whereas Ndt80 was not detectable upon transfer to rich medium. This suggested that meiotic controls, such as Ndt80, are dispensable for establishing the high-kinase state during return to growth. To confirm this idea, we induced *ndt80*Δ cells to synchronously enter meiosis. After six hours in SPM, return-to-growth was induced as before. Under these conditions, *ndt80*Δ cells kept in SPM remained stably arrested in prophase I. By contrast, upon transfer to rich medium, *ndt80*Δ cells budded and, within 2 hours, they assembled spindles, and started mitotic divisions (**Figure 23)**. Analysis of protein extracts showed that (1) Ndd1 and M-phase proteins accumulated rapidly upon transfer to rich medium. (2) Several proteins present in prophase I, such as securin/Pds1, Dbf4, Rec8, Clb5, Red1, and Zip1 persisted until, roughly, the onset of the first division. (3) Remarkably, the important meiotic regulators Spo13 and the kinase Ime2 were destroyed as abruptly as Ama1 upon transfer to rich media. This result showed that main meiotic controls, such as Ndt80 or Ime2, were not required for the transition from the low-kinase state of prophase I to the high-kinase state of mitosis. We concluded that return to growth involves the suppression of meiotic regulators and the specific selection of mitotic controls for the entry into M-phase.

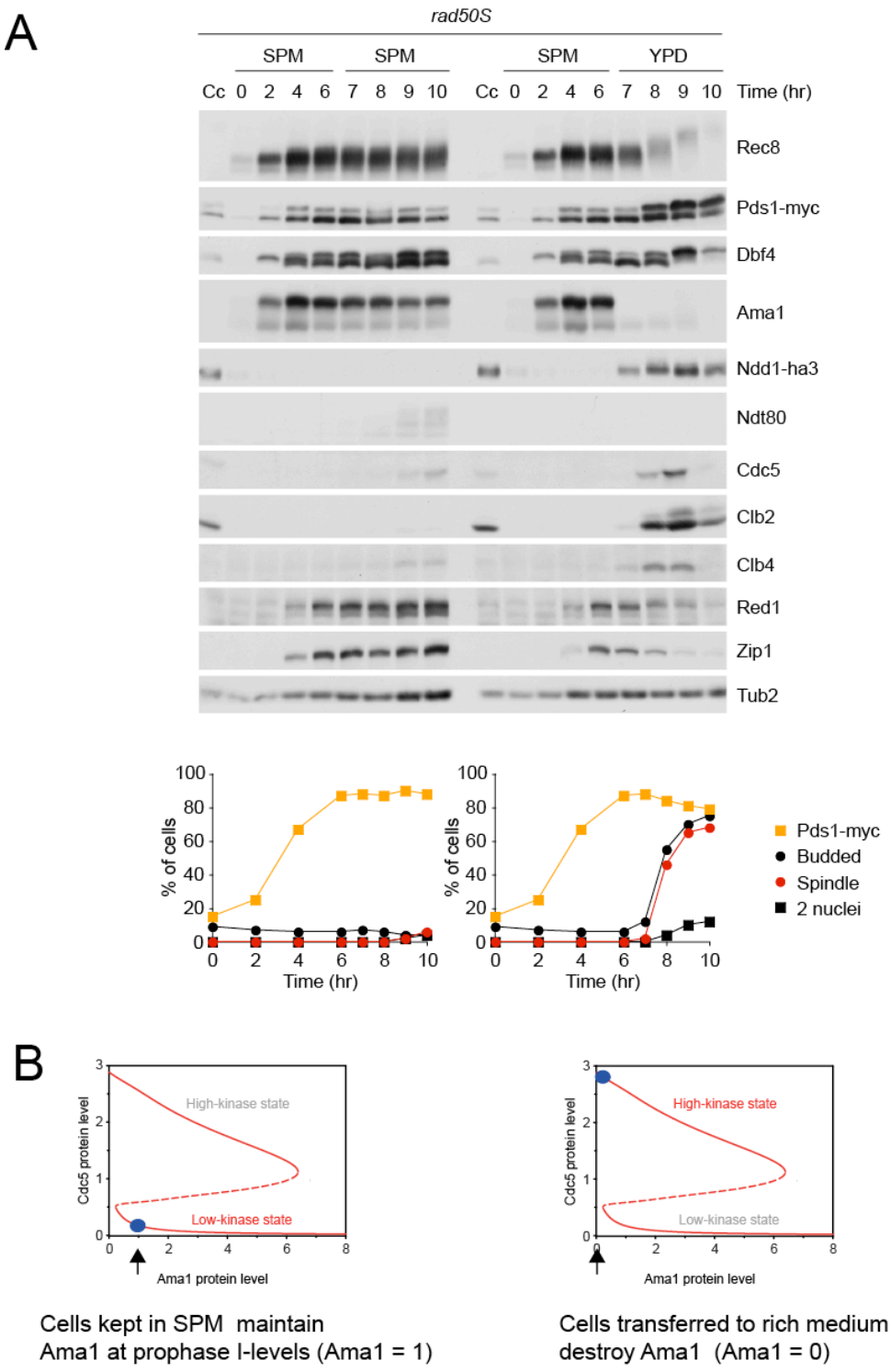

Figure 22. Return to growth involves the destruction of Ama1. Synchronous meiotic time course and return-to-growth of *rad50S* strains (Z12463). At 6 hr into meiosis, the culture was split, one half was re-suspended in sporulation medium (SPM) and the other half in rich medium (YPD). **(A)** Top, immunoblot analysis of protein levels. Bottom, immunofluorescence detection of budding, securin/Pds1-myc, mitotic spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. **(B)** Graphical interpretation of the experiment according to the model of the prophase I-to-metaphase I transition. Left, cells kept in SPM maintain prophase I-levels of Ama1 and remain in the low-kinase state. Right, cells transferred to rich medium establish the high-kinase state because Ama1 disappears, allowing the accumulation of Ndd1 and M-phase proteins.

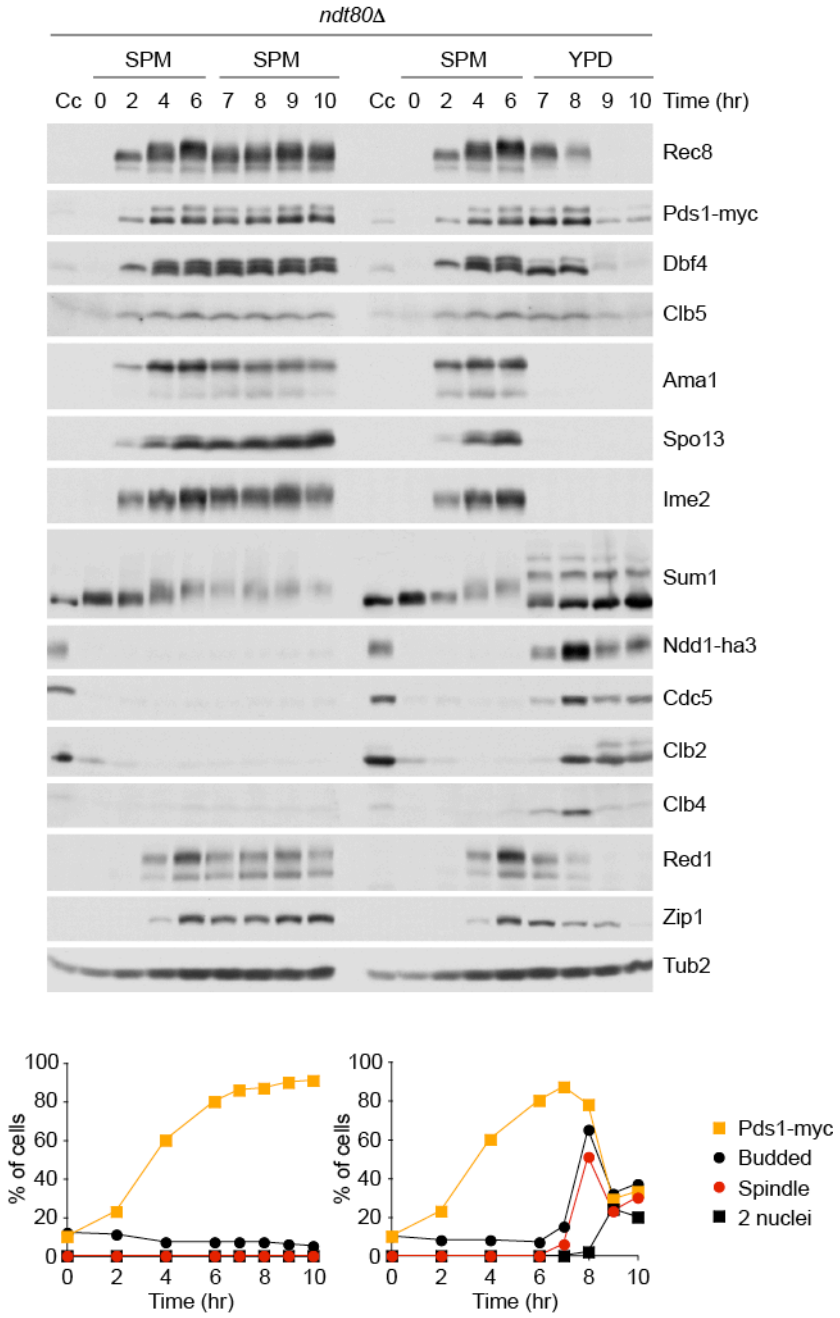

Figure 23. Meiotic controls are not required for return-to-growth. Synchronous meiotic time course of *ndt80*Δ strains (Z17725). At 6 hr into meiosis, the culture was split. One half was re-suspended in sporulation medium (SPM) and the other half in rich medium (YPD). Top, immunoblot analysis of protein levels. Bottom, immunofluorescence detection of budding, securin/Pds1-myc, mitotic spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells.

2.6. APC/C-Ama1 is sufficient, but not essential, to trigger the exit from the high-kinase state after metaphase I

Wild-type cells do not remain locked in the high-kinase state forever. After precisely two meiotic divisions, a low-kinase state is established and sporulation, the yeast equivalent of gametogenesis, occurs. During mitosis, the mechanisms that control the exit from the high-kinase state relay heavily on the Cdk1-opposing phosphatase Cdc14. Pathways, such as the MEN network, ensure that Cdc14 counteracts Cdk1 activity after the mitotic division occurs. It has been puzzling that this mechanism is largely dispensable for meiotic exit (Attner and Amon, 2012; Pablo-Hernando et al., 2007). Indeed, it is not understood how meiotic cells exit the high-kinase state after precisely two divisions. The model of the prophase I-to-metaphase I transition proposed the interesting possibility that the exit from the high-kinase state in meiosis could require a very different and meiosis-specific mechanism: the accumulation of high levels of Ama1 to reactivate APC/C-Ama1.

This idea predicts that the exit from the high-kinase state reached at metaphase I can be triggered by a strong increase in the levels of Ama1. We first analyzed P_{SCC1}-*CDC20* cells arrested in metaphase I and found, interestingly, that Ama1 is strongly up-regulate at later time points (**Figure 24. Left panel**). Assuming that very high levels of Ama1 are sufficient to reactivate APC/C-Ama1, P_{SCC1}-*CDC20* cells should degrade M-phase proteins once Ama1 accumulates. However, M-phase proteins in P_{SCC1}-*CDC20* cells remained almost constant throughout the time course, despite the strong accumulation of Ama1 at later time points. This observation suggested that the exit from the high-kinase state requires mechanisms in addition to the increased Ama1-levels. Another requirement for the exit from the high-kinase state can be the down-regulation of APC/C-Ama1 inhibitors. Since we have identified Clb1-Cdk1 as a specific inhibitor of APC/C-Ama1, we decided to analyze cells arrested in metaphase I lacking Clb1 (**Figure 24**). In a synchronous meiotic time course, P_{SCC1}-*CDC20* cells arrested at metaphase I with constant levels of M-phase proteins and meiosis I spindles. *clb1*Δ P_{SCC1}-*CDC20* cells reached metaphase I but, upon the up-regulation of Ama1 protein levels, M-phase proteins were efficiently destroyed, the spindle disassembled, and cells underwent a single nuclear division which led to the production of dyads. This effect was Ama1-dependent since the triple mutant *ama1*Δ

