Coordination of meiotic divisions and spore differentiation in yeast

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Vorgelegt von
Valentina Mengoli
Geboren am 16.02.1984 in Castel San Pietro Terme, Italien
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Valentina Mengoli

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Erstgutachter: Prof. Dr. Nicolas Gompel
Zweitgutachter: Prof. Dr. Marc Bramkamp

Curriculum Vitae

Valentina Mengoli

Email: mengoli@biochem.mpg.de

Education

2011-today Ph.D. student, Max Planck Institute of Biochemistry, Germany

2007-2012 Student, M. Gatti Laboratory (cell division)

2010 M.Sc. (Genetics and Molecular Biology), Sapienza University of Rome, Italy

2009 B.Sc. (Biology), Alma Mater Studiorum, Bologna

Publications

1. *Mengoli V., *Bucciarelli E., Lattao R., Piergentili R., Gatti M., Bonaccorsi S. The analysis of mutant alleles of different strength reveals multiple functions of topoisomerase 2 in regulation of Drosophila chromosome structure. PLoS Genetics 10. e1004739 * Authors contributed equally to this work.
2. Arguello-Miranda O., Zagoriy I., Mengoli V., Rojas J., Jonak K., Oz T., Graf P., Zachariae W. Casein Kinase 1 coordinates cohesin cleavage, gametogenesis, and exit from M phase in meiosis II. Developmental cell 40, 37-52

3. Jonak K., Zagoriy I., Oz T., Graf P., Rojas J., Mengoli V., Zachariae W. APC/C-Cdc20 mediates deprotection of centromeric cohesin at meiosis II in yeast. Cell cycle 16, 1145-1152

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Abstract

Gametogenesis involves two crucial tasks: reduction of the genome content from diploid to haploid by meiosis, and the subsequent differentiation of meiotic products into gametes. While meiosis consists of an evolutionarily conserved sequence of events, gamete differentiation varies dramatically, not only among species, but also between male and female. Possibly because gametogenesis in multicellular organisms is the result of a multitude of cues coming from different cell types, its study is often addressed from the point of view of the morphogenesis of the gamete, which is very specific. A remarkable characteristic of gametogenesis common to many organisms is that mRNAs encoding key proteins required for meiosis and gamete differentiation are produced before meiotic divisions begin, raising the question of how the two processes are coordinated. Keeping gamete differentiation in register with meiotic nuclear divisions is essential for the packaging of a haploid genome into the gametes and, thus, for the maintenance of a constant ploidy. In this study, we used yeast as a model to address how sporulation, the yeast equivalent of gametogenesis, is coordinated with the conserved pathway of meiotic nuclear divisions. We found that in wild-type cells, the initiation of sporulation is restricted to meiosis II because it requires four conditions to be satisfied: (i) induction of the genes required for spore formation, (ii) activation of the anaphase-promoting complex (APC/C) toward meiosis I-specific proteins, (iii) elevated activity of Cdk1, and (iiii) activation of casein-kinase 1δ (Hrr25). All these molecules are evolutionarily conserved regulators, suggesting that in both yeast and higher eukaryotes, the cell cycle machinery plays a prominent role in coordinating meiosis and gamete formation.

Table of contents

1. Introduction

Sexually reproducing organisms rely on faithful chromosome segregation in both mitosis and meiosis to procreate. Mitosis is the form of cell division that cells adopt to multiply, while meiosis is the specialized type of cell division that produces gametes. Errors in chromosome segregation in mitosis generate changes in chromosome content, producing aneuploid progeny. Errors in meiotic chromosome segregation can lead to miscarriage and consequent birth defects. It has been estimated that 10-30% of fertilized human eggs carry the "wrong" number of chromosomes, which represents a very high incidence of aneuploidy in comparison to other species (Hassold and Hunt, 2001). Gametogenesis consists of meiosis and gamete differentiation. The meiotic cell division process is evolutionarily conserved, with meiosis in humans following a similar program as in most other eukaryotes. Due to extensive studies of meiosis in budding yeast, fission yeast, flies, and mice, we now have a basic understanding of how genome haploidization is achieved. Gamete differentiation, by contrast, appears more variable, leading to the creation of eggs, sperm cells, or spores. Whereas genome haploidization and gamete differentiation both require components produced early in meiosis, they start at different times, raising the question of how the two processes are coordinated. Here, I have investigated how spore formation, the yeast equivalent of gamete differentiation, is coordinated with the two meiotic divisions. To start, I will introduce the principles of meiosis, followed by the basics of gamete differentiation in various organisms, and conclude with what we know about the coordination of the two processes and how we can further investigate this question in budding yeast.

1.1. Principles of genome haploidization

Maintenance of ploidy in sexually reproducing organisms requires meiosis to generate haploid gametes from diploid germ cells **(Figure 1)** (Petronczki et al., 2003). Gametes contain new combinations of genetic material, since meiotic DNA replication is followed by synapsis and recombination. At this stage,

homologous chromosomes (one maternal and one paternal) become tightly associated along their entire length and exchange homologous pieces of DNA

in a process called crossing over. Reciprocal recombination between homologous chromosomes provides the physical link that allows bivalents to be bi-oriented on the meiosis I spindle. At meiosis I sister kinetochores are clamped together, so that maternal and paternal centromeres are pulled to opposite spindle poles.

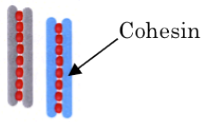
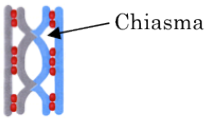
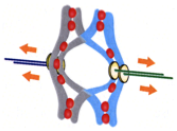
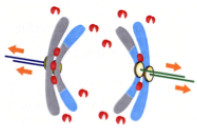
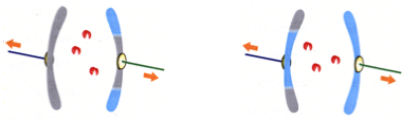
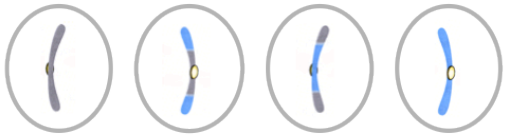
Meiotic stage	Chromosome organization	Landmark
S-phase		DNA replication
Prophase I		Recombination
Metaphase I		Monopolar attachment
Anaphase I		Cleavage of arm cohesion
Metaphase II		Bipolar attachment
Anaphase II		Cleavage of centromeric cohesion
Differentiation		Gamete formation

Figure 1. Meiotic cell divisions. Pre-meiotic S-phase is characterized by the loading of cohesins (red dots), containing the meiosis-specific Rec8 subunit, on chromatin. In prophase I, reciprocal recombination between homologous, non-sister molecules of DNA creates chiasmata, which provide the physical link between maternal and paternal chromosome through cohesion on chromosomal arms. In metaphase I, monopolar attachment of sister kinetochores ensures that maternal and paternal centromeres are pulled to the opposite poles of the spindle. Cleavage of Rec8 on chromosomal arms at anaphase I onset allows segregation of homologs. Cohesins around the centromeres are protected from cleavage, thereby providing attachment of sister kinetochores, which is required for bipolar attachment of sister chromatids in metaphase II.

Cleavage of centromeric cohesion finally triggers segregation of sister chromatids and the generation of haploid gametes.

Mono-orientation of sister chromatids is a unique feature of meiosis, and it is essential to halve the number of chromosomes in the first, reductional meiotic division. In budding yeast, bi-orientation of sister chromatids is suppressed in meiosis I by a four-protein complex, called monopolin, which associates with sister kinetochores and clamps them together (Petronczki et al., 2006; Rabitsch et al., 2003; Toth et al., 2000). So far, the monopolin complex has been found only in budding yeast, and how mono-orientation is achieved in other organisms remains poorly understood. Bivalents can be resolved due to the cleavage of the molecules that hold them together, namely cohesins, which are loaded on chromatids during DNA replication. They consist of three subunits: Smc1, Smc3, and Scc1 (replaced in meiosis by Rec8), that entrap sister DNA molecules within a proteinaceous ring (Gruber et al., 2003; Klein et al., 1999). The meiosis-specific α-kleisin subunit Rec8 is cleaved by separase (Buonomo et al., 2000), and separase activation is triggered by the ubiquitination of securin (together with cyclin B) by the anaphase-promoting complex (APC/C) at anaphase onset in both meiotic divisions. Notably, during meiosis, chromosomal cohesins are lost in two steps. In meiosis I, only cohesins along chromosome arms are cleaved to allow the segregation of homologous chromosomes, while cohesin complexes localized around centromeres are protected from cleavage, to allow a second division without another round of cohesin loading (Nasmyth and Haering, 2005). Complete loss of cohesion during meiosis I results in early separation of sister chromatids and random segregation in meiosis II, which is detrimental for gamete survival. For example, mutating Rec8 in order to make all molecules susceptible to cleavage in meiosis I causes the premature separation of sister centromeres (Katis et al., 2010). Furthermore, meiotic cells expressing the mitotic α-kleisin subunit Scc1 fail to protect centromeric cohesins in meiosis I (Toth et al., 2000), indicating that centromeric protection requires Rec8. Recent studies carried out in budding yeast, fission yeast, flies, plants, and mice showed that conserved shugoshin proteins are targeted to kinetochore to protect cohesins from separase cleavage in meiosis I (Hamant et al., 2005; Katis et al., 2004a; Kitajima et al., 2004; Lee et al., 2008; Marston et al., 2004; Rabitsch et al., 2004; Tang et al., 1998). Budding yeast Sgo1 protects centromeric Rec8 by recruiting a specific

form of protein phosphatase 2A bound to the regulatory B-type subunit Rts1 to centromeres (Riedel et al., 2006). In order to be cleaved by separase, Rec8 must be phosphorylated by the kinases Hrr25 and Cdc7-Dbf4 (Katis et al., 2010). PP2A-Rts1 opposes Rec8 phosphorylation at centromeres, resulting in phosphorylated Rec8 along chromosomes arms and unphosphorylated Rec8 at centromeres. Due to this difference, only arm-cohesins are cleaved by separase at anaphase I to resolve chiasmata, while centromeric Rec8 persists, holding together sister chromatids until meiosis II. To allow sister chromatid segregation during anaphase II, Sgo1 disappears from centromeres (Katis et al., 2004a). The removal of Sgo1 from centromeres is promoted by the Rec8-kinase Hrr25, which phosphorylates Rec8 and at the same time subjects Sgo1 to APC/C-Cdc20-dependent proteolysis. This removes PP2A from centromeres and enables Hrr25 to phosphorylate Rec8. Both Hrr25 functions are essential for the cleavage of centromeric cohesin at meiosis II (Arguello-Miranda et al., 2017).