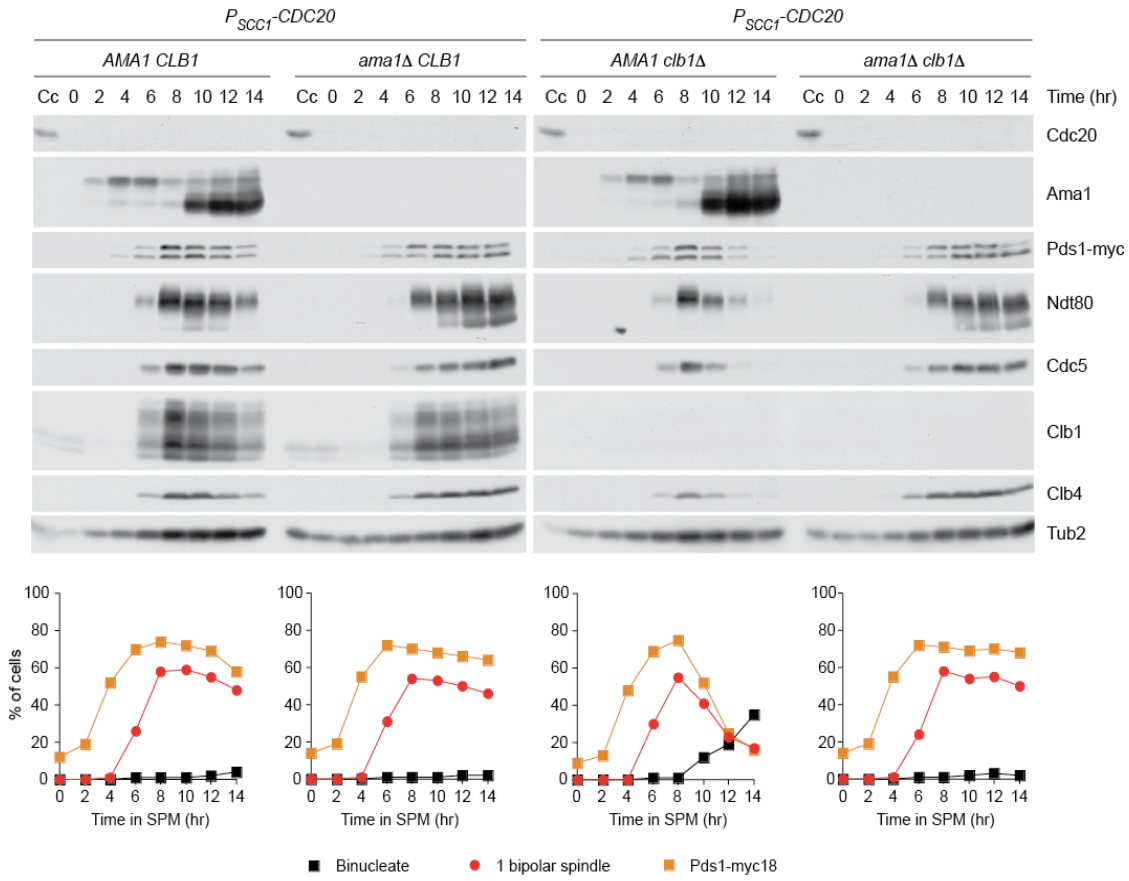

Figure 24. The re-activation of APC/C-Ama1 is sufficient to exit from the high-kinase state. Synchronous meiotic time course of the strains P_{SCC1}-*CDC20* (Z18334), *ama1*Δ P_{SCC1}-*CDC20* (Z18333), *clb1*Δ P_{SCC1}-*CDC20* (Z18332), and *clb1*Δ *ama1*Δ P_{SCC1}-*CDC20* (Z18331). Top, immunoblot analysis of protein levels. Bottom, immunofluorescence detection of securin/Pds1-myc, meiosis I spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown.

*clb1*Δ P_{SCC1}-*CDC20* stayed stably arrested in metaphase I. The reactivation of APC/C-Ama1 was a specific consequence of deleting *CLB1*, since the deletion of *CLB4* did not trigger the destruction of M-phase proteins **(Figure 25)**. Consistent with the role of Cdk1 as an APC/C-Ama1 inhibitor, the degradation of M-phase proteins in P_{SCC1}-*CDC20* cells was also triggered by inhibition of Cdk1 activity, in an Ama1-dependent manner **(Figure 26)**. By contrast, M-phase protein levels remained constant after inhibition of Cdc5 **(Figure 27)**. We concluded that a strong increase in the levels of Ama1, together with the down-regulation of APC/C-Ama1 inhibitors, is sufficient to trigger the exit from the high-kinase state of metaphase I.

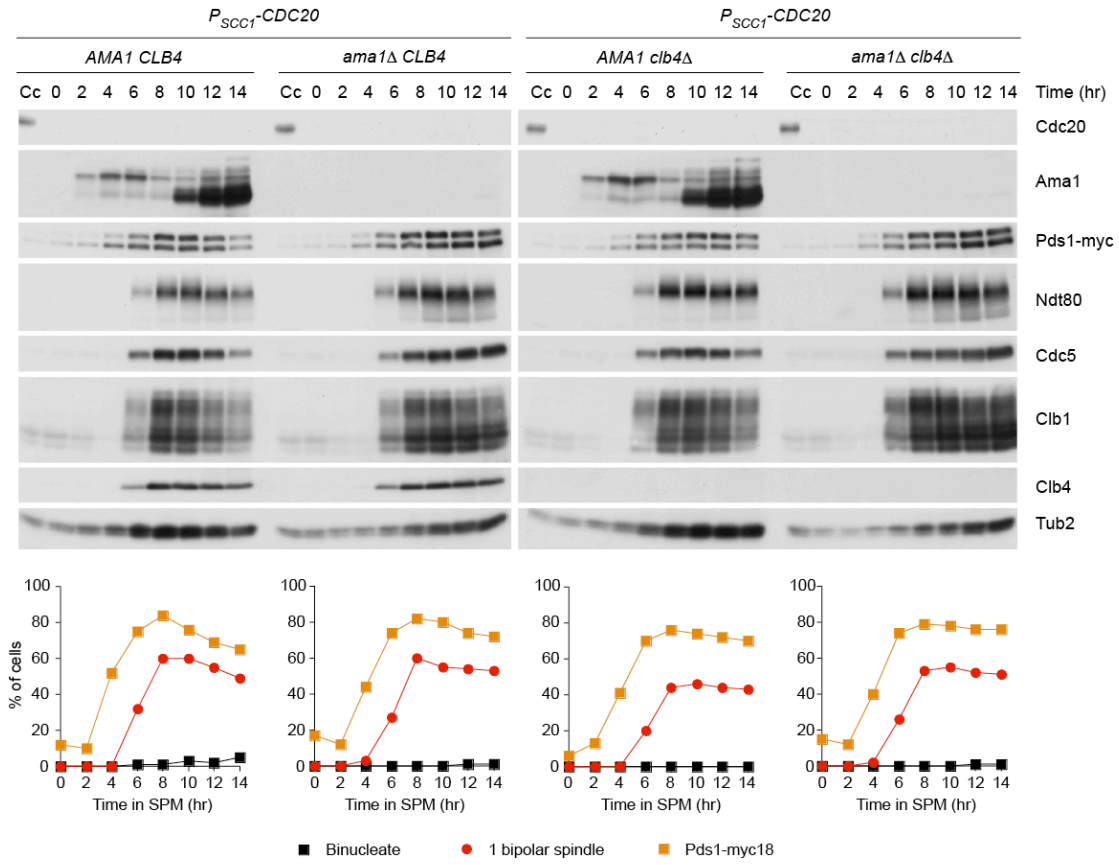

Figure 25. Deletion of *CLB4* does not trigger exit from the high-kinase state of metaphase I .
Synchronous meiotic time course of the strains P_{SCC1}-*CDC20* (Z18334), *ama1*Δ P_{SCC1}-*CDC20* (Z18333), *clb4*Δ P_{SCC1}-*CDC20* (Z18359), and *clb4*Δ *ama1*Δ P_{SCC1}-*CDC20* (Z18358). Top, immunoblot analysis of protein levels. Bottom, immunofluorescence detection of securin/Pds1-myc, meiosis I spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown.

*clb1*Δ P_{SCC1}-*CDC20* cells showed that the re-activation of APC/C-Ama1 during metaphase I terminated the high-kinase state preventing a second nuclear division. This implies that the re-activation of APC/C-Ama1 in wild-type cells must be tightly controlled, since a premature exit from the high-kinase state could bypass the second meiotic division.

The presence of APC/C-Ama1 inhibitors could block APC/C-Ama1 re-activation during nuclear divisions. However, It has been reported that Clb1 is a substrate of

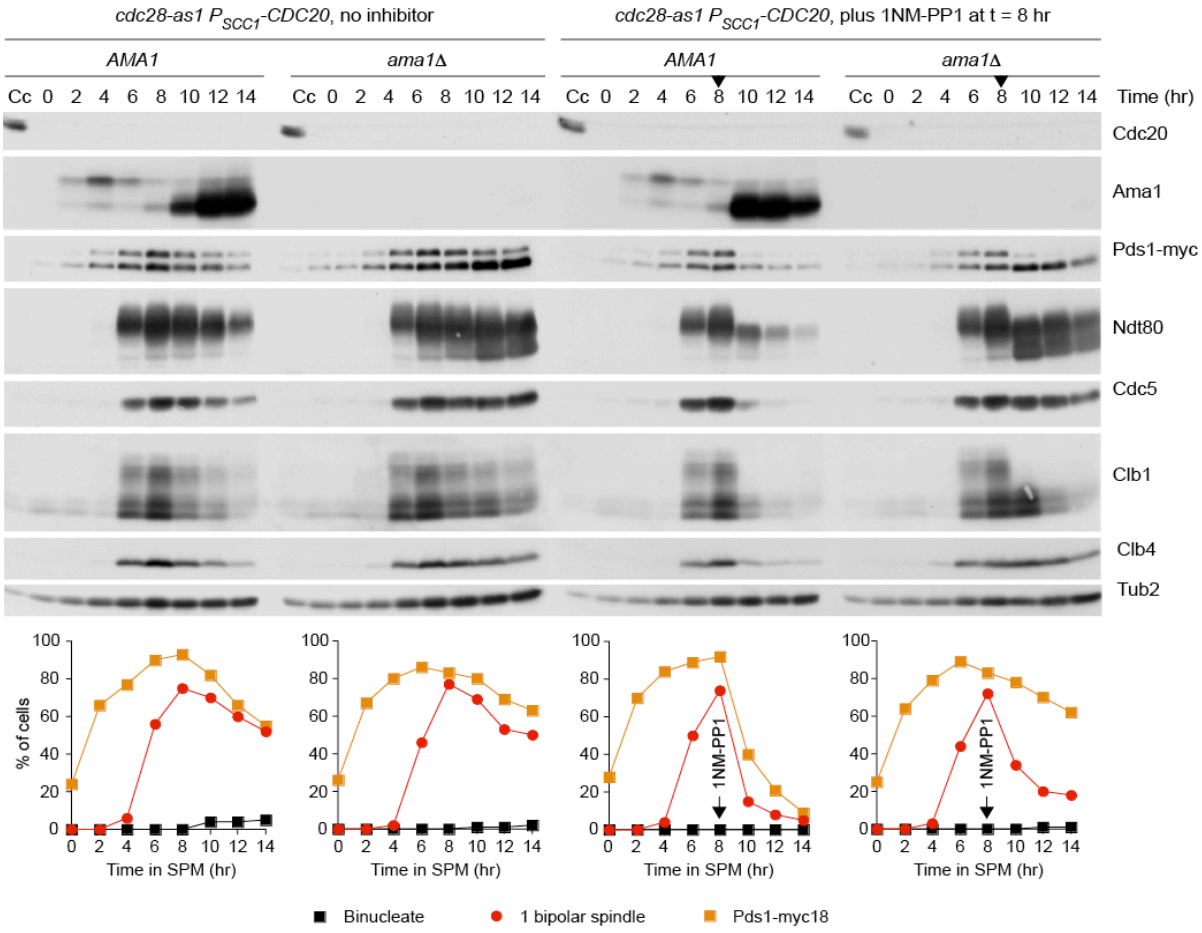

Figure 26. Down-regulation of Cdk1 triggers the reactivation of APC/C-Ama1. Synchronous meiotic time course of the strains *cdc28-as1 P_{SCC1}-CDC20* (Z17972), *ama1Δ cdc28-as1 P_{SCC1}-CDC20* (Z17971). At 8 hr into meiosis, the culture was split and cells were treated with DMSO or 5μM 1NM-PP1. Top, immunoblot analysis of protein levels. Bottom, immunofluorescence detection of securin/Pds1-myc, meiosis I spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown.

APC/C-Cdc20 during meiosis I. If APC/C-Ama1 inhibitors, such as Clb1, are down-regulated during meiotic divisions, how can wild-type cells ensure that APC/C-Ama1 does not trigger a premature exit from the high-kinase state? APC/C activators can be controlled by post-translational modifications, the regulation of their sub-cellular localization, and protein levels (Pesin and Orr-Weaver, 2008). Among these mechanisms, the model of the prophase I-to-metaphase I transition, predicts that the control of Ama1 levels should be relevant. We decided to analyze the protein levels

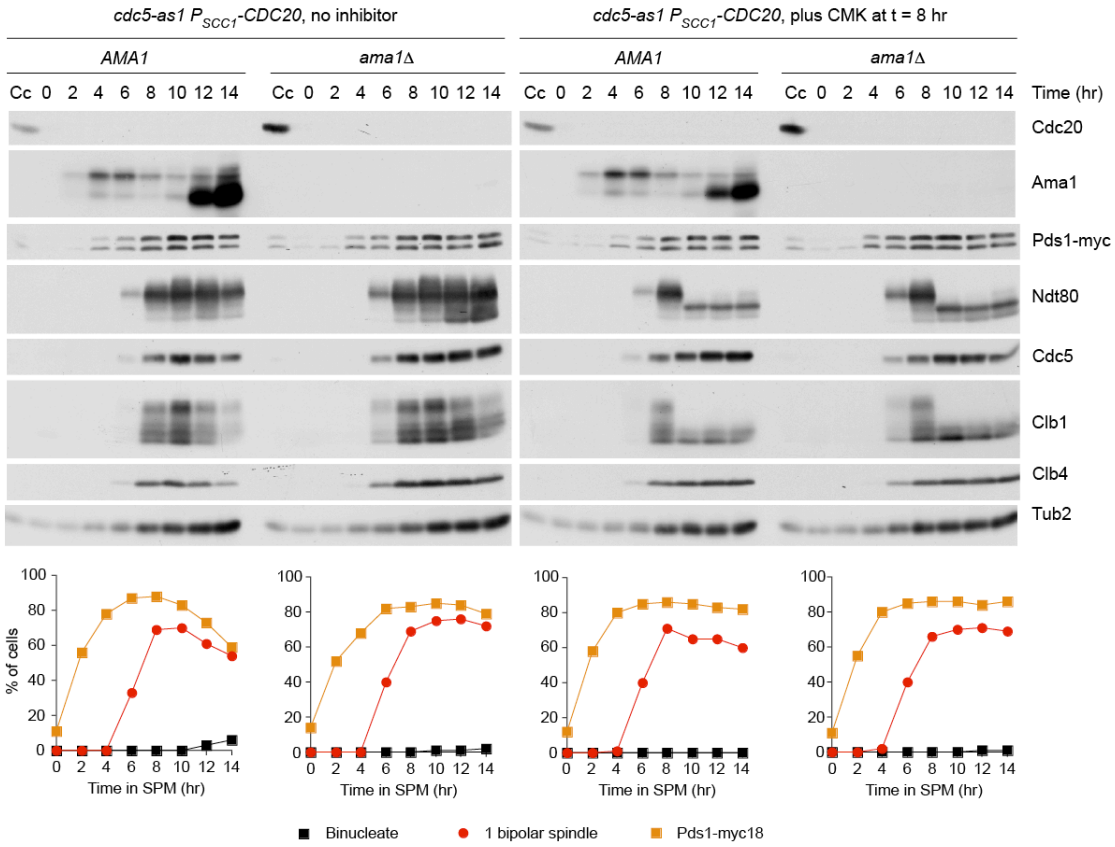

Figure 27. Inhibition of Cdc5 does not trigger the reactivation of APC/C-Ama1. Synchronous meiotic time course of the strains *cdc5-as* P_{SCC1}*-CDC20* (Z18209), *ama1*Δ *cdc5-as* P_{SCC1}*-CDC20* (Z18210). At 8 hr into meiosis, the cultures were treated with DMSO or 20 µM CMK. Top, immunoblot analysis of protein levels. Bottom, immunofluorescence detection of securin/Pds1-myc, meiosis I spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown.

and localization of N-terminally Myc18-tagged Ama1 during a synchronous meiotic time (**Figure 28)**. The onset of metaphase I, the progression through nuclear divisions, and the formation of viable spores, were unaffected by the tag. Consistent with previous reports (Oelschlaegel et al., 2005; Penkner et al., 2005), Ama1 was detectable since early stages of meiosis. Remarkably, the protein accumulated to very high levels towards the end of the time course. To determine the specific meiotic stage at which Ama1 is up-regulated, we detected Myc18-Ama1 by immunofluorescence. The immunostaining revealed that Ama1 displayed nuclear

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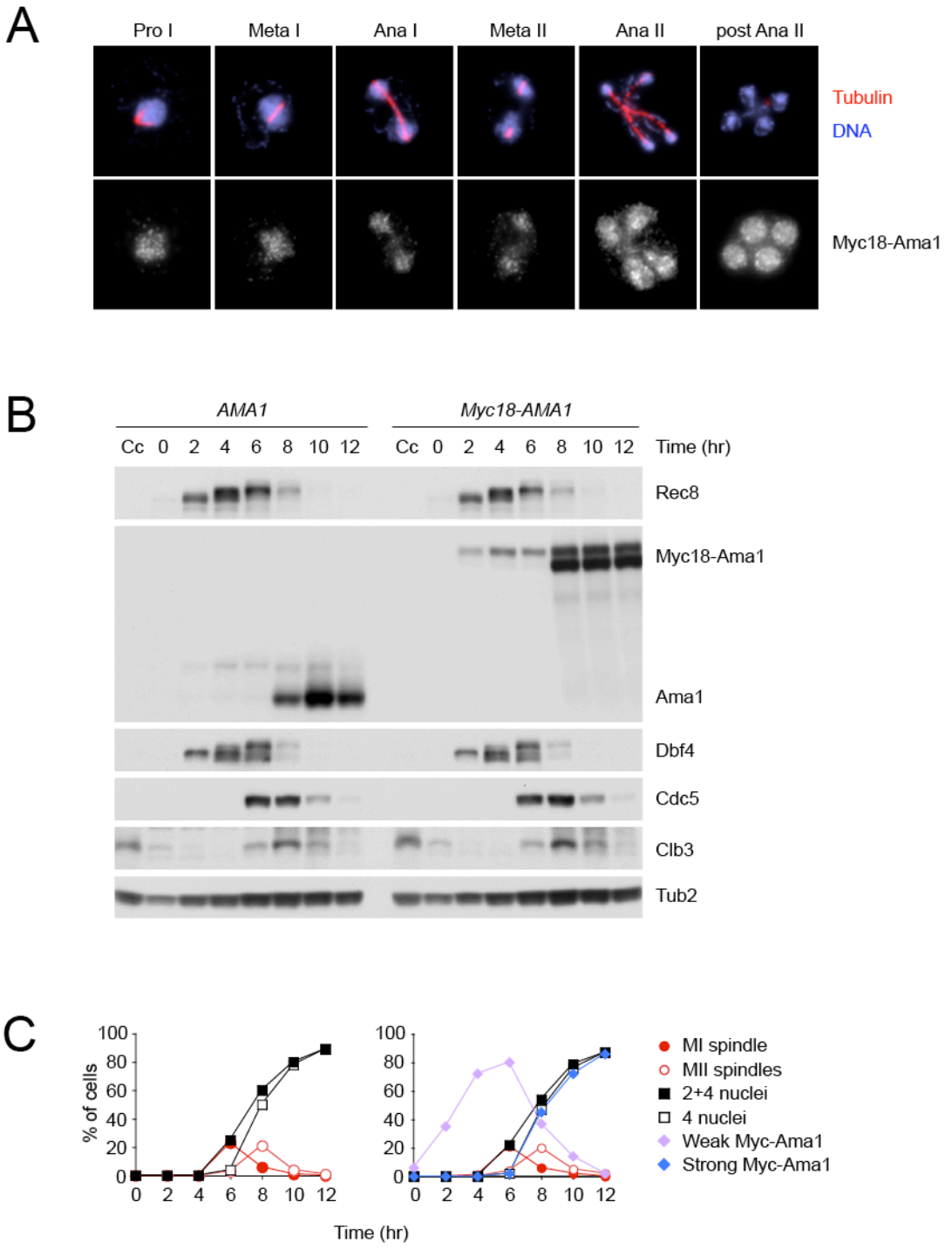

Figure 28. Ama1 levels are up-regulated at the end of the second meiotic division. Synchronous meiotic time course of wild-type (Z12185) and *Myc18-AMA1* (Z3086) cells. **(A)** Immunoblot analysis of protein levels. **(B)** Immunofluorescence detection of Myc18-Ama1 (weak or strong signal), meiosis I or meiosis II spindles (α-tubulin), and divided nuclei (DAPI) during the time course in fixed cells. Percentage of cells per time point is shown. **(C)** Representative immunostained cells for each meiotic stage are shown.

localization and its levels were almost unchanged from prophase I until metaphase II. Crucially, the signal of Ama1 became strongly up-regulated at the onset of anaphase II. We conclude that the increase in the levels of Ama1 occurs precisely at the end of the second meiotic division. By setting the up-regulation of Ama1 at this stage, the exit from the high-kinase state can take place after two nuclear divisions, and not before. These observations indicated that, in wild-type meiosis, the re-activation of APC/C-Ama1 could be crucial for the termination of the high-kinase state in late meiosis

To analyze the contribution of APC/C-Ama1 to the exit from the high-kinase state, we analyzed the behavior of *ama1*Δ cells in late meiosis (**Figure 29)**. We considered the disassembly of meiosis II-spindles as the landmark event for the exit from the high-kinase state. In a wild-type meiotic time course, the appearance of M-phase proteins and the assembly of meiosis I-spindles coincided with the abrupt accumulation of the transcription factor Ndt80. After the second nuclear division, meiosis II-spindles were abruptly disassembled and Ndt80 and its products were destroyed **(Figure 29. Left panel)**. Consistent with our previous results, *ama1*Δ cells assembled meiosis I-spindles before Ndt80 accumulated; however, meiosis II-spindles disassembled with almost wild-type kinetics. This suggested that *ama1*Δ cells exited the high-kinase state after the second meiotic division. To confirm that *ama1*Δ cells established the low-kinase state characteristic of the meiotic exit, we analyzed the levels of cylins during the time course. Consistent with the timely disassembly of spindles, cyclins were destroyed with almost no delay **(Figure 29. Right panel)**. Thus, Ama1 was not essential from the exit from the high-kinase state. Nevertheless, we observed that several events that mark the end of the meiotic program were blocked in *ama1*Δ cells. (1) Ndt80 and its products, such as Cdc5 and Cdc20, persisted even after nuclear divisions were completed. (2) The APC/C activator Cdh1 remained as a highly modified species, which correspond to its inactive form (Jaspersen et al., 1999; Zachariae et al., 1998). (3) The Cdk1 inhibitor Sic1 failed to accumulate (Benjamin et al., 2003; Schwob et al., 1994). (4) The transcriptional repressor Sum1 was not reactivated, since the protein remained highly modified, which corresponds to its inactive state (Corbi et al., 2014). (5) The meiosis-specific kinase Ime2 was not down-regulated. Taken together, these observations indicated that *ama1*Δ cells exited from the high-kinase state but failed to carry out other

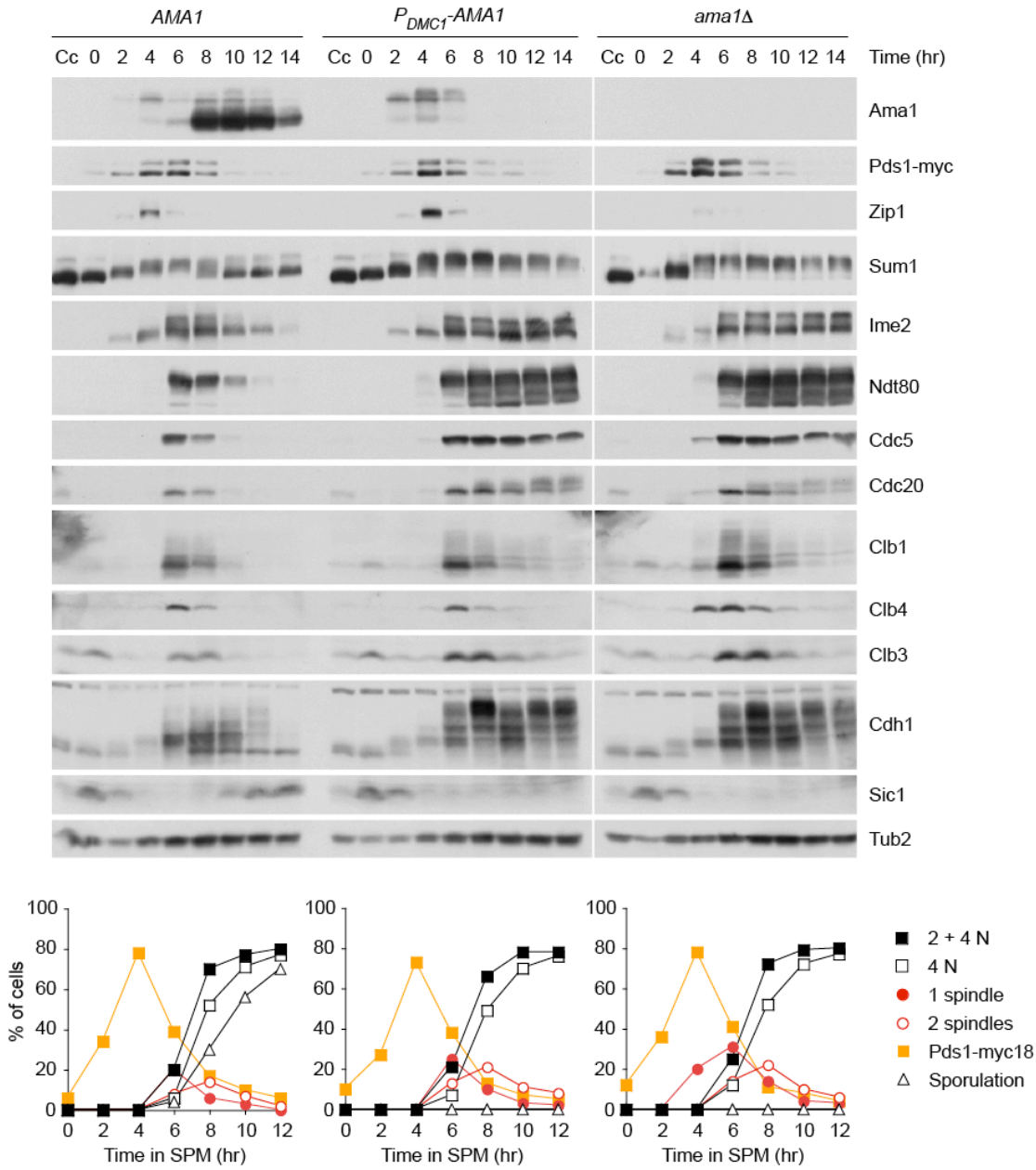

Figure 29. APC/C-Ama1 is required for timely exit from meiosis. Synchronous meiotic time course of wild-type (Z20217), *ama1*Δ P_{DMC1}-*AMA1* (Z20218) and *ama1*Δ (Z20219) strains. Top, immunoblot analysis of protein levels. Bottom, immunofluorescence detection of securin/Pds1-myc, meiosis I or meiosis II spindles (α-tubulin), divided nuclei (DAPI), and sporulation in fixed cells. Percentage of cells per time point is shown.

processes relevant for meiotic exit. To exclude the possibility that this phenotype originates from an early defect in *ama1*Δ cells, we sought to create a mutant that abolishes specifically the late functions of Ama1. We found that, when expressed from the promoter of the early meiotic gene *DMC1*, Ama1 was restricted to early stages (**Figure 29. Middle panel)**. In synchronized meiotic time courses, *ama1*Δ *DMC1p-AMA1* cells displayed a wild-type phenotype during the first stages of meiosis, as judged by the levels of synaptonemal proteins, such as Zip1, and the Ndt80-dependent accumulation of spindles. *ama1*Δ *DMC1p-AMA1* cells then completed the two meiotic divisions and disassembled spindles with wild-type kinetics. The first detectable defect of these cells occurs at the exit from the second meiotic division. At this point, *ama1*Δ *DMC1p-AMA1* cells recapitulated the *ama1*Δ phenotype. Ndt80, Cdc5, and Cdc20 were not destroyed. Cdh1, Sum1, and Ime2, remained as highly modified species. Sic1 did not re-accumulate. Taken together, our observations indicated that APC/C-Ama1 is essential for several meiotic exit processes, but is dispensable for the exit from the high-kinase state. In wild-type cells, the re-activation of APC/C-Ama1 in late meiosis can contribute to link the destruction of the high-kinase state, after nuclear divisions have taken place, with the final events of the meiotic program.