1.2. Diversities and similarities of gametogenesis across various organisms

Transmission of the single copy genome into the zygote requires coordination between meiosis and differentiation into a gamete. While meiotic chromosome segregation follows an evolutionary conserved sequence, gamete differentiation is much more diverse, creating gametes with very different features. A common feature of all gametes is to rely during gametogenesis on the temporal and spatial coordination of meiotic nuclear divisions and gamete differentiation, which might therefore require conserved molecules. Although budding and fission yeast diverged from a common ancestor more than a billion years ago, both yeasts share a tightly controlled spore formation program that starts only after the completion of meiosis I (Balasubramanian et al., 2004; Shimoda, 2004). Yeast sporulation shares some features with animal spermiogenesis too, such as: (i) meiosis and differentiation proceed continuously, without cell cycle arrest, and (ii) each haploid nucleus generates a gamete. By contrast, primary oocytes arrest at prophase I for days or years depending on the species, to start differentiation before meiotic divisions, and the result of gametogenesis is only one oocyte containing one haploid nucleus. Both sporulation and spermiogenesis are regulated by a transcriptional

program, which produces mRNAs encoding cell cycle regulators and differentiation factors prior to the first meiotic division (Chu and Herskowitz, 1998; Eddy, 1998). This raises the question of how the differentiation program starts at the right time relative to meiotic nuclear divisions. *Drosophila* male gametogenesis is the best-characterized model for the study of the coordination between cell cycle and differentiation. In wild-type flies, meiotic divisions precede spermatogenesis, but nuclear division is not required for the activation of spermatid differentiation (Lin et al., 1996). Indeed, some fly mutants skip crucial events of meiosis but nevertheless initiate spermiogenesis (Sigrist et al., 1995; Stern et al., 1993; White-Cooper et al., 1993), suggesting that spermiogenesis is independent of progression through meiosis. A different category of mutants, encoding testis-specific components of the transcription factor TFIID, cause both arrest of meiotic cell cycle progression and failure to initiate spermiogenesis (Hiller et al., 2004; Hiller et al., 2001). It has been proposed that the genes encoding testis-specific components of the transcription factor TFIID activate the transcription program that drives spermatid differentiation (White-Cooper et al., 1998). In budding yeast, the induction of the meiosis-specific transcription factor Ndt80 sets in motion multiple pathways, required for both meiotic divisions and sporulation. Spore formation initiates precisely during metaphase II in wild-type conditions (Neiman, 2011), so it is likely to be somehow dependent on progression through meiotic divisions. Surprisingly, in yeast, as in flies, mutants defective in sporulation progress normally through meiosis (Nag et al., 1997), and mutants defective in nuclear divisions can nevertheless produce spores (Schild and Byers, 1980). However, with few exceptions, these spores contain the wrong number of chromosomes, suggesting that in order to produce haploid gametes, it is important to coordinate sporulation with meiosis II, so that each spore can enclose a haploid nucleus. How this is achieved is currently unclear, but yeast might be a promising model to understand how meiosis and gamete differentiation are coordinated.

1.3. Cell cycle control of meiosis

Progression through the cell cycle is controlled by the periodic activation and inactivation of two types of enzymes: cell cycle kinases (Cdks) and the

anaphase-promoting complex (APC/C), a ubiquitin ligase (Nigg, 2001). Cdks are a family of serine/threonine kinases, which phosphorylate key regulators of cell cycle progression, thereby triggering DNA replication, spindle assembly, chromosome condensation and segregation. Cdk activity requires the binding of a regulatory cyclin subunit (Murray, 2004). Budding yeast encodes a single Cdk (Cdk1/Cdc28), which can be bound to 6 regulatory B-type cyclins (Clb1-6). All known cyclins are targeted to the proteasome by the addition of ubiquitin chains, and mitotic and meiotic cyclins are ubiquitinated by the APC/C (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995). APC/C activity is governed by three activators, which also function as substrate adaptors: Cdc20, Cdh1, and the meiosis-specific Ama1. During the mitotic cell cycle, APC/C-Cdh1 is required for targeting cyclins for degradation in order to allow the completion of mitotic exit, and to maintain cells in the subsequent G1 phase (Yeong et al., 2000), whereas in meiosis, its activity is restricted to the premeiotic G1 phase (Oelschlaegel et al., 2005). The main functions of APC/C-Cdc20 in mitosis are (a) to trigger the degradation of securin, thereby allowing cohesin cleavage by separase and thus chromosome segregation, and (b) to target cyclins for degradation, thereby promoting the exit from mitosis. In meiosis, APC/C-Cdc20 is activated twice, due to the mutual regulation with Cdk1/Clbs: activation of Cdk1-Clbs at entry into meiosis I induces spindle assembly and it is required for activation of APC/C-Cdc20. At anaphase I, APC/C-Cdc20 mediates the degradation of Clbs, leading to the lowering of Cdk-Clbs activity, which in turn triggers the decline of APC/Cdc20 activity. Low APC/C-Cdc20 activity allows the re-accumulation of Clbs required for entry into meiosis II **(Figure 2)** (Arguello-Miranda et al., 2017). It is unclear how the periodic activations are stopped after meiosis II, and how cells proceed to gametogenesis, rather than entering a third division. The meiosis-specific APC/C activator Ama1 is thought to play a role in stopping the oscillations of Cdk1/Clbs; it accumulates to high levels in meiosis II and induces the degradation of Ndt80, the transcription factor that drives the transcription of M-phase genes, including cyclins (Okaz et al., 2012).

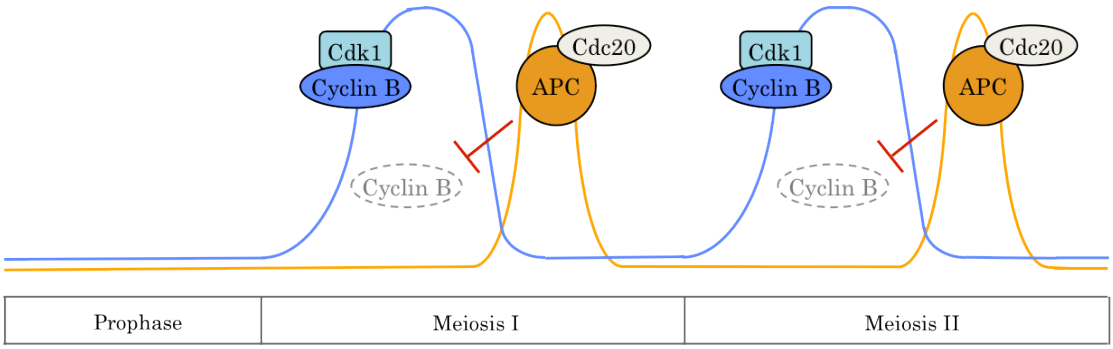


Figure 2. Cdk1 and APC/C-Cdc20 activity in meiosis. After a long prophase characterized by the absence of M-phase Cdk1 activity, two waves of Cdk1 activity are generated, due to the accumulation of B-type cyclins. Cdk1/Clbs activates APC/C-Cdc20, which in turns inactivates Cdk1 by destroying cyclins, thereby leading to a decrease of APC/C-Cdc20 activity.

Although the periodic activation of Cdk1-Clbs and APC/C-Cdc20 is similar in meiosis I and in meiosis II, the outcome of the two divisions is dramatically different. This is due to additional mechanisms and molecules that prepare chromosomes for segregation. For example, reductional segregation of chromosomes follows pre-meiotic S-phase, during which DNA replication, recombination, and monopolar attachment of sister chromatids occur in this precise order. S-phase kinases such as Cdk1 bound to S-phase specific cyclins Clb5 and Clb6 (Smith et al., 2001) and Dbf4-dependent Cdc7 kinase are crucial for the induction of recombination and monopolar attachment (Matos et al., 2008) and their regulatory subunits are degraded at anaphase I, thereby rendering recombination and monopolar attachment hallmarks of meiosis I. By contrast, very little is known about how meiosis II-specific events, namely equational division, de-protection of centromeric cohesins, exit from meiosis II, and gametogenesis are confined to meiosis II. In principle, these events could be either inhibited in meiosis I, or activated only in meiosis II. To date, the corresponding mechanisms are not fully understood. Here, I investigated how the initiation of spore formation is confined to meiosis II. This is crucial to ensure that only single copy genomes are enclosed into spores.

1.4. Sporulation in budding yeast

In the presence of a non-fermentable carbon source and in the absence of nitrogen, budding yeast diploid cells enter meiosis and package their nuclei into sturdy structures, called spores, which are able to survive harsh conditions for a long period of time (Neiman, 2011). Meiosis occurs within the boundaries

of the mother cell, and each haploid nucleus that arises from anaphase II is enveloped in a double-layer of the newly formed prospore membrane. Within the two membrane layers, a thick spore wall is deposited, and the mother cell becomes the ascus in which the four spores are enclosed. These morphogenetic events require a re-organization of the vegetative cell cycle control, which is achieved in two ways: first, mitotic regulators are replaced by meiotic counterparts and second, mitotic regulators acquire a new function in meiosis. For example, generating new membranes to enclose the nuclei requires the fusion of post-Golgi secretory vesicles to be directed to the prospore membrane instead of the plasma membrane as occurs in mitosis. Such changes are achieved by replacing the mitotic t-SNARE complex subunit Sec9 with its meiosis-specific counterpart, Spo20 (Neiman et al., 2000). The details of the stages of sporulation are described below.

1.4.1. Modification of the spindle pole bodies: assembly of a meiotic plaque

Formation of a prospore membrane initiates in meiosis II at the sole microtubule-organizing center of yeast cells, the spindle pole body (Moreno-Borchart and Knop, 2003). The spindle pole body (SPB) is embedded in the nuclear envelope and its structure has been investigated extensively by electron microscopy. In a diploid cell, the spindle pole body has a diameter of 160 nm, and is composed of three plaques of electron-dense material, the central, inner, and outer plaque. Due to its complexity, not all SPBs components have been localized (Jaspersen and Winey, 2004). The core SPB **(Figure 3)** consists of a crystal of Spc42, an essential coiled-coil protein that assembles into a hexagonal matrix (Bullitt et al., 1997). The N-terminus of Spc42 is oriented toward the nuclear face and associates with two other coiled-coil proteins: Spc29, a structural component with a role in SPB duplication, and Spc110, the γ-tubulin complex receptor on the nuclear face of the SPB. A fourth protein, calmodulin, has been proposed to regulate the binding between Spc110 and Spc29 (Adams and Kilmartin, 1999; Elliott et al., 1999). The C-terminus of Spc42 faces the cytoplasm and binds to the C-terminus of Cnm67, the coiled-coil spacer between central plaque and outer plaque (Adams and Kilmartin, 1999). Cnm67 binds to the outer plaque protein Nud1, which is not a structural component of the SPB but has a signaling function in mitotic exit (Adams and Kilmartin, 1999;

Elliott et al., 1999; Gruneberg et al., 2000). Another coiled-coil protein that is part of the SPB core is Spc72, which constitutes the γ-tubulin complex receptor of the cytoplasmic face of the SPB (Knop and Schiebel, 1998). The γ-tubulin complex is required for the nucleation of microtubules on both sides of the SPB, and it includes Tub4, Spc98, and Spc97 (Geissler et al., 1996; Knop and Schiebel, 1997; Spang et al., 1996). In meiosis, the SPBs duplicate twice: first during pre-meiotic S-phase, and then again in meiosis II.

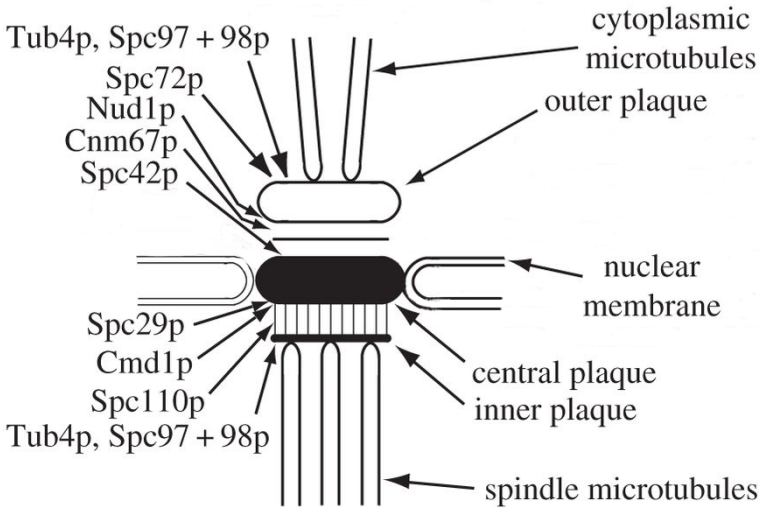


Figure 3. Spindle pole body architecture. The spindle pole body is a polarized organelle representing the only microtubule-organizing center of yeast cells. It is organized in plaques: the inner plaque is the area where the spindle microtubules dock to the SPB and harbors the γ-tubulin complex (consisting of Tub4, Spc97 and Spc98) and the N-terminus of Spc110. The central plaque is composed of a crystal of Spc42 around which structural proteins Spc110, Spc29 and Cmd1 assemble. The outer plaque consists of two coiled-coil proteins, such as Cnm67 and Spc72, and a third component with signaling functions, named Nud1. Modified from (Kilmartin, 2014).

While in meiosis I the spindle pole bodies are similar in shape to those of mitotic cells (Moens and Rapport, 1971a), in meiosis II, they appear more prominent, due to the recruitment of several meiosis-specific proteins, which form a further electron-dense layer, named meiotic plaque (MP). The main MP components are Mpc70/Spo21, Mpc54, and Spo74 (Bajgier et al., 2001; Knop and Strasser, 2000; Nickas et al., 2003). The MP proteins Mpc70 and Mpc54 are coiled-coil proteins, arranged with their N-termini toward the cytoplasm, and the C-termini located toward the N-terminus of a constitutive component of the SPB outer plaque, Cnm67 (Mathieson et al., 2010). Prior to the assembly of

the meiotic plaque, the outer plaque protein Spc72 is removed from the SPBs, presumably by proteolysis (Knop and Strasser, 2000; Nickas et al., 2004).

1.4.2. Formation of the prospore membrane (PSM)

Meiotic plaques mediate the docking and fusion of membrane precursors at the SPBs **(Figure 4)**. In the *MPC54* deletion mutant, membrane vesicles appear loosely tethered to the MP, which prevents their fusion, suggesting that engagement of the vesicles with the MP surface is critical to prospore membrane initiation (Mathieson et al., 2010). After docking on the MP, membrane vesicles undergo fusion, which leads to the formation of a membrane cap, a precursor of the PSM (Moens and Rapport, 1971a). Vesicle fusion requires a SNARE complex that acts specifically during sporulation in order to divert secretory vesicles from the plasma membrane to the prospore membrane (Neiman, 1998). Once formed on the MP, the PSM rapidly grows laterally from the spindle pole. During this expansion phase, the prospore membrane remains in contact with the spindle pole, but its growth is unlikely to require the meiotic plaque, since it occurs at sites that are increasingly more distant from it. Time-lapse microscopy experiments have shown that while it expands, the PSM changes shape to engulf the emerging nuclei (Diamond et al., 2009). Two cytoskeletal systems have been found to be associated with the growing membrane: septins and the leading edge complex. Septins are a family of conserved filament-forming proteins that are required during budding to compartimentalize the cortex of yeast cells by assembling as a ring at the bud neck (Barral et al., 2000). Septins were also found to localize at the prospore membrane, but in this case, they lack their characteristic ring-shaped structure (Fares et al., 1996). Despite their striking behavior during prospore membrane formation, septins are not required for spore formation and their function remains unclear (De Virgilio et al., 1996; Fares et al., 1996). It is possible that septins play a role in prospore membrane growth together with some other, redundant mechanisms. The second protein complex associated with the growing prospore membrane was named the leading edge complex (LEP) due to its localization (Moreno-Borchart et al., 2001). Its components Don1, Ady3, and Ssp1 are meiosis-specific gene products, identified in various two-hybrid screens for interactors of the MP proteins. Don1, the first component identified,

is a coiled-coil protein. Don1 localizes at the four poles of meiotic spindles during the second meiotic division. At anaphase II, it decorates four ring-like structures, two for each spindle. At later stages, just

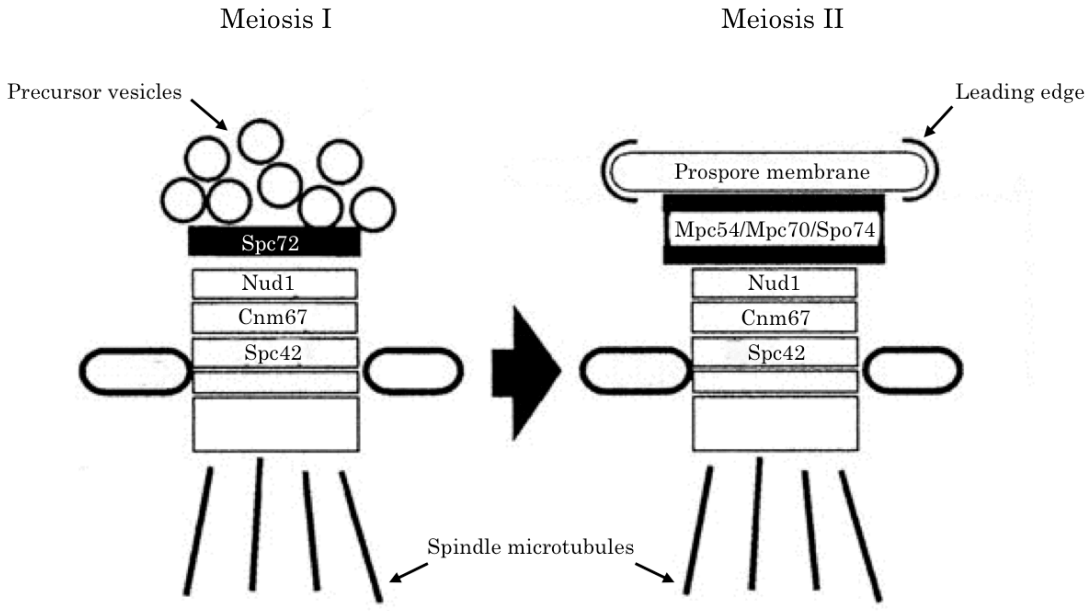


Figure 4. Prospore membrane assembly at the meiotic plaque. In meiosis I, the spindle pole bodies are, like in mitosis, composed by constitutive subunits such as Spc42, Cnm67, Nud1, and Spc72. Formation of a prospore membrane initiates in meiosis II at the spindle pole body and it requires the removal of Spc72 and the subsequent recruitment of the meiotic plaque proteins Mpc54, Mpc70 and Spo74. The growing edges of the prospore membrane are decorated by a protein complex named leading edge. Adapted from (Knop and Strasser, 2000).

after meiosis II, the Don1 rings are smaller and closer to each other, localizing toward the middle of the disassembling spindles. Finally, when spores become visible, Don1 is dispersed along the prospore membrane (Knop and Strasser, 2000). In cells lacking MP components, Don1 forms cytoplasmic foci, which do not correspond to SPBs. Nevertheless, the deletion of Don1 has no effect on sporulation, suggesting that Don1's proper localization is not required for sporulation. Ady3 and Ssp1 are, instead, required for sporulation, but to different extent (Moreno-Borchart et al., 2001; Nag et al., 1997). Ady3 co-localizes with Don1, and both proteins depend on Ssp1 for their localization. While Ady3 loss leads to a modest sporulation defect, Ssp1 deletion leads to the complete absence of spores, without affecting meiotic progression. In the *SSP1* deletion mutant, prospore membrane growth is abnormal, and when observed by electron microscopy, the prospore membrane remains tightly

associated with the surface of the nuclear envelope. As a result, little cytoplasm is engulfed by the growing membrane, and no spores are formed (Moreno-Borchart et al., 2001). Thus, Ssp1 seems to be the only LEP component essential for sporulation. Ssp1 degradation has been proposed to be an essential step in prospore membrane closure, and it is likely dependent on APC/C-Ama1 (Diamond et al., 2009; Maier et al., 2007).