3. Discussion

3.1. The transition from prophase I into metaphase I

Late prophase I is a crucial decision point. At this stage, *S. cerevisiae* cells must decide whether (1) to prolong prophase I, (2) to enter metaphase I, or (3) to return to mitosis. Higher eukaryotes also need to make decisions during prophase I. Mammalian oocytes can remain arrested at this stage for years and then continue the meiotic program (Mehlmann, 2005). In female humans, a primary oocyte, that reaches prophase I by the time of birth, can finally decide to progress into metaphase I decades later (Hunt and Hassold, 2002). What mechanisms control whether the cells stay in prophase I or continue into metaphase I? Our work in yeast shows that both prophase I and metaphase I are two mutually exclusive stable steady states. Prophase I is a low-kinase state characterized by the MeiRC repression of Ndt80 and the proteolysis of M-phase factors mediated by APC/C-Ama1. During metaphase I, both mechanisms are inactivated: the MeiRC machinery is destroyed and APC/C-Ama1 is inhibited. By analyzing the main biochemical players of these processes, we found that (1) M-phase Cdk1 activity is kept low during prophase I by APC/C-Ama1-mediated proteolysis of cyclins. (2) The kinase Cdc5 is able to inactivate the MeiRC and must also be excluded from prophase I by APC/C-Ama1-dependent proteolysis. (3) Once recombination is completed, the MeiRC is silenced and Ndt80 triggers the accumulation of M-phase promoting factors and APC/C-Ama1 inhibitors. (4) At metaphase I, APC/C-Ama1 is inactivated by Ndt80-dependent inhibitors, such as Clb1-Cdk1, allowing stabilization of M-phase proteins. Mathematical modeling of this regulatory network revealed bi-stability as an emergent property of the system. Experimental evidence showed that the decision to abandon prophase I and to enter metaphase I is, indeed, controlled by a bi-stable switch between the low-kinase state of prophase I and the high-kinase state of metaphase I. Furthermore, the model allowed us to discover that the dynamic control of Ama1 levels is a main feature of return-to-growth and an essential requirement for the coordinated exit from meiosis.

3.2. APC/C-Ama1 is required for maintaining the low-kinase state of prophase I

A conserved feature of meiosis in all sexually reproducing organisms is a long prophase I (Von Stetina and Orr-Weaver, 2011). Prophase I in *S. cerevisiae* lasts approximately 4 hours, almost 50% of the time a cell needs for the completion of meiosis (Padmore et al., 1991). By contrast, mitotic M-phase is induced approximately 15 minutes after S-phase (Lim et al., 1996). How can the cells maintain such a long period without M-Cdk1 activity during prophase I?

It has been suggested that prophase I is maintained mainly by transcriptional or post-transcriptional repression of Ndt80 (Corbi et al., 2014; Lindgren et al., 2000; Pak and Segall, 2002b; Shin et al., 2010; Tung et al., 2000). Our results change this view by identifying proteolysis of M-phase proteins as another requirement for prophase I. Cells lacking *AMA1* activate M-phase Cdk1 activity prematurely and advance spindle assembly. The early activation of Cdk1 is caused by the stabilization of the cyclin Clb4 and the normally mitosis-specific transcription factor Ndd1, which produces Clb1 and Clb2 (Okaz et al., 2012). Remarkably, the APC/C-Ama1-mediated supprression of M-phase factors during prophase I is implemented as a coherent feed-forward loop. APC/C-Ama1 not only marks cyclins for degradation, but also their source, Ndd1. This mechanism prevents any unscheduled accumulation of M-phase Cdk1 activity during prophase I. Thus, APC/C-Ama1-driven destruction of M-phase proteins is the key factor that keeps Cdk1 low at this stage, not the lack of transcription of cyclins. Although Ama1 is only found in other ascomycete fungi (Cooper et al., 2000), the strategy of using proteolysis to maintain long periods of low Cdk1 activity might be conserved and executed by related APC/C activators, such as Cdh1. Interestingly, mice oocytes were unable to maintain a prolonged prophase I-arrest when the APC/C activator Cdh1 was knocked-out (Holt et al., 2011). Terminally differentiated cells, such as neurons, take advantage of similar mechanisms. Cdh1 depletion in post-mitotic neurons, for instance, has been observed to cause the exit from the low-kinase state of G0 and to force cells into an aberrant S phase (Almeida et al., 2005). Thus, proteolysis of M-phase-promoting regulators might be a conserved feature of any low kinase state, be it G1, G2, prophase of meiosis, or the G1-like state of terminally differentiated or quiescent cells.

3.3. APC/C-Ama1 is inactivated by Cdk1-Clb1 at the prophase I-to-metaphase I transition

Our lab has shown that during prophase I, M-phase proteins are actively destroyed by APC/C-Ama1-mediated proteolysis (Okaz et al., 2012). At metaphase I, we found that the situation changes dramatically. Ndt80 produces inhibitors of APC/C-Ama1, such as the cyclin Clb1, which lead to the inactivation of APC/C-Ama1 and the stabilization of M-phase proteins.

Clb1 has been described as an activator of APC/C-Cdc20 in mitosis (Rahal and Amon, 2008). By contrast, it acts as an inhibitor of APC/C-Ama1 during meiosis. The role of Clb1, as a regulator of a meiosis-specific form of APC/C, shows how cyclins can undertake important functions outside the mitotic cell cycle. This supports the view that the large number of related cyclins in the genomes might be a requirement for producing more elaborated programs of cell division or development (Fitch et al., 1992; Grandin and Reed, 1993). Clb1 has no major role during mitosis, but it is the most important cyclin for meiosis. Why this is the case, has been unclear. Our results provide an explanation: Clb1 is a key regulator of a meiosis-specific APC/C. Clb1 is a powerful inhibitor of APC/C-Ama1 that allows the stabilization of all M-phase proteins tested so far. This is in sharp contrast to the other well-known APC/C-Ama1 inhibitor, Mnd2, which protects a small set of substrates, most notably securin/Pds1 (Oelschlaegel et al., 2005). The use of a general APC/C-Ama1 inhibitor at the prophase I-to-metaphase I transition helps to create a robust exit from prophase I. However, cells lacking Clb1 still produce M-phase proteins and enter metaphase I almost with wild-type kinetics. Remarkably, even cells lacking Cdk1 activity accumulate stable M-phase proteins once Ndt80 appears. These results imply that additional, non-Cdk1 inhibitors of APC/C-Ama1 are produced by Ndt80. The nature of such inhibitory mechanisms is at the present unknown. We predict that their inactivation in *clb1*Δ cells should keep APC/C-Ama1 fully active and block the entry into metaphase I. By producing additional non-Cdk1 inhibitors, Ndt80 ensures that APC/C-Ama1 can be effectively inactivated once DSBs are repaired.

3.4. Cdc5 is a strong inhibitor of the MeiRC

The prophase I-to-metaphase I transition is accompanied by the disassembly of the synaptonemal complex (SC). The polo-like kinase, Cdc5 in budding yeast, was identified as the Ndt80 product required for the dissociation of the synaptonemal complex proteins from chromatin (Clyne et al., 2003; Sourirajan and Lichten, 2008). However, the mechanism of action of Cdc5 was unclear. Here, we showed that Cdc5 triggers the destruction of the axial element protein Red1 and the synaptonemal complex protein Zip1. Both proteins are extremely stable in the absence of Cdc5, implying that their abrupt disappearance is caused by proteolysis.

The Cdc5-dependent destruction of Red1 has a remarkable consequence. It provides a mechanism to silence the MeiRC independently of the repair of DSBs. Red1 is required for the chromatin association of all the other SC proteins (Page and Hawley, 2004), its destruction inactivates the MeiRC checkpoint machinery. Cdc5 is therefore an inhibitor of the MeiRC that must be tightly controlled during prophase I. An example of the consequences of a premature appearance of Cdc5 is observed in *ama1*Δ cells. In wild-type meiosis, the silencing of the MeiRC leads to Ndt80-dependent Cdc5 accumulation, which destroys the SC at the onset of metaphase I. Cdc5 does not appear before the MeiRC is silenced because it is actively destroyed by APC/C-Ama1. In *ama1*Δ cells, however, Cdc5 is stabilized prematurely and inactivates the MeiRC by destroying Red1. Once the MeiRC is disabled, the transcription factor Ndt80 produces more Cdc5. This positive feedback loop creates ever more Cdc5 and *ama1*Δ cells are forced to enter metaphase I even in the presence of DSBs. As a fatal consequence, *ama1*Δ cells show 23% missegregation per chromosome in meiosis I(Okaz et al., 2012).

We confirmed that Cdc5 inactivates the MeiRC even in the presence of unrepaired DSBs by analyzing the consequences of ectopically expressing small amounts of HA-tagged Cdc5 in the *dmc1*Δ *rad51*Δ background. These cells lack the recombinases required for DSB repair. During meiosis, *dmc1*Δ *rad51*Δ cells are stably arrested in prophase I because the unrepaired DSBs keep the MeiRC active at repressing Ndt80. A very different outcome is observed when even small amounts of Cdc5 are introduced into this system. Ectopic induction of HA-tagged Cdc5 in *dmc1*Δ *rad51*Δ cells leads to the silencing of the MeiRC by the destruction of Red1. The following

activation of Ndt80 produces more endogenous Cdc5 engaging the positive feedback loop. *dmc1*Δ *rad51*Δ cells are then forced into the high-kinase state and progress through meiosis in the presence of unrepaired DSBs. Although the Cdc5-Ndt80-positive feedback loop can promote a robust entry into metaphase I, it must be suppressed as long as DSBs are being repaired. By destroying Cdc5, APC/C-Ama1 maintains the conditions in which the MeiRC can work. The risk of a premature firing of the Cdc5-Ndt80-positive feedback loop during prophase I justifies why the cells need the ATP-demanding process of proteolysis to control it. To keep such a positive feedback loop only under transcriptional control would be dangerous, since transcription can be a rather leaky process (Spitz and Furlong, 2012). As we have shown in *dmc1*Δ *rad51*Δ cells, even a small amount of Cdc5 is sufficient to fire the loop and to establish the high-kinase state before DSBs are repaired. Therefore, the energetic cost of sustained APC/C-Ama1-mediated proteolysis throughout prophase I is the price of keeping the positive feedback loops under a tight control.

Our results provide a new mechanism for silencing the MeiRC independently of DSB repair and identify Cdc5 a potent inhibitor of the MeiRC. The role of Polo-like kinases in the disassembly of the synaptonemal complex is likely to be a conserved feature of the exit from prophase I. In mouse spermatocytes, the Polo-like kinase 1 (Plk1) co-localizes with the SC and is required for removal of the central element proteins SYCP1 and TEX12 (Jordan et al., 2012). The proteolytic machinery responsible for the Cdc5-dependent destruction of the synaptonemal complex remains to be identified.

3.5. The prophase I-to-metaphase I transition is a bi-stable switch

In collaboration with Dr. Vinod Unni and Prof. Dr. Béla Novák, we integrated our results to analyze the prophase I-to-metaphase I transition as a network. Mathematical modeling showed that the progression from prophase I into metaphase I is a transition between two stable steady states controlled by a bi-stable switch. The key prediction of the model, that both prophase I and metaphase I can be reached with the same level of Ama1, has been experimentally confirmed. In wild-type cells, the switch to metaphase I is flipped by the repair of DSBs. A bi-stable control system explains several properties of this transition.

3.5.1. A rapid transition between two stable steady states

Why is prophase I a stable steady state? By destroying not only Ndd1, but also its products, APC/C-Ama1 suppresses M-phase-promoting positive feedback loops. The destruction of cyclins blocks the positive feedback loop that activates Ndd1. The destruction of Cdc5 prevents the inactivation of the MeiRC and the production of Ndt80. Thus, cells can remain in prophase I for long periods of time because APC/C-Ama1-driven coherent feed-forward loops block the entry into M-phase until DSBs are repaired.

Why is metaphase I also a stable state? Several positive feedback loops, triggered by Ndt80, produce and maintain this high-kinase state: (1) the production of Cdk1-Clb1 inhibits APC/C-Ama1 leading to the stabilization of more Clb1 and other M-phase proteins, such as Cdc5. (2) Cdc5 inactivates the MeiRC triggering the production of more Ndt80. (3) Ndt80 induces its own transcription. (4) Ndt80 and Ndd1 also enhance their own activity by the production of cyclins. Once DSBs are repaired, the MeiRC is silenced, triggering the accumulation of Ndt80, which fires all the positive feedback loops that lead to the high-kinase state. While prophase I and metaphase I are stable steady states, the transition between the two is an abrupt and rapid process.

3.5.2. The progression from prophase I into metaphase I is an all-or-none process resistant to noise

During this transition, cells can be kept stably either in prophase I or in metaphase I, but not in an intermediate state. Small changes in the components of the system or external conditions can produce small deviations from the stable steady states, but the system will rapidly return to one of the stable states. Small variations in the activity of APC/C-Ama1, MeiRC, or Cdk1 are expected in living cells, since they are a noisy system. However, before DSB repair, any perturbed state will be forced to settle in the low-kinase state. Once DSBs are repaired, any perturbation will be forced to settle in the high-kinase state.

3.5.3. A bi-stable switch provides directionality to the meiotic program

It is never observed that wild-type cells in metaphase I return to prophase I. The irreversibility of the prophase I-to-metaphase I transition is another emergent property of the protein network. In the presence of DSBs, the cells have the potential to rest in two steady states over a range of Ama1 values. In wild-type cells, prophase I occurs before metaphase I, because the cells activate the MeiRC and produce APC/C-Ama1 before Ndt80 can accumulate. Once DSBs are repaired, the bi-stable switch is flipped, and the low-kinase state is no longer available at prophase I-levels of Ama1. The cells are then forced into metaphase I. Once the "jump" to the high-kinase state has occurred, the cells cannot go back simply because prophase I ceases to exist as a stable steady state.

3.5.4. The MeiRC is embedded in a bi-stable switch

The MeiRC must be sensitive enough to block the progression into metaphase I even in the presence of a single unrepaired DSB (Hochwagen and Amon, 2006; Ira et al., 2004). It has been proposed that the signal of DSBs must be amplified extensively in order to generate the inhibitory power to block metaphase I. This assumption fails to explain how such a strong checkpoint can be rapidly silenced after the last DSB has been repaired. The paradox of an extremely sensitive, but easy-to-silence, checkpoint can be explained in the context of a bi-stable switch.

The MeiRC is strong because APC/C-Ama1 destroys actively its inhibitor, Cdc5, during prophase I. We have shown that even small amounts of prematurely stabilized Cdc5 can lead to the silencing of MeiRC. Therefore, by destroying Cdc5, APC/C-Ama1 ensures that the checkpoint remains functional, capable of inhibiting Ndt80. This allows the cells to stay locked in the low-kinase state as long as DSBs are present. Both the MeiRC and APC/C-Ama1 work together to block all the M-phase-promoting positive feedback loops. Under these conditions, the MeiRC has the power to maintain the low-kinase state of prophase I for long periods of time.

Once the last DSB has been repaired, the MeiRC can be easily silenced because Ndt80 fires several M-phase promoting positive feedback loops. As a consequence, APC/C-Ama1 is inhibited, Cdc5 accumulates and Ndt80 increases its own transcription. All of these processes converge in the production and stabilization of

more Cdc5, which destroys Red1, thereby dismantling the MeiRC. In this way, the cells are forced into the high-kinase state while the sensitive MeiRC is abruptly inactivated by several positive feedback loops.

3.5.4. The bi-stable switch as an ubiquitous control mechanism

The bi-stable switch can be a ubiquitous mechanism to create robust irreversible transitions during mitosis, meiosis, and other processes, such as cell differentiation (Tyson and Novak, 2010). Bi-stability emerges in very different biological systems because it is created by the interactions in a network, and not by specific biochemical activities. Biological bi-stable systems usually contain a combination of positive and double-negative feedback loops. A simple example of bi-stability is the conserved network composed of Wee1, Cdc25 and Cdk1, which control entry into M phase (Domingo-Sananes et al., 2011; Nurse, 1990). The biochemical reactions in this system differ remarkably from the prophase I-to-metaphase I transition; for instance, there is no proteolysis. Nevertheless, the basic ingredients to create a bi-stable switch are present (**Figure 30**). On the one hand, there is mutual inhibition between Cdk1 and Wee1, a double negative feedback loop. Both kinases inhibit one another by direct phosphorylation. On the other hand, the mutual activation between Cdk1 and the phosphatase Cdc25 is a positive feedback loop. Cdc25 contributes to Cdk1 activation by removing inhibitory phosphorylations. This allows Cdk1 to activate more Cdc25, which engages the positive feedback loop. This system can rest in two stable steady states. In the low-kinase state, Wee1 gets the upper hand by inhibiting Cdk1, which also keeps Cdc25 inactive. However, if cyclin levels rise above a certain threshold, Cdk1 activity builds up. Wee1 is then inhibited and Cdc25 is activated, which leads to the production of more active Cdk1. Although the example shown here is over-simplified, the Wee1-Cdk1-Cdc25 system is a key component of the regulatory network that governs the transition from G2 into M-phase in several organisms. The progression through G1-S, G2-M, metaphase-anaphase and the mitotic exit are also likely to be controlled by bi-stable switches (He et al., 2011; Verdugo et al., 2013).

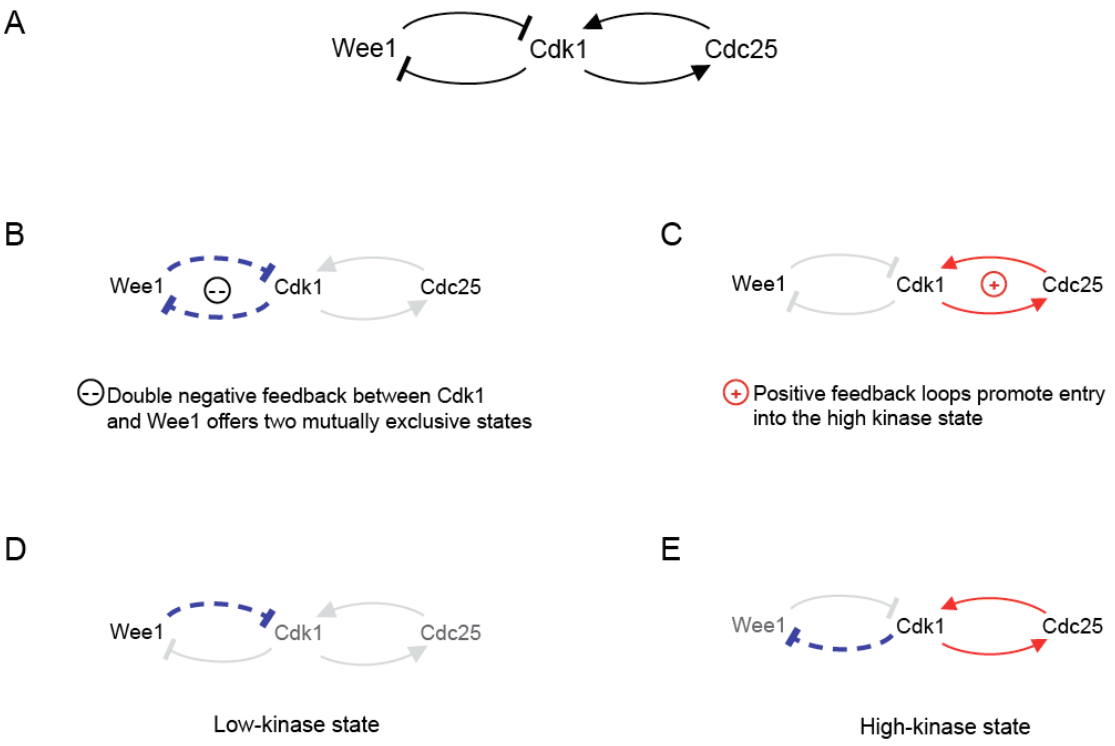

Figure 30. Bi-stability in the Wee1-Cdk1-Cdc25 network. (A) Wiring diagram of the protein regulatory network. Arrows mean activation; bar-headed lines represent inhibition. This system is characterized by **(B)** a double-negative feedback loop between Cdk1 and Wee1, which creates two mutually exclusive states. **(C)** A positive-feedback loop between Cdk1 and Cdc25, which promotes rapid activation of Cdk1. In the low-kinase state Wee1 gets the upper hand first, keeping Cdk1 and Cdc25 inactive **(D)**. If Cdk1 builds up, the positive feedback loop between Cdc25 and Cdk1 promotes the inactivation of Wee1 and the establishment of a high-kinase state **(E)**. Modified from Domingo-Sananes *et al.* (2011).

3.6. The bi-stable switch at the prophase I-to-metaphase I transition provides a mechanistic insight for return-to-growth

Our model of the prophase I-to-metaphase I transition provides a new interpretation for return-to-growth. Yeast cells that have entered prophase I can still opt to return to the mitotic program if they are transferred to rich medium. Therefore, during return-to-growth, the cells go from the low-kinase state of prophase I to the high-kinase state of mitotic M-phase. Our model successfully predicted that such a transition requires

the down-regulation of the APC/C-Ama1. Once Ama1 is destroyed, the cells are able to accumulate Ndd1 and the M-phase proteins required for entering mitosis. Accordingly, the down-regulation of APC/C-Ama1 seems to be the first priority during return to growth. Ama1 levels dropped beyond the limit of western blotting detection within one hour from transfer to rich medium. Other meiosis-specific proteins such as Zip1 and Red1 are not degraded as abruptly and persist, probably until Cdc5 triggers their destruction at the onset of the first mitotic metaphase.

Interestingly, key meiosis-specific controls are excluded from return-to-growth process. The Ime2 kinase, for instance, is essential for establishment the meiotic program and plays a complementary role to Cdk1 in several processes (Honigberg, 2004). However, along with the meiosis I-specific protein Spo13, Ime2 disappears as abruptly as Ama1 after transfer to rich media. Proteolysis of major meiotic regulators is a landmark of return to growth. It has been shown that Ime2 is degraded by the SCF-Grr1-ligase in the presence of glucose (Purnapatre et al., 2005). Whether a similar proteolytic mechanism is responsible for the degradation of Ama1 and Spo13 upon return to growth remains to be explored. Another main feature of return to growth is the choice of M-phase promoting transcription factors. Upon transfer to rich media, *rad50S* cells can potentially use Ndd1 or Ndt80 to establish the high-kinase state. However, cells selectively accumulated Ndd1. How Ndt80 is excluded from return to growth remains unclear. However, a potential mechanism is transcriptional repression, since we observed that Sum1 is extremely modified upon transfer to rich media (**Figure 23)**.

Return-to-growth can be envisioned as a transition between two mutually exclusive states: during prophase I APC/C-Ama1 is fully active and suppresses Ndd1, the mitotic source of M-phase Cdk1 activity. However, if the cells are presented with nutrients, APC/C-Ama1 is down-regulated allowing Ndd1 to produce cyclins and to establish a high-kinase state. This could explain why the cells afford to simultaneously synthesize and destroy Ndd1 during prophase I. By maintaining Ndd1 under the control of APC/C-Ama1-mediated proteolysis, a quick return to the mitotic cell cycle is ensured, if nutrients appear. This hypothesis predicts that return to growth should be hindered if APC/C-Ama1 is maintained active.

3.7. The reactivation of APC/C-Ama1 after metaphase I is sufficient to trigger exit from the high-kinase state

APC/C-Ama1 can be reactivated after metaphase I when the levels of Ama1 are strongly increased and APC/C-Ama1 inhibitors, such as Clb1, are eliminated. This result reveals that the metaphase I-arrest in P_{SCC1}-*CDC20* cells is not solely caused by the absence of APC/C-Cdc20 activity. Our experiments show that cells depleted for Cdc20 can be maintained in metaphase I because they contain enough inhibitory power to counteract the eventual increase of Ama1 protein levels. P_{SCC1}-*CDC20* cells lacking a potent APC/C-Ama1inhibitor, such as Clb1, enter metaphase I but, once Ama1 strongly accumulates, they are unable to prevent the re-activation of APC/C-Ama1. The ensuing destruction of M-phase proteins causes *clb1*Δ P_{SCC1}-*CDC20* cells to exit the high-kinase state and then finish meiosis.