1.4.3. Spore wall assembly

The closure of the prospore membrane is required for the subsequent deposition of spore wall precursors in the lumen of the PSM (Coluccio et al., 2004). A time course analysis of sporulating cells followed various fluorescent cell wall components, and revealed that the different spore wall layers are deposited in a temporal order that matches their order from inside to outside within the mature cell wall: mannan, β-1,3-glucan, chitosan, and dityrosine (Tachikawa et al., 2001). While haploid cells exposed to starvation tend to arrest, diploid cells undergo sporulation, a much more complicated response. One ecological advantage of sporulation has been suggested to be the dispersal in different environments by other organisms, such as insects. While this would explain why the spore wall is built under starvation conditions, it raises the question of why sporulation is associated with meiosis, rather than being limited to the building of a more robust cell wall under adverse conditions (Neiman, 2011).

1.5. On the coordination of meiosis and sporulation

Spore differentiation starts at metaphase II, when cells contain four spindle pole bodies as poles of two meiosis II spindles, which are pulling on sister chromatids. How MP formation, the first hallmark of sporulation, occurs at this precise time is not yet known. At entry into metaphase I, Ndt80 already promotes the transcription of mRNAs encoding MP proteins and other proteins required at later stages of prospore membrane formation. However, these proteins do not become active until entry into metaphase II. It is not clear yet whether an activating mechanism promotes recruitment of MP proteins to the spindle pole bodies precisely at metaphase II, or their recruitment is inhibited in meiosis I, or both. Remarkably, cells depleted of separase can form spores

with undivided nuclei. Thus, chromosome segregation is not required for sporulation (Buonomo et al., 2000). In contrast, eliminating Cdc20 activity in meiosis leads to a block of sporulation, even though proteins required for sporulation, including MP proteins, have been expressed (Simchen, 1974), indicating a link between sporulation and the cell cycle machinery. However, it is unclear whether APC/C-Cdc20 plays a direct role in sporulation, or whether it merely promotes progression to meiosis II. Other cell cycle regulators are required for some stages of spore formation, but either their role is not known or, as in the case of Ama1, they act downstream of MP assembly. It has been reported that prior to the loading of the meiotic plaque, the outer plaque protein Spc72 is removed from the SPBs, presumably by proteolysis, shortly after SPBs reduplication (Knop and Strasser, 2000; Nickas et al., 2004). Spc72 is also a mitotic component of the outer plaque and it is required for microtubule nucleation, being the ϒ-tubulin complex receptor on the cytoplasmic face of the SPBs (Knop and Schiebel, 1998). The function of cytoplasmic microtubules in meiosis I is not known, and neither is the mechanism by which Spc72 is removed from the outer plaque. Whether Spc72 turnover is relevant for MP formation has not been tested to date.

1.6. Yeast mutants that make only two-spored asci

Any model attempting to explain how the onset of spore formation is confined to meiosis II in wild-type yeast should take into account mutants that make only two spores, instead of four. As mentioned before, mutants defective in completing meiotic divisions can still produce spores, despite containing the "wrong" number of chromosomes (Buonomo et al., 2000; Schild and Byers, 1980). Relevant to the coordination of meiosis II with gametogenesis are those mutants that undergo recombination followed by a single equational division, such as the deletions of Spo12 or Spo13 (Klapholz and Esposito, 1980a). Spo12 is a protein with nuclear and nucleolar localization, whose precise function is unknown. Spo12 is cell cycle regulated and it acts as a regulator of mitotic exit (Shah et al., 2001). Cells lacking Spo12 enter meiosis I normally and divide chromosomes along a single spindle axis, but they show substantial segregation of sister chromatids in this division. It has been shown that partial segregation of sister chromatids is caused not by any alteration of centromeres

in meiosis I, but more likely by the inability of cells to downregulate Cdk1/Clbs kinase in anaphase I. As a consequence, meiosis I spindles disassembly is delayed, while loss of monopolin from centromeres or degradation and re-accumulation of Pds1 proceed normally (Buonomo et al., 2003). Spo13 is a meiosis I-specific protein, and its homologues in fission yeast and mammalian cells have been identified only recently (Kim et al., 2015). *spo13Δ* cells undergo a single meiotic division in which sister chromatids partially segregate (Klapholz and Esposito, 1980b). In contrast to the *SPO12* deletion mutant, cells lacking Spo13 show a delay in APC-C/Cdc20 activation, resulting in a delay to degrade Pds1 with respect to meiosis I entry. Anaphase I is also longer than in wild-type cells, and re-accumulation of Pds1 does not occur, suggesting that APC/C is activated only once in the absence of Spo13. Surprisingly, when *spo13Δ* cells are depleted of Cdc20 they can still perform a division, thereby bypassing the need of Cdc20 for progression into anaphase I. This suggested that Spo13 might be an enhancer of APC activity, possibly together with an APC/C activator different from Cdc20 (Katis et al., 2004b). Even though the *spo13Δ* mutant undergoes only one division and one round of APC/C activation, it forms spores, indicating a defect in the coordination between cell cycle and sporulation.

1.7. Aims of this study

In wild-type budding yeast, spore differentiation starts at metaphase II when cells contain four spindle poles ready to segregate chromatids. Nevertheless, the transcriptional cascade that drives meiosis, initiated by Ndt80, produces the genes products required for meiosis and for gamete differentiation at the same time, before the meiotic divisions. How spore differentiation is confined to meiosis II is still unknown. One possibility is that cells have an inhibitory mechanism that prevents sporulation from starting in meiosis I. In alternative, there may be an activation signal for spore differentiation that is triggered upon entry into meiosis II. Therefore, the aim of this work was to unravel the network of regulators of both meiotic progression and sporulation. In this study, we took advantage of those mutants that produce asci containing two haploid spores instead of four, which might display a defect in the coordination of meiotic divisions and gamete differentiation. We partially elucidated the

mechanisms that restrict sporulation to a defined stage of meiosis. Because the onset of gamete differentiation relies on conserved cell cycle regulators, such as such as the APC/C, Cdk1, and Hrr25, this work might provide a framework of how different types of gametes start to differentiate only when genome haploidization has occurred, to make sure that a single haploid genome will be engulfed in the mature gamete.

1.8 Contributions

My colleague Tugce Oz performed the experiment shown in Figure 20B, and Julie Rojas contributed to the experiment shown in Figure 18A.

2. Results

Here, we have investigated how spore formation, the yeast equivalent of gametogenesis, is coordinated with the two meiotic divisions. We have synchronized meiotic cultures of yeast to analyze the function of essential cell cycle regulators in meiosis and sporulation. We show that Hrr25 is not only required for centromeric cohesin cleavage in meiosis II, and exit from meiosis II, but also for the first step of sporulation, namely the assembly of the meiotic plaque. However, Hrr25 is present throughout meiosis, thus it does not explain why sporulation occurs only in meiosis II. We find that the mutual regulation of APC/C and Cdk1/Clbs is important to restrict sporulation to meiosis II.

2.1 Hrr25 activity is required after anaphase I for several meiosis II events

The study of meiosis II is not a trivial task, mainly due to the short time between the two meiotic divisions, and to the dependence of both divisions on the same set of basic cell cycle regulators. To manipulate protein's functions only in meiosis II without perturbing meiosis I, the *CDC20*-meiotic-arrest/release (*CDC20-mAR*) system was constructed (Arguello-Miranda et al., 2017). The *CDC20-mAR* system consists of the endogenous *CDC20* gene controlled by the mitosis-specific *CLB2* promoter, and an additional copy of *CDC20* expressed by the copper-inducible *CUP1* promoter. After induction of meiosis, *CDC20-mAR* cells arrest in metaphase I due to the lack of Cdc20. Addition of $CuSO_4$ induces progression through anaphase I, and meiosis II within 120 minutes. Hrr25 is one of the kinases that phosphorylate Rec8 to promote cohesin cleavage by separase in meiosis. In contrary to Cdc7-Dbf4, which becomes inactive after anaphase I due to the degradation of Dbf4 by APC/C-Cdc20, Hrr25 is expressed at constant levels throughout the whole meiosis, and it is required for chromosome segregation in meiosis I (Katis et al., 2010). Furthermore, *hrr25* mutants exhibit a defect in forming spores. However, at which stage of spore formation Hrr25 is required remained unclear (Petronczki et al., 2006). This prompted us to explore Hrr25's functions in meiosis II, combining the *CDC20-mAR* system with an *HRR25* allele that is sensitive to kinase inhibition upon addition of the ATP analog 1NM-PP1 (*hrr25-as*) (Petronczki et al., 2006). Our

laboratory has shown recently that Hrr25 is required in meiosis II for de-protection and cleavage of centromeric Rec8, for the exit from meiosis, and for prospore membrane formation (Arguello-Miranda et al., 2017). A key experiment in this regard was the imaging of Rec8-GFP and RFP-tagged tubulin in *CDC20-mAR HRR25* and *CDC20-mAR hrr25-as* cells **(Figure 5)**. We induced the cultures to enter meiosis, and released cells from the metaphase I arrest by adding $CuSO_4$(10 μM). We added 1NM-PP1 (5 μM) 40 min later, when majority of cells in both cultures were in anaphase I. In both strains, Rec8-GFP accumulates during prophase and in metaphase I it decorates the entire chromatin. Upon entry into anaphase I, two dots at the poles of the spindle become brighter, while the nuclear signal becomes weaker. The dots represent centromeric Rec8, which in meiosis II is localized between the poles of each metaphase II spindle. In *HRR25* cells, the centromeric Rec8 dots were visible for 40±12 min, and they disappeared when metaphase II spindles started to elongate. Furthermore, shortly after the removal of centromeric Rec8, meiosis II spindles were disassembled, thereby living for 65±13 min. By contrast, in *hrr25-as* cells, centromeric Rec8 persisted longer (141±116 min) than in control cells in the presence of meiosis II spindles. The lifetime of MII spindles in *hrr25-as* cells was also prolonged (180±108 min). Therefore, Hrr25 activity in meiosis II is required for centromeric cohesin cleavage, and also for the disassembly of meiosis II spindles, which is one of the hallmarks of exit from meiosis II. The exit from meiosis II relies on Hrr25 to inactivate M-phase kinases that promote spindle formation, such as Cdk1 and Cdc5. Hrr25 inactivates the kinases though activation of Cdc20- and Ama1-mediated proteolysis of Clb1, Cdc5, and Ndt80 (Arguello-Miranda et al., 2017).

2.2 Hrr25 mediates the recruitment of meiotic plaque proteins at the spindle pole bodies in meiosis II

Due to its roles in both meiosis II and spore formation, we considered Hrr25 as a promising candidate to study the coordination of the two processes. We started from asking at which stage of the gamete differentiation pathway Hrr25 was required. Yeast spores are formed at the end of meiosis II inside the mother cell's boundaries. This differentiation process involves the formation of a

prospore membrane (PSM), the plasma membrane equivalent that encapsulates the spores (Moreno-Borchart et al., 2001).

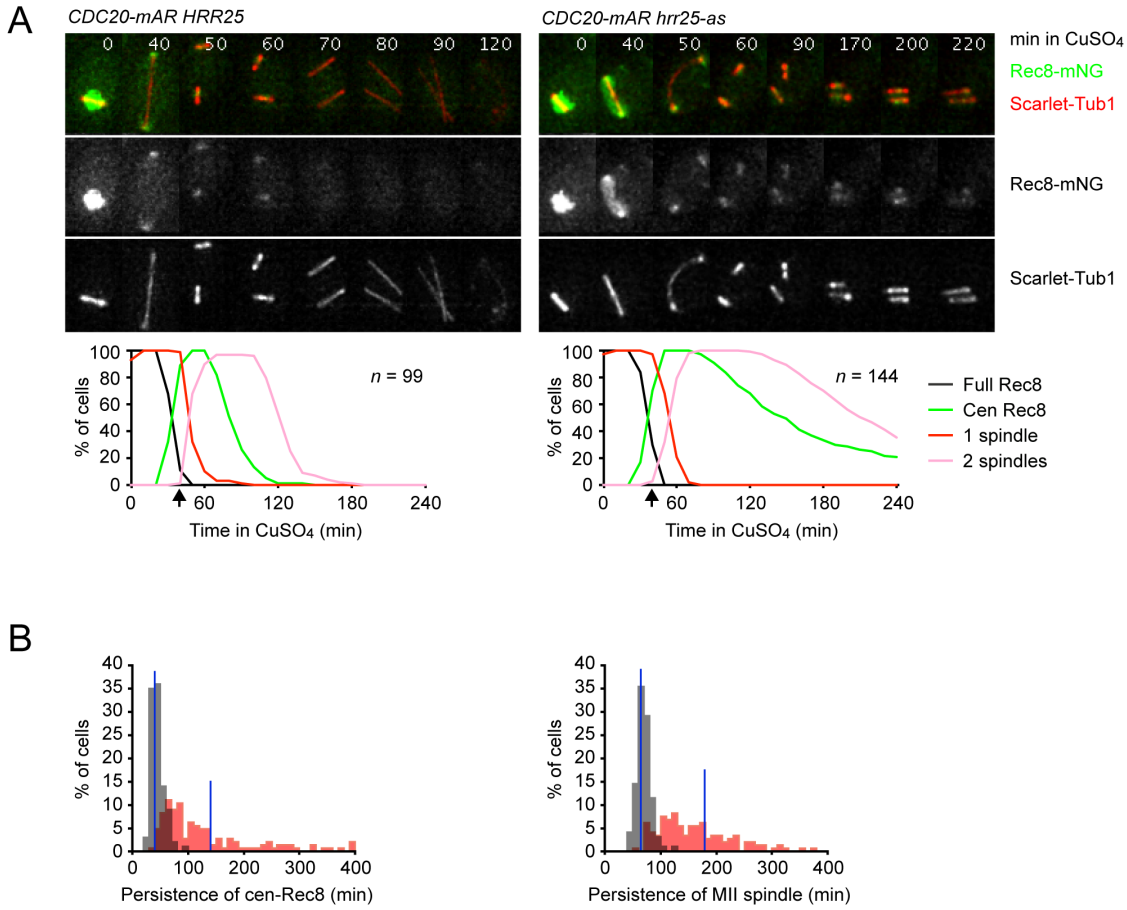


Figure 5. Hrr25 regulates centromeric Rec8 cleavage and exit from meiosis II. Live-cell imaging of *CDC20-mAR HRR25* (Z23834) and *CDC20-mAR hrr25-as* (Z23833) strains undergoing meiosis. Cells expressing RFP-tubulin, and Rec8-GFP were arrested in metaphase I by Cdc20 depletion, imaging was started at 7 hours in SPM, and cells were released into anaphase by addition of $CuSO_4$ (10 μM) and treated with 1NM-PP1 (5 μM) 40 minutes later. (A) Top panels display representative time-lapse series. Below images: graphs showing the percentage of cells with full Rec8, cen-Rec8, 1 spindle, or 2 spindles. (B) Histograms of the lifetimes of centromeric Rec8 and of meiosis II spindles in *HRR25* (grey) and *hrr25-as* (red) cells. Blue lines indicate mean values.

Therefore, the first question we addressed was whether Hrr25 is involved in shaping the PSM. We imaged *HRR25* and *hrr25-as* strains, expressing GFP-tagged histone H2B (Htb1-GFP) to visualize nuclei, and the PSM-marker Spo20-RFP **(Figure 6)**. Strains were induced to synchronously enter meiosis in the presence of 1NM-PP1 (5 μM). In *HRR25* cells, Htb1-GFP nuclear signal divided into two masses in meiosis I, and into four equal masses in meiosis II. While the two nuclei generated by the first division started to elongate, due to the pulling forces of the spindles, discrete foci of Spo20-RFP appeared in

proximity of the DNA. These foci represent the prospore membrane precursors that are recruited to the SPBs. In a few minutes, the foci became circles, growing around the dividing nuclei. When the second nuclear division was completed, the circles surrounded and enclosed each nucleus. By contrast, *hrr25-as* cells failed to divide, and membrane precursors did not concentrate in four discrete foci in the vicinity of the DNA. Instead, they accumulated in the cytoplasm and did not form any structure. We concluded that PSM docking to the SPBs is severely impaired in cells lacking Hrr25 activity. Therefore, PSM cannot grow around chromatin in meiosis II, and cells fail to form spores.

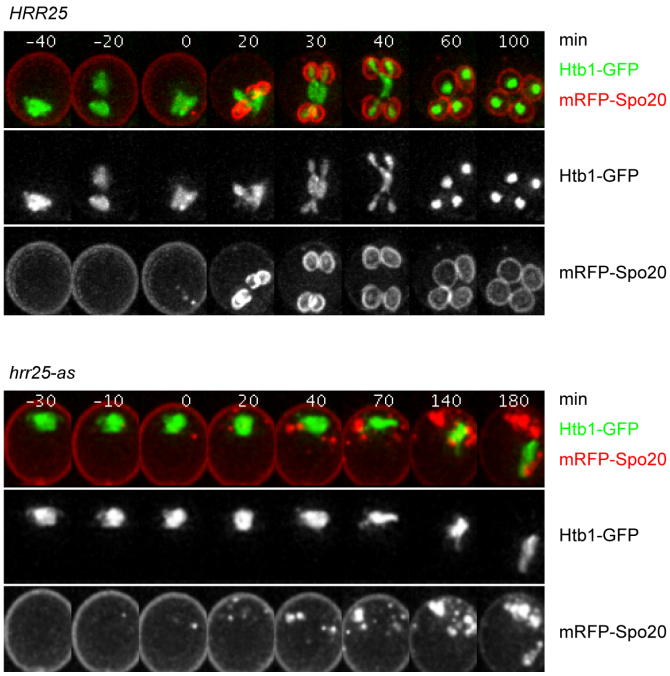


Figure 6. Hrr25 is required for the docking of prospore membrane precursors at the SPBs. Live-cell imaging of *HRR25* and *hrr25-as* strains undergoing meiosis. *HRR25* (Z23794) and *hrr25-as* (Z23795) expressing Spo20-RFP and Htb1-GFP were filmed every 10 minutes for 12 hours. 1NM-PP1 (5 μM) was added after 3 hours in SMP. Representative time-lapse series are shown.

The recruitment of membrane precursors at the SPBs in meiosis II depends on a meiosis-specific structure, called meiotic plaque (MP), composed of three proteins: Mpc54, Mpc70, and Spo74. To analyze whether the recruitment of MP proteins at the SPBs required Hrr25, we tested the localization of each MP protein in *HRR25* and *hrr25-as* strains, expressing Mpc54, or Mpc70, or Spo74 tagged with GFP. To visualize SPBs and to follow meiotic progression, the outer plaque component Cnm67 was tagged with RFP **(Figure 7)**. Prophase is