In wild-type meiosis, a premature reactivation of APC/C-Ama1 can have disastrous consequences. As observed in *clb1*Δ P_{SCC1}-*CDC20* cells, APC/C-Ama1 is sufficient to precipitate the exit from the high-kinase state even before a second meiotic division takes place. Therefore, the stage in which Ama1 increases must be tightly controlled. Remarkably, we found that wild-type cells "set" the up-regulation of Ama1 precisely to the end of the second meiotic division. Thus, APC/C-Ama1 is only reactivated after two nuclear divisions are completed. How can Ama1 be specifically accumulated to high levels at this stage? Transcriptional regulation could be a mechanism. *AMA1* mRNA levels are maintained relatively constant during the first stages of meiosis, but then increase enormously (Chu et al., 1998; Primig et al., 2000). Interestingly, *AMA1* has been characterized as a direct target of Ndt80 (Chu and Herskowitz, 1998). Consistent with this finding, Ama1 up-regulation is never seen in *ndt80*Δ cells. However, Ndt80 appears at the entry into metaphase I whereas Ama1 only accumulates at the onset of anaphase II. This delay implies that, after Ndt80 is activated, another event is responsible for the up-regulation of Ama1. The activation of APC/C-Cdc20 could play such a role, since P_{SCC1}-*CDC20* cells present a strong delay in the up-regulation of Ama1 when compared to wild-type cells. Taken together, these observations indicate that the production of high levels of Ama1 is an Ndt80-dependent process that can be delayed, but not prevented, by inactivating

APC/C-Cdc20. The trigger for the strong accumulation of Ama1 at the end of meiosis remains to be identified.

3.8. APC/C-Ama1 and the exit from meiosis

APC/C-Ama1 has been implicated in the execution of post-meiotic events such as spore wall formation (Cooper et al., 2000; Diamond et al., 2009). Previous work has also shown that the efficient degradation of the APC/C activator Cdc20 required Ama1 (Tan et al., 2011). Nevertheless, the specific function of APC/C-Ama1 in late meiosis has remained controversial. Here we showed that Ama1 is a crucial regulator of characteristic events of meiotic exit, such as (1) the destruction of Ndt80, Cdc5, and Cdc20, (2) the reactivation of APC/C-Cdh1, (3) the re-accumulation of Sic1, (4) the reactivation of Sum1, and (5) the down-regulation of Ime2. APC/C-Ama1, however, was not essential for the elimination of high-kinase state in late meiosis, as judged by the disassembly of meiosis II-spindles and the destruction of cyclins. How is the high-kinase state eliminated in *ama1*Δ cells? Proteolysis of cyclins, mediated by the APC/C, is a suitable option. Since Cdh1 remains highly modified in *ama1*Δ cells, which corresponds with its inactive form (Zachariae et al., 1998), the most likely trigger of the exit is APC/C-Cdc20. This theory implies that APC/C-Cdc20 is also tightly controlled to produce the exit from the high-kinase state precisely after the second division. However, it is not known whether the activity or specificity of APC/C-Cdc20 could be so dynamically controlled during meiosis in yeast.

The elimination of the high-kinase state after the second meiotic division is accompanied by the events required for the exit of the meiotic program: (1) the transcription of cyclins and other M-phase proteins is terminated because Ndt80 is destroyed and its transcriptional inhibitor, Sum1, is reactivated. (2) proteolysis of cyclins and other M-phase proteins is promoted by the reactivation of APC/C-Cdh1. (3) the Cdk1-inhibitor Sic1 is re-accumulated. These processes are thought to be the result of the concerted regulation of Cdk1 and Ime2 (Benjamin et al., 2003; Irniger, 2011). In wild-type cells, the activities of Cdk1 and Ime2 drop coordinately after the second division, creating a link between the destruction of the high-kinase state and the events of meiotic exit. Interestingly, in *ama1*Δ cells this coordination is lost.

Although Cdk1 is inactivated, Sum1 and Cdh1 remain as slow migrating bands, Sic1 is not re-accumulated and Ndt80 is not destroyed. Persistent Ime2 activity could explain why these processes are blocked in *ama1*Δ cells. This suggests that meiotic exit requires the simultaneous down-regulation not only of Cdk1, but also of Ime2. These two kinases share a common set of substrates, which includes Sic1, Cdh1, and Sum1, among others (Bolte et al., 2002; Moore et al., 2007; Shin et al., 2010). However, Ime2- and Cdk1-consensus sites are different and, more importantly, the main Cdk1–counterating phophatase, Cdc14, cannot act on sites phosphorylated by Ime2 (Holt et al., 2007). Therefore, to reverse the phosphorylation state of proteins that are dual substrates, cells must counteract both kinases separately. Cdk1 can be inactivated by the destruction of cyclins and by the activity of Cdc14 at the onset of the second meiotic division. By contrast, the mechanisms that inactivate Ime2 are not understood. For instance, no physiologically relevant Ime2-counteracting phosphatase has been described so far. Our results suggest that APC/C-Ama1 is involved in the inactivation of Ime2 at the end of meiosis. Interestingly, Ime2 and Ndt80 seem to create a positive feedback loop, because they mutually enhanced their activities (Benjamin et al., 2003; Sopko et al., 2002). Thus, by triggering the destruction of Ndt80 in late meiosis, APC/C-Ama1 could stop the Ime2-Ndt80 positive feedback loop and down-regulate Ime2 activity. It is tempting to envision that Ndt80 is a direct substrate of APC/C-Ama1 in late meiosis, but this remains to be tested. Alternatively, APC/C-Ama1 could trigger the activation of an as yet unknown Ime2-counteracting phosphatase. Potential candidates are the phosphatase PP2C, which has been shown to preferentially de-phosphorylate Ime2 sites *in vitro* (Holt et al., 2007), and the Glc7/PP1 phosphatase, which has a meiosis-specific regulatory subunit, Gip1, also involved in post-meiotic events (Tachikawa et al., 2001).

The requirement of APC/C-Ama1 for meiotic exit suggests that this process differs radically from the mitotic exit. At the onset of mitotic anaphase, the Mitotic Exit Network (MEN) terminates the high-kinase state by increasing the activity of the Cdk1-counteracting phosphatase Cdc14. The dephosphorylation of Cdk1 substrates, the accumulation of Sic1, and the reactivation APC/C-Cdh1 are the main effects of the full Cdc14 release (Bosl and Li, 2005). In meiosis, however, this pathway is largely dispensable (Attner and Amon, 2012). Our results favor a view in which the exit of meiosis is controlled by a different network, dependent of APC/C-Ama1,

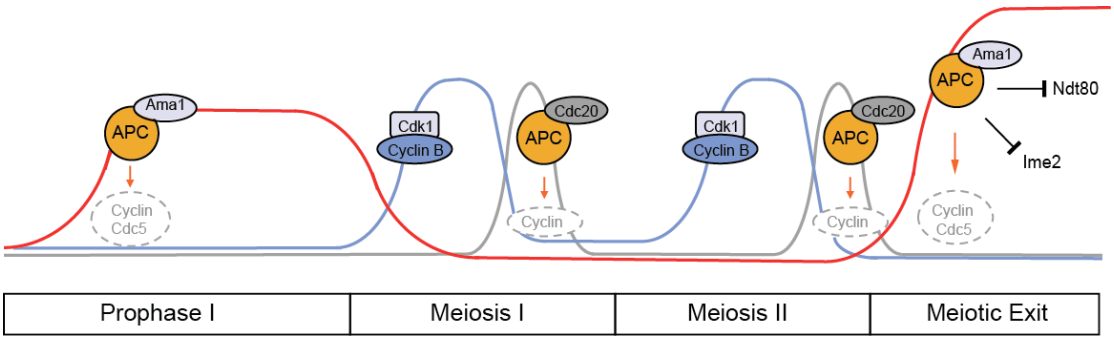

Figure 31.The role of APC/C-Ama1 in meiosis. Prophase I is a long period devoid of M-phase Cdk1 activity because of APC/C-Ama1-mediated destruction of B-type cyclins and mitotic controls. As cells exit prophase I, B-type cyclins accumulate abruptly, and APC/C-Ama1 is inactivated. The two ensuing waves of Cdk1 activation are timely counteracted by APC/C-Cdc20, producing meiosis I and II. At the end of the second meiotic division, APC/C-Ama1 is reactivated and triggers not only the ultimate destruction of cyclins and Cdc5, but also the down-regulation of important meiotic regulators such as the Ime2 kinase and M-phase promoting transcription factor, Ndt80. The precise mechanism used by APC/C-Ama1 to control meiotic exit remains to be characterized.

which counteracts the effects of the kinases Cdk1 and Ime2 (**Figure 31**). In wild-type cells, the re-activation of APC/C-Ama1 in late meiosis is essential to coordinate the elimination of the high-kinase state, produced by Cdk1, with the Ime2-regulated events that mark the end of the meiotic program.

3.8. How do cells make decisions?

In higher eukaryotes, decisions that resemble the prophase I-to-metaphase I transition are found during cell differentiation. Although the context and the biochemical pathways involved certainly differ, both processes correspond to a series of decisions between mutually exclusive states (Bolouri and Davidson, 2002; Sunadome et al., 2014). One of the most influential ideas to conceptualized cellular decision-making is the "epigenetic landscape" (**Figure 32**), proposed by C. Waddington in 1957 (Baedke, 2013; Ferrell, 2012; Waddington, 1957). In his metaphor, an undifferentiated cell is like a ball at the top of a hill. Once the ball starts to roll downwards, it finds a landscape composed of bifurcating valleys. Each bifurcation represents a decision point. Depending on its trajectory, the ball will finally settle in a specific valley,

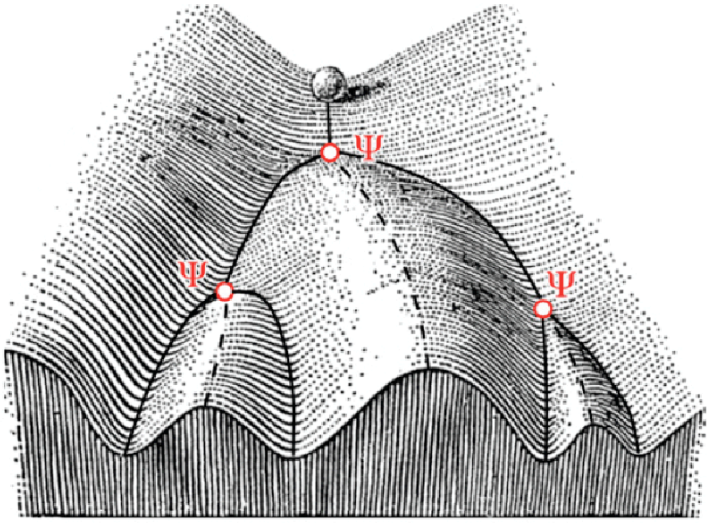

Figure 32. Waddington's epigenetic landscape. A cell, represented by a ball, rolls downhill in a landscape composed of valleys that correspond to different states. Each bifurcation represents a point of decision (Ψ). Once the ball settles into a valley, the mountain ridges prevent it from going into the alternative states. The landscape is fixed and remains unchanged during the decision process. The mechanism for decision-making is not explained. Modified from Ferrell (2012).

which corresponds to a discrete differentiated state. Once this happens, other valleys or differentiation states are not available anymore to our cell, because the mountains surrounding its final valley confine it. Although the metaphor offered a grasp of how the differentiation process proceeds, it failed to explain how the cells irreversibly make decisions at a given bifurcation.

This is not a problem if we consider that the decision-making process is done with bi-stable switches. To continue the metaphor, a bi-stable system represented as a bifurcation diagram can also be depicted as a landscape by introducing a parameter, called potential (**Figure 33.A** work by Dr. Vinod Unni and Prof. Dr. Béla Novák). This potential can be intuitively defined as what determines the speed at which the system moves toward a stable steady state (Ferrell, 2012). The greater the difference in potential between a point in our landscape and a stable steady state, the faster the system will move, from the high-potential point towards the stable steady state.

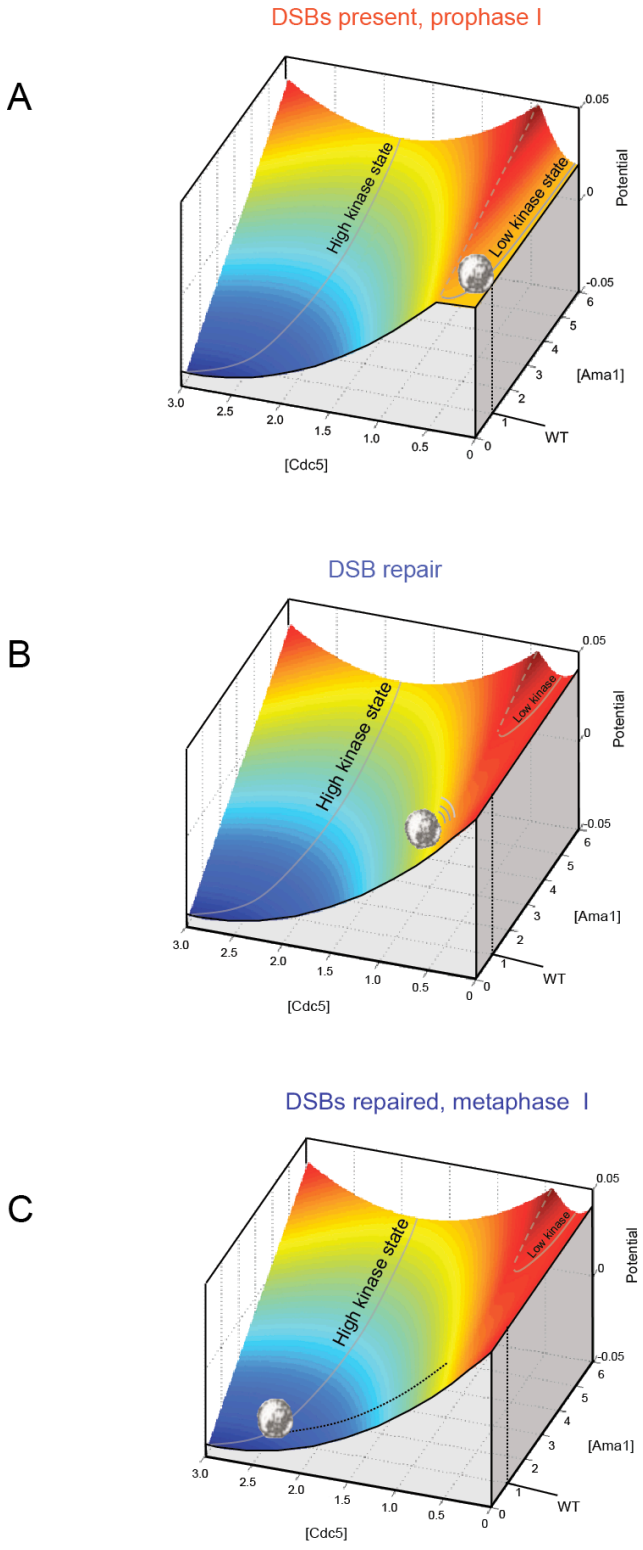

Figure 33. Bi-stable switch potential landscape. Work by Dr. Vinod Unni and Prof. Dr. Béla Novák. **(A)** The bi-stable system of the prophase I-to-metaphase I transition can be depicted as a landscape composed two valleys (stable steady states, solid lines) separated by a ridge (unstable steady states, dashed line). A cell, represented by a ball, sits on the upper valley or low-kinase state. **(B)** Once the bi-stable switch is flipped, the landscape changes. The ridge between the valleys disappears and the ball rolls downhill finally settling in the only valley left, the high kinase state. **(C)** Initially, the cell has two options, but once the bi-stable switch is activated, only one stable steady state is left.