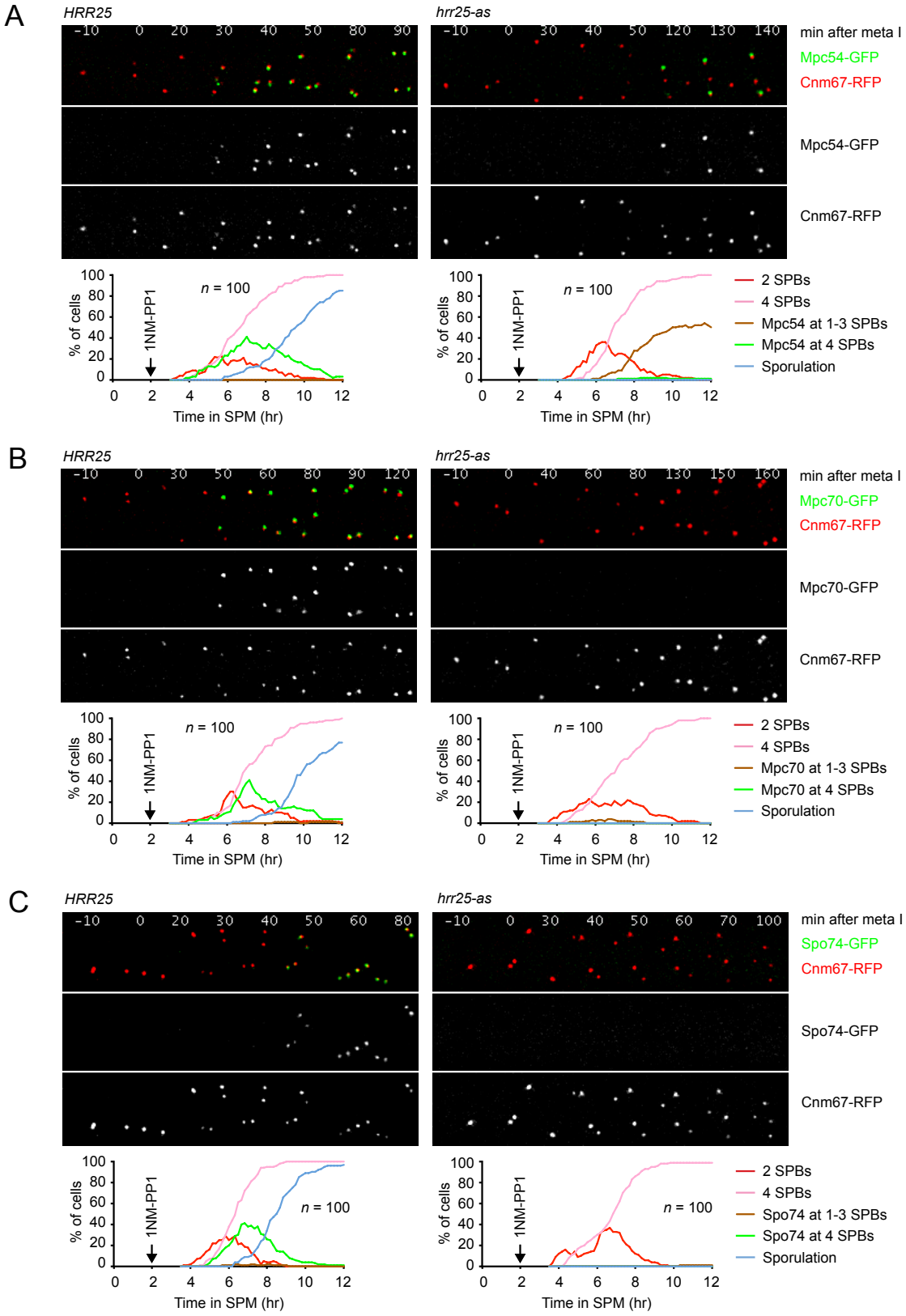


Figure 7. Hrr25 regulates the localization of Mpc54, Mpc70, and Spo74 at the SPBs in meiosis II. Live cell imaging of *HRR25* and *hrr25-as* strains undergoing meiosis. (Legend on the next page)

characterized by duplicated but closely apposed SPBs, which form a single, dot-like Cnm67-RFP signal. SPB separation is the landmark of metaphase I, which is followed by their further separation due to spindle elongation characteristic of anaphase I. Subsequently, a second round of SPB duplication occurs, a landmark of metaphase II. In wild-type cells, Mpc54-GFP, Mpc70-GFP, and Spo74-GFP co-localized with Cnm67-RFP upon SPB re-duplication (Bajgier et al., 2001; Knop and Strasser, 2000; Nickas et al., 2003). Furthermore, we observed that in almost every cell, each MP protein localized to all four SPBs. The high temporal resolution of the experiment allowed us to calculate that the recruitment of Mpc54-GFP, Mpc70-GFP, and Spo74-GFP at the SPBs occurs at the same time, namely 50 minutes after SPBs separation. By contrast, *hrr25-as* cells showed little if any localization of Mpc70-GFP and Spo74-GFP at the SPBs, while Mpc54-GFP was localized only at two of the four SPBs in 60% of the cells, albeit with a considerable delay compared to wild-type cells. We concluded that Hrr25 mediates the recruitment of MP proteins at the SPBs in metaphase II of meiosis. Even though Mpc54-GFP might be less sensitive to Hrr25 activity, the absence of any one of the three proteins was reported to be sufficient to prevent the formation of a functional meiotic plaque (Bajgier et al., 2001; Knop and Strasser, 2000; Nickas et al., 2003). Since previous studies reported that the tagging of MP proteins might reduce their functionality (Bajgier et al., 2001), we sought to analyze the behavior of MP proteins in *hrr25-as* cells without labeling proteins with fluorescent tags. Therefore, we used electron microscopy to compare the SPBs of *HRR25*, *hrr25-as*, and *mpc54Δ mpc70Δ* cells **(Figure 8)**. MP assembly occurs at metaphase II, and with conventional synchrony the proportion of cells expressing any of the three MP proteins at a given time-point is not higher than 30%. Thus, we used

Figure 7. Hrr25 regulates the localization of Mpc54, Mpc70, and Spo74 at the SPBs in meiosis II. (A) *HRR25* (Z21830) and *hrr25-as* (Z21831) expressing Cnm67-RFP and Mpc54-GFP were filmed every 10 minutes for 12 hours. Top panels display representative time-lapse series. Below images: graphs showing the percentage of cells with 2 or 4 Cnm67-RFP signals and with Mpc54-GFP at the SPBs. (B) *HRR25* (Z21840) and *hrr25-as* (Z21841) expressing Cnm67-RFP and Mpc70-GFP were filmed every 10 minutes for 12 hours. Top panels display representative time-lapse series. Below images: graphs showing the percentage of cells with 2 or 4 Cnm67-RFP signals and with Mpc70-GFP at the SPBs. (C) *HRR25* (Z28716) and *hrr25-as* (Z28717) expressing Cnm67-RFP and Spo74-GFP were filmed every 10 minutes for 12 hours. Top panels display

representative time-lapse series. Below images: graphs showing the percentage of cells with 2 or 4 Cnm67-RFP signals and with Spo74-GFP at the SPBs.

the *CDC20-mAR* system to improve the synchronous progression of the meiotic cultures upon induction of anaphase I. We induced sporulation in *CDC20-mAR HRR25*, *hrr25-as*, and *mpc54Δ mpc70Δ* cells. Samples were taken every 20 minutes for indirect immunofluorescence staining on fixed cells of α-tubulin to visualize spindles, and DNA staining to monitor nuclear division **(Figure 8A)**. Upon release into anaphase I, *HRR25* cells went through the first nuclear division. Next, they disassemble meiosis I spindles and substitute them with meiosis II spindles, which pulled nuclei apart in the second meiotic division. At t=600 min in SPM, all cells that entered meiosis were tetranucleate. Consistent with previous reports (Knop and Strasser, 2000), *CDC20-mAR mpc54Δ mpc70Δ* cells progressed through meiosis with wild-type kinetics. By contrast, *hrr25-as* cells kept meiosis II spindles much longer than wild-type cells, and these spindles failed to elicit a nuclear division (Arguello-Miranda et al., 2017). We collected cells for electron microscopy at various time points, and after scoring the progression of the cultures, we analyzed t=560 min in SPM, the time point that shows the peak of meiosis II spindles in all three strains **(Figure 8B)**. More than 40 SPBs were examined by TEM in each strain, and scored for the presence or absence of the MP. 93% of the SPBs scored in *HRR25* cells had a characteristic meiosis II morphology, with a defined bilayer electron-dense structure above the central plaque (Moens and Rapport, 1971b), representing the meiotic plaque. As expected, all the SPBs scored in *mpc54Δ mpc70Δ* cells showed abnormalities in the outer plaque: the defined bilayer was absent but amorphous material connected to the central plaque was evident (Bajgier et al., 2001). The SPBs of *hrr25-as* cells, similarly to *mpc54Δ mpc70Δ* cells, showed only the central plaque and a thin line of amorphous material on the top (corresponding to the unmodified outer plaque), thereby confirming that Hrr25 activity is essential for the formation of meiotic plaques in metaphase II. Finally, since the localization of MP proteins depends on Hrr25, we tested whether Hrr25 was required for the expression of the MP proteins **(Figure 9)**. We induced cells of *HRR25* and *hrr25-as* strains expressing either Mpc54-GFP, or Mpc70-GFP, or Spo74-GFP to synchronously enter meiosis in the presence of 1NM-PP1 and collected samples every two hours. Immunofluorescence staining of α-tubulin was used to visualize the spindles

in fixed cells **(Figure 9A)**. Protein levels were analyzed by immunoblotting of whole cell extracts **(Figure 9B-C-D)**.