Let us assume that the low-kinase state valley is the starting point for our cell. This location is a point of high potential in comparison to the high-kinase state valley. However, the cell stays in the low-kinase state because of the ridge made by the unstable steady states, which prevents it from rolling downhill. Thus, before flipping the switch, there are two valleys (stable steady states, solid lines), separated by a ridge (unstable steady states, dashed line). Once the bi-stable switch is flipped, the landscape changes (**Figure 33.B**). The mountain ridge separating the two valleys is retracted and the cell, previously sitting in a valley, now finds itself on a mountain slope at a point of much higher potential than the high-kinase state valley. There is no option but to roll downhill and settle in the only valley left.

In Waddington's interpretation, the landscape is fixed and the cells must somehow decide which way to take at a bifurcation point. In a bi-stable switch system, the landscape changes at the moment of decision, reducing the number of available stable states to one. This renders the process irreversible, the cells cannot return to previous alternative states because they no longer exist. Indeed, a cell can only move into a new valley once its previous valley disappears. Decision-making is accomplished by eliminating the alternatives states, reducing the number of steady stable states to one.

3.9 Concluding Remarks

In our work we have shown that yeast cells use a bi-stable switch mechanism to decide whether to stay in prophase I, with the option of return to growth, or continue to metaphase I and complete meiosis. Bi-stability is an emergent property of the regulatory protein network that controls the prophase I-to-metaphase I transition. It provides an irreversible, all-or-none and robust passage from the low-kinase state of prophase I into the high-kinase state of metaphase I. This proposes the following view of the meiotic program: in wild-type meiosis, the cells will remain in prophase I until the last DSB is repaired. During this time, APC/C-Ama1 triggers the destruction of Ndd1, B-type cyclins and Cdc5, preventing both the unscheduled appearance of a spindle and the premature silencing of the MeiRC. If the cells sense nutrients during prophase I, they can return to growth by destroying Ama1. This stabilizes the transcription factor Ndd1, which produces the cyclins and M-phase proteins required for the return to mitotic proliferation. If the starvation conditions persist, cells will flip

the bi-stable switch and enter the high-kinase of metaphase I once DSBs are repaired. After completing two rounds of chromosome segregation, APC/C-Ama1 is reactivated and coordinates the exit from meiosis. Taken together, our results provide a framework that explains how yeast cells can choose the right program of cell division. Our model of the prophase I-to-metaphase I transition has the possibility to be expanded into a mathematical description of the entire meiosis. Furthermore, it could also be adapted to model other processes of cellular decision-making.

4. Materials and methods

4.1. Yeast strains

Fast sporulating SK1-background strains (*ho::LYS2 lys2 ade2::hisG trp1::hisG leu2::hisG his3::hisG ura3*) were used in this work (Kane and Roth, 1974). Diploid strains were generated by mating of the corresponding haploid strains. Mutations are homozygous unless otherwise stated. The following mutations have been previously described: *CDC20* under the control of the mitotic *SCC1* promoter, *ndt80Δ::HIS3*, and the *clb1Δ::NatMX4* mutation (Okaz et al., 2012), the analog sensitive alleles *cdc28-as1* (Bishop et al., 2000) and *cdc5-as* (Snead et al., 2007), the mutations *PDS1myc18::KlTRP1* and *ama1Δ::NatMX4* (Oelschlaegel et al., 2005). The strains bearing the estradiol-inducible expression system (Benjamin et al., 2003) contain a pRS303 or pRS304 plasmid (Sikorski and Hieter, 1989) that produces a *GDP1*-promoter-driven Gal4 fusion to the hormone-binding domain of the human estrogen receptor. YIplac plasmids (Gietz and Sugino, 1988) containing B-type cyclins or *AMA1* under the control of a *GAL1* promoter were integrated at the *ura3*, *leu2* or *trp1* loci (Okaz et al., 2012). Table 1 describes in detail the full genotypes of the strains used in this work. The next section describes the construction of the strains.

4.2. Construction of plasmids and yeast strains

One-step PCR C-terminal epitope tagging was used to generate the strains containing Myc or Ha C-terminally tagged proteins (Knop et al., 1999; Wach et al., 1994). The resulting PCR-amplified cassette contains the *TRP1* gene from *Kluyveromyces lactis,* which complements the *trp1* mutation in SK1.

The deletions of genes *CLB1*, *NDT80*, *AMA1* were obtained by one-step gene replacement by amplifying by PCR the appropriate antibiotic resistance cassette

conferring resistance to the kanamycin derivative G418, nourseothricin, or hygromycin B (Goldstein and McCusker, 1999). The *rad51Δ::LEU2*-containing strains were obtained by crossing the corresponding haploids to the SK1 strain *rad51Δ::LEU2* obtained from N. Kleckner (Harvard University, USA). The *rad50S::URA3* mutation was introduced in the SK1 background by backcrossing a strain containing *rad50S::URA3* obtained from Vicent Geli (CRCM, France) to wild-type SK1 at least six times. The *rad50S* mutation is lysine 81 to isoleucine.

To specifically deplete the Cdc20 protein during meiosis, the promoter of *CDC20* (-341 to -1, ATG = +1) was exchanged with the promoter of the mitosis-specific *SCC1* gene (-840 to -1), which was amplified by PCR from the plasmid c4035 as a cassette conferring resistance to G418 (Clyne et al., 2003).

To restrict *AMA1* expression to early meiosis, *AMA1* was put behind the early meiosis-specific *DMC1* promoter (-340 to -1) by the triple ligation of a *Bgl*II/*Eco*RI fragment containing the N-terminus of *AMA1*, an *Eco*RI/*Hin*dIII fragment containing the C-terminus of *AMA1* and a YIplac204 carrying the *DMC1* promoter cut with *Bgl*II/*Hin*dIII. The resulting P_{DMC1}-*AMA1* plasmid was cut with *Bsu*36I to integrate at the *trp1* locus in an *ama1Δ::CaURA3* strain. N-terminally tagged Myc18-Ama1 was made by integrating at the endogenous locus a pRS306 plasmid, cut with *Eco*RI, containing N-terminally tagged Ama1 (Oelschlaegel et al., 2005). To obtain *CLB1*, *CLB2*, *CLB3*, *CLB4*, *CLB6* and *CDC5* under the control of the *GAL1* promoter, or *CLB5* and *AMA1* under the control of the shortened *GALL* promoter, the corresponding ORF was cloned behind the *GAL1* or *GALL* promoter (Mumberg et al., 1994) in the yeast integrative plasmids YIplac128 (*LEU2*), YIplac211 (*URA3*) or YIplac204 (*TRP1*) described in(Okaz et al., 2012). The resulting P_{GAL}-Protein YIplac plasmids were cut with *Pfl*MI, *Apa*1, or *Bsu*36I to integrate them at the *leu2*, *ura3* or *trp1* locus, respectively.

4.3. Meiotic time course experiments

Meiotic time courses were prepared and carried out at 30 °C. Healthy zygotes obtained with the appropriate haploid strains were streaked to single colonies on glycerol plates (YPG). The single colonies were picked after 40 hours and transferred to yeast extract peptone dextrose (YPD) plates, making a patch of circa 2 cm^2. After

a period no longer than 23 hours, the resulting patch was plated vigorously to an approximately one-cell thick homogeneous lawn on YPD plates with a dry, smooth surface. Simultaneously, a loop-full of the patch was put on solid sporulation medium (SPM, 2% K-acetate). After a period of no longer than 23 hours, the meiotic proficiency of the diploids on the sporulation plate was evaluated by looking at the cells on a phase-contrast microscope. The best diploids were then inoculated into 250 ml of YEPA medium (YP plus 2% K-acetate) in 2.8 l flasks to an OD_{600} between 0.3 - 0.35. The cultures were shaken at 200 rpm for 11-12 hours at 30 °C in an orbital shaker. At the end of this period, the OD_{600} reached 1.5-1.7 and cells arrested in G1, with less than 15% budded cells. The cultures were then concentrated by centrifugation at 3500 rpm for 3 min, washed once with 150 ml of SPM, centrifuged one more time, and finally resuspended in 100 ml of SPM, resulting in an final OD_{600} of between 3 and 3.5.

In time courses, including *cdc28-as1* strains, the inhibitor 1NM-PP1 (Cayman Chemicals) was added to a final concentration of 5 μM from a stock solution of 5 mM in DMSO, stored at -20°C until use. In time courses including *cdc5-as* strains, the inhibitor CMK (AccendaTech, Tianjin, P.R. China) was added to a final concentration of 20 μM from a stock solution of 20 mM in DMSO, stored at -20°C until use. Whenever the estradiol-inducible system was used, the expression of proteins under the control of the *GAL1* promoter was triggered with 5 μM ß-estradiol (Sigma). For measuring the half-life of proteins, cycloheximide (Sigma) was added to meiotic time course cultures to a final concentration of 500 μg/ml from a stock solution of 10 mg/ml in DMSO. At the indicated time points, samples were collected for trichloroacetic acid (TCA) protein extracts and immunofluorescence.

4.4. Return-to-growth experiments

Healthy zygotes were prepared and synchronized as for the meiotic time course experiments. 6 hours after transfer to sporulation medium (SPM), the cultures were quickly split in two and centrifuged at 4000 rpm for 2 min. One half of the culture was resuspended in four volumes of SPM medium, the other half in four volumes of rich medium, YPD (for example 60 ml of culture were pelleted and resuspended in

240 ml of SPO or YPD, OD_{600} immediately after resuspension = 0.7). The thoroughly resuspended cultures were transferred to 2.8 l flasks, each containing no more than 60 ml of culture.

4.5. Indirect immunofluorescence of meiotic time course and return to growth samples

Immunostaining was performed according to (Salah and Nasmyth, 2000). Briefly, 900 µl of cells were fixed by adding 100 µl of 35% formaldehyde. Return to growth samples were fixed for 20 min at room temperature and then kept overnight at 4°C. Meiotic time course samples were fixed overnight at 4 °C. Samples were then washed four times with 1 ml of 0.1 M potassium phosphate buffer pH 6.4, one time with 1 ml spheroplasting buffer (0.1M potassium phosphate buffer pH 7.4, 1.2 M sorbitol, 0.5 mM magnesium chloride) and finally resuspended in 200 µl of spheroplasting buffer. 6 µl of a freshly prepared 10 % solution of β–mercaptoethanol were added to each sample. After incubation at 30 °C for 15 min, samples were incubated with 10 µl of zymolase solution (Zymolyase 100T from amsbio, 1 mg/ml in spheroplasting Buffer) for around 10 min, and then, the refractivity of the cells was assessed at the phase-contrast microscope. When about 75% of the fixed cells looked as a dark rounded mesh with fuzzy edges, the digestion was stopped by adding 1 ml of cold spheroplasting buffer. After gentle centrifugation, the spheroplasts were resuspended in 200 µl of spheroplasting buffer. 5 µl of spheroplasts per time point were deposited on a polylysine-covered 15-well slide. Spheroblasts were allowed to adhere to the surface for 5 min, the excess volume was aspirated and the cells were dehydrated by incubating the slides 3 min in methanol and 10 s in acetone, both at -20 °C. The slides were rehydrated by incubating with 5 µl of PBS (0.04 M monohydrogen potassium phosphate, 0.01 M dihydrogen potassium phosphate, 0.15 M sodium chloride, 0.1 % sodium azide) per well, and then blocked with PBS containing 1 % bovine serum albumin (PBS-BSA). Primary antibodies were incubated for one hour. Slides were washed four times with PBS-BSA for 5 min. Secondary antibodies were incubated for one hour and after four washes with PBS-BSA, the wells were covered with 4 µl of 4',6-diamidino-2-phenylindole (DAPI) to stain DNA, and the slides sealed with nail polisher.