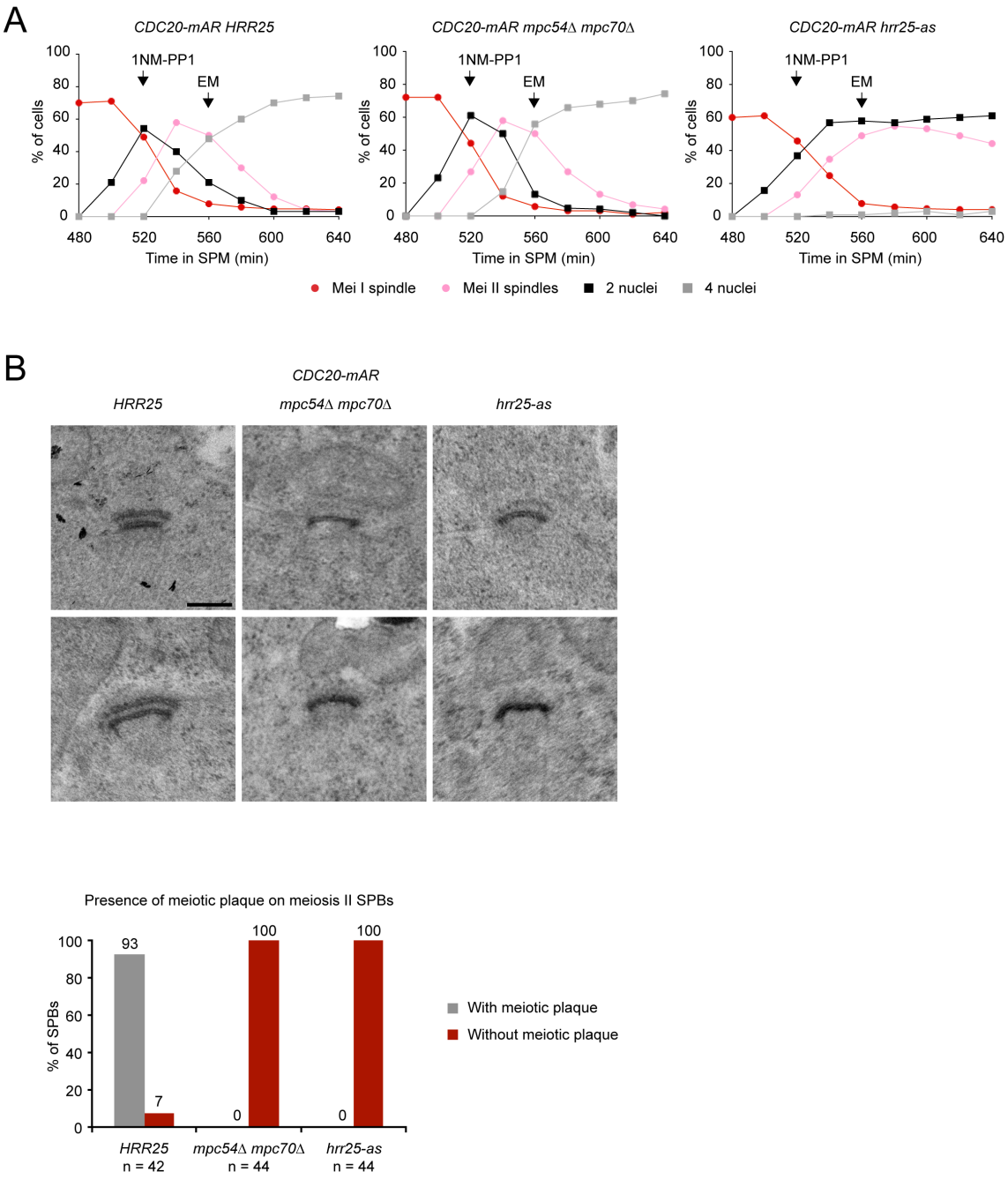


Figure 8. Hrr25 is required for the formation of meiotic plaques. *CDC20-mAR* strains containing *HRR25* (Z23218), *hrr25-as* (Z23219) or *mpc54Δ mpc70Δ* (Z29809) were arrested in metaphase I by Cdc20 depletion, released into anaphase by addition of $CuSO_4$ (10 μM) and treated with 1NM-PP1 (5 μM) 40 minutes later. (A) Immunoflurescence samples were taken every 20 minutes, stained by indirect immunofluorescence to detect α-tubulin, and DNA. A sample at time point 560 minutes in SPM was processed for electron microscopy. (B) Representative pictures of spindle pole bodies are displayed (scale bar, 200 nm), and the histogram below the images shows the percentage of spindle pole bodies with (grey) or without (red) a meiotic plaque.

The appearance of meiosis-I spindles is a landmark of metaphase I, and in both *HRR25* and *hrr25-as* strains, meiosis I spindles can be observed at t=6 hours after induction of meiosis. Progression into anaphase is marked by the appearance of bi-nucleated cells, which accumulate in wild-type cells at t=8 hours. By contrast, in *hrr25-as* cells, while entry into meiosis occurs with similar kinetics compared to wild-type cells, nuclear division is blocked because sister kinetochores are bi-oriented and centromeric Rec8 is protected (Petronczki et al., 2006). Nevertheless, both *HRR25* and *hrr25-as* cells disassemble meiosis I spindles at t=10 hours, and subsequently assemble meiosis II spindles. By the end of the time-course, 95% of wild-type cells disassembled meiosis II spindles and were tetra-nucleate, while 90% of *hrr25-as* cells remained mono-nucleate with two meiosis II spindles (Petronczki et al., 2006). Consistent with normal entry into M-phase, *hrr25-as* cells start expressing MP proteins at the same time as *HRR25* cells. However, *hrr25-as* cells accumulated Mpc54, Mpc70-GFP and Spo74-GFP at high levels, whereas *HRR25* cells degraded Mpc70-GFP and Spo74-GFP after 10 hours from the induction of meiosis. This suggests that expression of Mpc54, Mpc70, and Spo74 does not require Hrr25. Instead, the marked decline of the levels of Mpc70-GFP and Spo74-GFP occurs only in the presence of Hrr25 kinase activity, indicating that Hrr25 not only mediates the recruitment of MP proteins at the SPBs, but also promotes the degradation of MP proteins prior to final stages of spore formation. Whether the degradation of MP proteins after meiosis II is necessary for sporulation remains unclear.

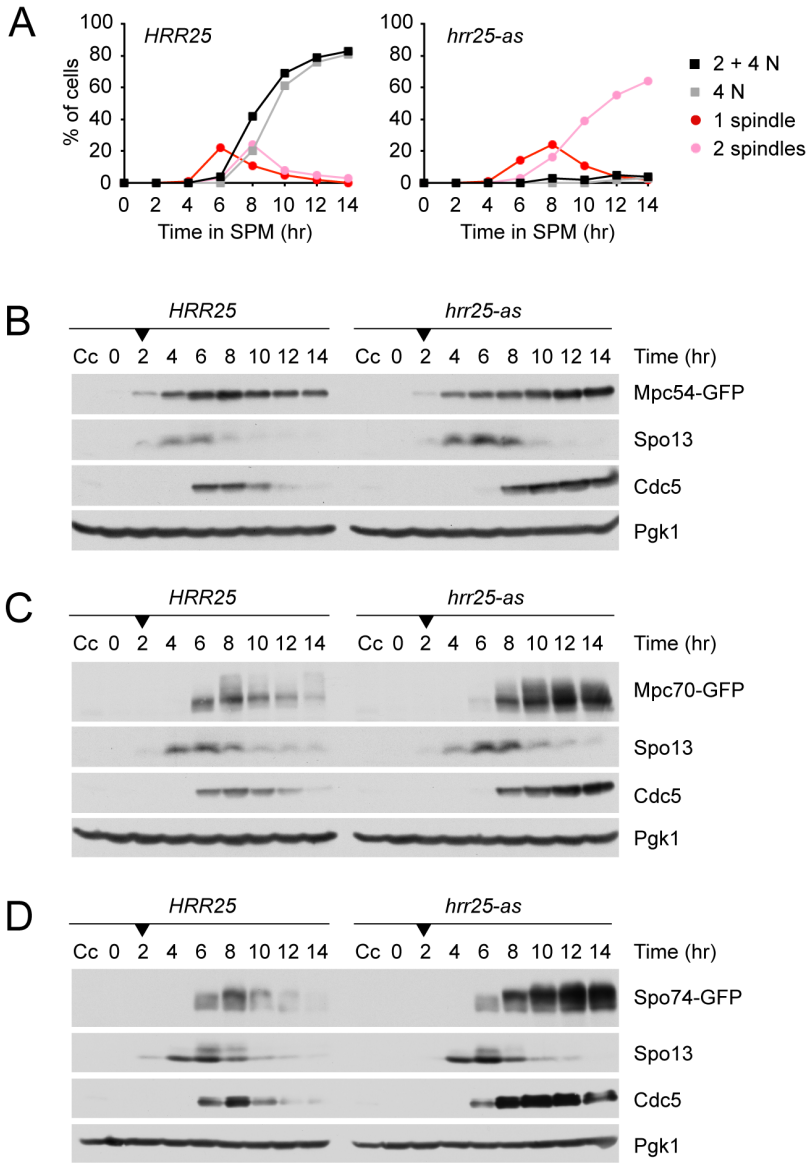


Figure 9. Expression of Mpc54, Mpc70 and Spo74 occurs normally in *hrr25-as* cells. *HRR25* and *hrr25-as* strains containing Mpc54-GFP, Mpc70-GFP, or Spo74-GFP were treated with 1NM-PP1 after 3 hours in SPM. Protein extracts prepared in trichloroacetic acid were analyzed by immunoblotting. (A) Immunofluorescence detection of meiotic spindles (α-tubulin), and nuclei (DAPI) in fixed *HRR25* cells (Z21830) and *hrr25-as* cells (Z21831) expressing Mpc54-GFP. Percentage of cells with 1 or 2 spindles and 2 or 4 nuclei is shown at each time point. (B) Immunoblot detection of protein levels along the time course. Cc indicates a sample from proliferating cells. (C) Immunoblot detection of protein levels in *HRR25* (Z21840) and *hrr25-as* (Z21841) cells expressing Mpc70-GFP. (D) Immunoblot detection of protein levels in *HRR25* (Z28716) and *hrr25-as* (Z28717) cells expressing Spo74-GFP.

2.3 Hrr25 is not required for the removal of Spc72 from the cytoplasmic face of the SPBs in anaphase I

Prior to the loading of the meiotic plaque, the outer plaque protein Spc72 is removed from the SPBs, presumably by proteolysis, shortly after anaphase I (Knop and Strasser, 2000). Whether the removal of Spc72 is a pre-requisite for

meiotic plaque assembly remains unclear. We speculated that a failure in recruiting the MP proteins at the SPBs in the absence of Hrr25 activity might be due to the stabilization of Spc72 on the cytoplasmic face of the SPBs. To test whether Spc72 expression levels or degradation require Hrr25 activity, we induced diploid *HRR25* and *hrr25-as* strains containing Spc72-GFP and Cnm67-RFP to synchronously enter meiosis in the presence of 1NM-PP1, and we collected samples for immunoblot detection of whole cell extracts every two hours **(Figure 10A)**. As expected, in wild-type cells, Spc72-GFP was detectable from the induction of meiosis to t=8 hours. With the time-resolution of two hours we can observe that Spc72-GFP levels declined at the same time as an APC/C-Cdc20 substrate, such as Spo13 (Sullivan and Morgan, 2007). In *hrr25-as* cells, the expression pattern of Spc72-GFP was very similar to that in the control strain. Strikingly, Cnm67 levels in control cells declined after t=10 hours in SPM, whereas in *hrr25-as* cells, Cnm67 levels remained high until the end of the time-course, similarly to Cdc5 levels. Next, we tested whether in *hrr25-as* cells Spc72 is also removed from the SPBs. We induced diploid *HRR25* and *hrr25-as* strains containing Spc72-GFP and Cnm67-RFP to synchronously enter meiosis in the presence of 1NM-PP1, and we observed the localization of Spc72 using live-cell imaging **(Figure 10B)**.