The following primary antibodies were used for immunodetection: monoclonal mouse anti-Myc 9E10 (1:5, Zachariae lab), monoclonal rat anti-tubulin YOL 1/34 (1:300, Serotec), polyclonal rabbit anti-Myc (1:300, Gramsch CM-100). The Secondary fluorophore-labeled antibodies were goat anti-mouse CY3 (1:400, Jackson ImmunoResearch), goat anti-rat Alexa 488 (1:300, Jackson ImmunoResearch), goat anti-rat CY3 (1:400, Jackson ImmunoResearch), goat anti-Rabbit Alexa 488 (1:200, Chemicon).

Cells were scored as Pds1myc18 positive when clear, bright nuclear staining was observed. The first nuclear division was counted when cells produced two distinguishable masses of DNA. The second nuclear division was scored when cells presented 4 masses of DNA. Cell counting was done on an Axioskop 2 epifluorescence microscope. A 100x α-Plan-Fluar 1.40 NA oil immersion was used as objective lens (Carl Zeiss). 100 cells per time point were counted. A CCD camera controlled by Quick Capture software was used to take the pictures and Adobe Photoshop was used to process them into images.

4.6. SDS-PAGE analysis of protein extracts obtained by TCA precipitation

9 ml from a meiotic culture were centrifuged at 4000 rpm for 2 min, resuspended in 1 ml of 10% TCA and transferred to a 1.5 ml safe-lock Eppendorf tube and centrifuged again at 8000 rpm for 2 min at 4 °C. The pellets were snap-frozen in liquid nitrogen and then stored at -80 °C. For breakage, pellets were thawed on ice, inside a 4 °C cold room. 200 µl of glass beads (diameter = 0.5 mm) and 200 µl of 10 % TCA were added and the samples were mechanically disrupted by shaking them on a bead beater set at max speed for 30 min. The resulting supernatant was transferred to a fresh safe-lock Eppendorf tube and spun at 3000 rpm at 4 °C for 10 min. The acidic pellets were thoroughly resuspended in 200 µl of 2X concentrated Laemmli buffer with freshly added β–mercaptoethanol (62.5 mM Tris-HCl pH 6.8, 10 % glycerol, 2 % SDS, 0.01 % bromophenol blue, 0.4 M β–mercaptoethanol) and then neutralized with 100 µl of 1M Tris Base. Samples were mixed thoroughly, boiled for 10 min at 95 °C and finally spun for 10 min at 13000 rpm. Protein concentration in the extracts was measured with the Bradford protein assay (BioRad) and 60 µg of total protein were loaded on SDS-8% polyacrylamide gels. For the analysis of the proteins Red1 and

Sum1, 100 µg of protein were loaded. For the analysis of the protein Sic1, 100 µg of protein were loaded on SDS-10% polyacrylamide gels.

4.7. Western blotting and immunodetection of proteins

Semidry western blotting (0.45 mA/cm^2 for 1 hr) was used to transfer proteins to a PVDF membrane (Immobilon P, Millipore). Membranes were then blocked for 1 hour in PBS buffer containing 0.1 % Tween 20 and 4% non-fat milk powder (PBS-T). The primary antibodies were incubated for 1 hour at room temperature. After four washes with PBS-T, the membrane was incubated for 1 hour, or overnight at 4°C, with secondary antibodies conjugated to horseradish peroxidase. After four washes with PBS containing only 0.1 % Tween 20, the membranes were incubated 20s with a light-generating substrate solution (ECL detection system, GE Healthcare) and developed on a Kodak X-omat machine.

Mouse monoclonal antibodies 12CA5 (1:500, Zachariae lab) and 9E10 (1:100, Zachariae lab) were used for the detection of HA and Myc tagged proteins, respectively. Rabbit polyclonal antibodies were used for the detection of Ama1 (1:2000; Oelschlaegel *et al*., 2005), Cdh1 (1:5000, Zachariae lab) Cdc5 (1:5000, Matos *et al*., 2008), Cdc20 (1:5000, Camasses *et al*., 2003), Clb2 (1:2000, Okaz *et al*., 2012), Clb3 (1:5000, Zachariae lab), Ndt80 (1:10000, a gift from Kirsten Benjamin), Rec8 (1:5000, Katis *et al*., 2010), Red1 (1:5000, a gift from Shirleen Roeder, Howard Hughes Medical Institute, USA), Tub2 (1:20000, a gift from Wolfgang Seufert, University of Regensburg, Germany), Sic1 (1:600, Santa Cruz sc-50441), Hop1 (1:5000, a gift from Franz Klein, Max F. Perutz Laboratories, Austria) and Dbf4 (1:5000, Matos *et al*., 2008). Goat polyclonal antibodies were used for the detection of Clb1 (1:300, Santa Cruz sc-7647), Clb4 (1:400, Santa Cruz sc-6702), Clb5 (1:100, Santa Cruz sc-6704), Clb6 (1:400, Santa Cruz sc-7166), Sum1 (1:200, Santa Cruz sc-26441), Ime2 (1:100, Santa Cruz sc-26444), Cdc14 (1:1000, sc-12045 Santa Cruz), and Zip1 (1:200, Santa Cruz sc-48716).

Table 1. List of *Saccharomyces cerevisiae* SK1 strains used in this work

Strain[1]	Genotype[2]
Z2828	*MATa/MATalpha PDS1myc18::KlTRP1*
Z3086	*MATa/MATalpha Myc18-AMA1::URA3*
Z12185	*MATa/MATalpha*
Z12463	*MATa/MATalpha rad50S::URA3 NDD1-HA3-KlTRP1 PDS1myc18::KlTRP1*
Z15630	*MATa/MATalpha ndt80Δ::NatMX4 PDS1myc18::KlTRP1*
Z17725	*MATa/MATalpha ndt80Δ::HIS3 NDD1-HA3-KlTRP1 PDS1myc18::KlTRP1*
Z17726	*MATa/MATalpha ndt80Δ::HIS3 ama1Δ::NatMX4 NDD1-HA3-KlTRP1 PDS1myc18::KlTRP1*
Z17971	*MATa/MATalpha cdc28-as1 ama1Δ::NatMX4 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KITRP1*
Z17972	*MATa/MATalpha cdc28-as1 cdc20::P_{SCC1}CDC20-KanMX4 PDS1myc18::KITRP1*
Z18209	*MATa/MATalpha cdc5L158G::HphMX4 cdc20::P_{SCC1}-CDC20-KanMX6 PDS1myc18::KITRP1*
Z18210	*MATa/MATalpha cdc5L158G::HphMX4 ama1Δ:: NatMX4 cdc20::P_{SCC1}-CDC20-KanMX6 PDS1myc18::KITRP1*
Z18331	*MATa/MATalpha clb1Δ::NatMX4 ama1Δ::CaURA3 cdc20::P_{SCC1}-CDC20-KanMX4 PDS1myc18::KITRP1*
Z18332	*MATa/MATalpha clb1Δ::NatMX4 cdc20::P_{SCC1}-CDC20-KanMX4 PDS1myc18::KITRP1*
Z18333	*MATa/MATalpha ama1Δ::NatMX4 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KITRP1*
Z18334	*MATa/MATalpha cdc20::P_{SCC1}-CDC20-KanMX4 PDS1myc18::KITRP1*
Z18515	*MATa/MATalpha ndt80Δ::HIS3 leu2::P_{GAL}-CLB1-LEU2 ura3::P_{GDP}-GAL4(484).ER-URA3 PDS1myc18::KlTRP1*
Z18358	*MATa/MATalpha clb4Δ::KanMX4 ama1Δ::NatMX cdc20::P_{SCC1}-CDC20-KanMX4 PDS1myc18::KITRP1*

Z18359	*MATa/MATalpha clb4Δ::KanMX4 cdc20::P_{SCC1}-CDC20-KanMX4 PDS1myc18::KITRP1*
Z18669	*MATa/MATalpha ndt80Δ::HIS3 trp1::P_{GAL}-CLB4ha3-TRP1 ura3::P_{GDP}-GAL4(484).ER-URA3 PDS1myc18::KlTRP1*
Z18883	*MATa/MATalpha ndt80Δ::HIS3 trp1/trp1::P_{GAL}-CLB5-TRP1 ura3/ura3::P_{GDP}-GAL4(484).ER-URA PDS1myc18::KITRP1*
Z18948	*MATa/MATalpha ndt80Δ::NatMX4 trp1::P_{GAL}-CDC5-TRP1his3::P_{GDP}-GAL4(484).ER-HIS3*
Z19211	*MATa/MATalpha ndt80Δ::NatMX4 his3::P_{GDP}-GAL4(484).ER-HIS3 trp1/trp1::P_{GAL}-CLB6-TRP1*
Z19050	*MATa/MATalpha ndt80Δ::HIS3 ama1Δ:: NatMX4 MEK1ha3::CaURA3 PDS1myc18::KITRP1*
Z19092	*MATa/MATalpha ndt80Δ::HIS3 cdc5L158G ama1Δ:: NatMX4 MEK1ha3::CaURA3 PDS1myc18::KITRP1*
Z19376	*MATa /MATalpha clb1Δ::NatMX4 PDS1myc18::KlTRP1*
Z19408	*MATa/MATalpha cdc28-as1 ndt80Δ::HIS3 leu2::P_{GAL}-CLB1-LEU2 ura3::P_{GDP}-GAL4(484).ER-URA3 cdc20::P_{SCC1}-CDC20-KanMX4 PDS1myc18::KITRP1*
Z19409	*MATa/MATalpha ndt80Δ::HIS3 leu2::P_{GAL}-CLB1-LEU2 ura3::P_{GDP}-GAL4(484).ER-URA3 cdc20::P_{SCC1}-CDC20-KanMX4 PDS1myc18::KITRP1*
Z19521	*MATa/MATalpha ama1Δ::CaURA3 PDS1myc18::KlTRP1*
Z19716	*MATa/MATalpha rad51Δ::LEU2 dmc1Δ::KanMX4 PDS1myc18::KlTRP1*
Z19731	*MATa/MATalpha rad51Δ::LEU2 dmc1Δ::KanMX4 PDS1myc18::KlTRP1 his3::P_{GDP}-GAL4(484).ER-HIS3 ura3::P_{GAL}-CDC5ha3-URA3*
Z19732	*MATa/MATalpha rad51Δ::LEU2 dmc1Δ::KanMX4 ndt80Δ::NatMX4 his3::P_{GDP}-GAL4(484).ER-HIS3 ura3::P_{GAL}-CDC5ha3-URA3 PDS1myc18::KlTRP1*
Z19785	*MATa/MATalpha rad51Δ::LEU2 dmc1Δ::CaURA3 ama1Δ::NatMX4 leu2::P_{GALL}-p-AMA1(cDNA)-LEU2 cdc20::P_{SCC1}-CDC20-KanMX4 his3/his3::P_{GDP}-GAL4(484).ER-HIS3 PDS1myc18::KITRP1*
Z20217	*MATa/MATalpha PDS1/PDS1myc18::KlTRP1*

Z20218	*MATa/MATalpha ama1Δ::CaURA3 PDS1/PDS1myc18::KlTRP1*
Z20219	*MATa/MATalpha ama1Δ::CaURA3 trp1/ trp1::P_{DMC1}-AMA1(intron)-TRP1 PDS1/PDS1myc18::KlTRP1*

[1] The genetic background of *S. cerevisiae* SK1 is*: ho::LYS2 ura3 leu2::hisG trp1::hisG his3::hisG ura3 leu2::hisG trp1::hisG his3::hisG*

[2] Each mutation is homozygous unless otherwise state

4.8 Abbreviations

AE - axial elements
AI – Additional Ndt80-dependent inhibitor of APC/C-Ama1
as - analog-sensitive
APC/C - anaphase-promoting complex/cyclosome
BSA - bovine serum albumin
CDK1 - cyclin-dependent kinase
CHX - cycloheximide
CMK - pyrrolopyrimidine chloromethylketone
CO – crossover
DAPI - 4',6-diamidino-2-phenylindole
DHJ - double Holliday junction
DMSO - dimethyl sulfoxide
DNA - deoxyribonucleic acid
DSB - double-strand break
M – molar
MDa – Megadalton
NA - numerical aperture
NCO - non-crossover
OD - optical density
PCR - polymerase chain reaction
MeiRC - meiotic recombination checkpoint
S – Svedberg
SAC - spindle assembly checkpoint
SC - synaptonemal complex
SCF - Skp1-cullin-F-box protein family of ubiquitin ligases
SDS - sodium dodecylsulfate
SPM - sporulation medium
TCA - trichloroacetic acid
YEPA - yeast peptone medium plus 2% K-acetate
YPD - yeast peptone dextrose medium

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Erklärung entsprechend §5.5 der Promotionsordnung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Die Dissertation wurde im Zeitraum von März 2009 bis Mai 2015 verfasst und von Dr. Wolfgang Zachariae (Max-Planck-Institut für Biochemie Martinsried), wie folgt betreut; von März 2009 bis März 2012 am Max-Planck-Institut für Molekulare Zellbiologie und Genetik Dresden sowie von März 2012 bis Mai 2015 am Max-Planck-Institut für Biochemie Martinsried.

Meine Person betreffend erkläre ich hiermit, dass keine früheren erfolglosen Promotionsverfahren stattgefunden haben.

Ich erkenne die Promotionsordnung der Fakultät für Mathematik und Naturwissenschaften, Technische Universität Dresden an.

München, Mai 2015.

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