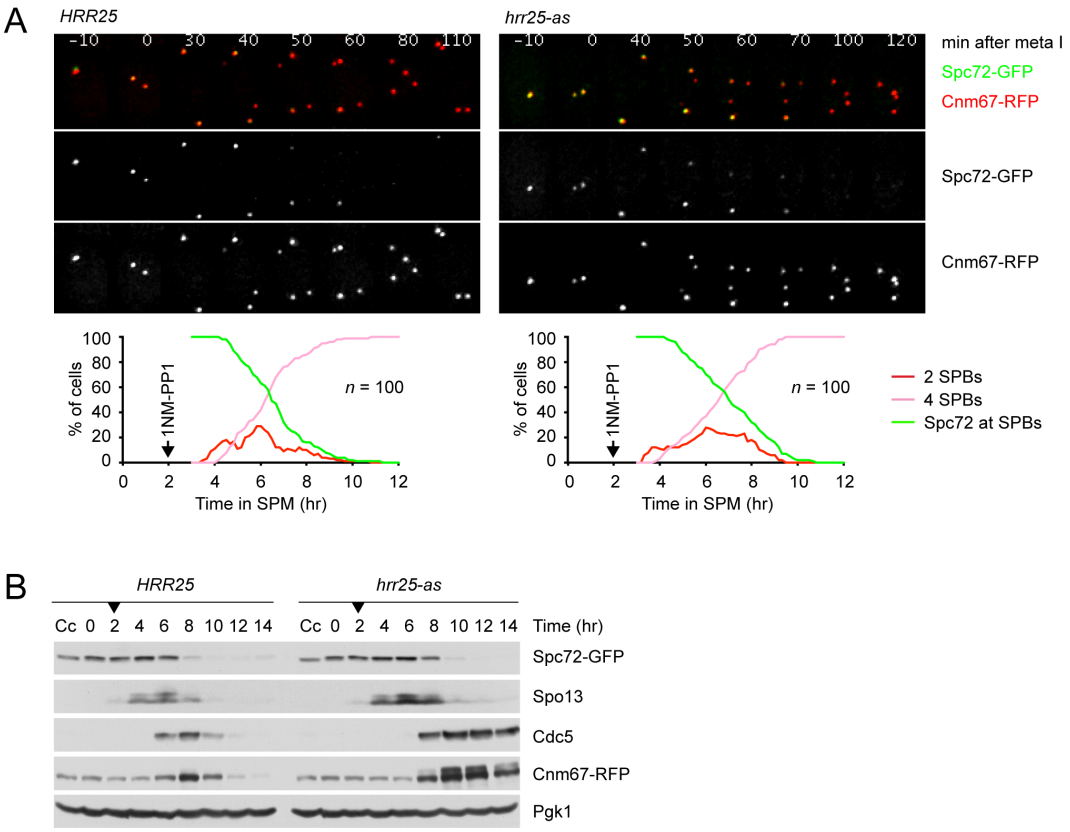


Figure 10. Spc72 localization and stability do not depend on Hrr25. Meiotic time course of *HRR25 SPC72-GFP* (Z22117) and *hrr25-as SPC72-GFP* (Z22118) strains. (A) Live cell imaging of *HRR25* and *hrr25-as* strains undergoing meiosis. Cells expressing Cnm67-RFP and Spc72-GFP were filmed every 10 minutes for 12 hours. Representative time-lapse series are shown. Below images: graphs displaying the percentages of cells with 2 or 4 Cnm67-RFP signals and with Spc72 at the SPBs. (B) Immunoblot detection of protein levels along the time course. Cc indicates a sample from proliferating cells.

Consistent with previous reports, in wild-type cells, Spc72 became undetectable at the SPBs shortly after anaphase I (Knop and Strasser, 2000). Similarly, in *hrr25-as* cells, the removal of Spc72 from the SPBs occurred with the same kinetics as in the *HRR25* strain. Since Spc72 behaves normally in *hrr25-as* cells, we inferred that Hrr25 regulates the assembly of the MP in meiosis II, without affecting Spc72 removal from the SPBs. The possibility that Hrr25 regulates only the assembly of the meiotic plaque, but not the structure or function of the constitutive components of the SPBs, such as Cnm67, or Nud1, is based on the following evidence: (i) Hrr25 in meiosis localizes from prophase to the end of meiosis diffusely in the nucleus, and it is not enriched at the SPBs at any meiotic stage (Petronczki et al., 2006), (ii) Hrr25 is not required for the duplication or the separation of the SPBs in meiosis (Petronczki et al., 2006),

and (iii) Hrr25 is not involved in Spc72 removal from the SPBs, which is the last event preceding MP formation. For these reasons, we decided to focus on the role of Hrr25 on the MP assembly and on understanding whether the defect in MP formation is a consequence of the failure of other meiosis-specific tasks of Hrr25.

2.4 Hrr25 interacts with Mpc54, Mpc70, and Spo74 at the time of their expression

In order to understand how Hrr25 promotes MP assembly, we tested whether Hrr25 interacts with Mpc54, Mpc70, and Spo74 in vivo, through co-immunoprecipitation experiments. We induced wild-type cells and cells expressing Hrr25 with a C-terminal ha3 tag (*HRR25-ha3*) to synchronously undergo meiosis, and we withdrew samples every one or two hours for anti-HA immunoprecipitation, followed by immunoblot detection of either Mpc54-GFP, or Mpc70-GFP, or Spo74-GFP **(Figure 11)**. In both wild-type cells and cells expressing Hrr25-ha3, Mpc54-GFP is expressed starting from 4 hours upon induction of meiosis, until 10 hours upon induction, which corresponds to the end of meiotic divisions. During this time we observed that Mpc54-GFP co-purifies with Hrr25-ha3 **(Figure 11A)**. Because Hrr25-ha3 co-purifies also with Rec8 at time point 4 and 6 hours, and Rec8 is cleaved in anaphase I, Hrr25-ha3 co-purifies with Mpc54-GFP even before anaphase I. In other words, Hrr25 interacts with Mpc54 before Mpc54 is recruited to the SPBs, suggesting that binding of Hrr25 to Mpc54 occurs prior to the recruitment of Mpc54 to the SPBs. We observed a similar pattern in the co-purification of Mpc70-GFP with Hrr25-ha3 **(Figure 11B)** and in the co-purification of Spo74-GFP with Hrr25-ha3 **(Figure 11C)**. We concluded that Hrr25 interacts with all three MP proteins in meiosis, even before the proteins bind to the SPBs, suggesting that Hrr25 might have a role in making the MP proteins competent to localize at the SPBs.

2.5 Hrr25 activity is required for MP formation independently of nuclear division

The inhibition of Hrr25 activity in meiosis II leads to a failure of nuclear division, due to the requirement of Hrr25 for Rec8 phosphorylation and for the removal of Sgo1 from centromeres (Arguello-Miranda et al., 2017). Since

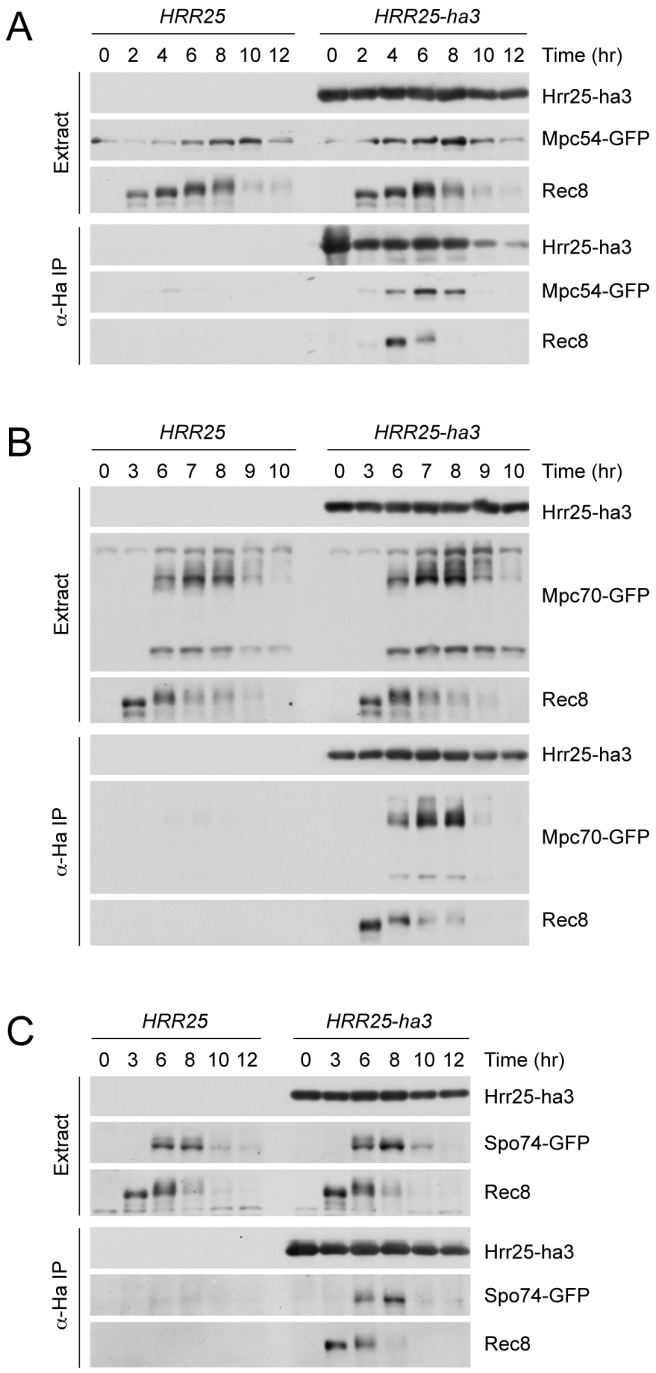


Figure 11. Hrr25 co-purifies with Mpc54, Mpc70, and Spo74 even before they are recruited to the SPBs. Co-immunoprecipitation of Hrr25 and MP proteins. Immunoblot analysis of whole cell extract and anti-Ha immunoprecipitates from (A) *HRR25 MPC54-GFP* (Z6344) and *HRR25-ha3 MPC54-GFP* (Z28477) strains, (B) *HRR25 MPC70-GFP* (Z28238) and *HRR25-ha3 MPC70-GFP* (Z28239) strains, and (C) *HRR25 SPO74-GFP* (Z30966) and *HRR25-ha3 SPO74-GFP* (Z30967) strains.

sporulation starts at meiosis II, we asked whether cells lacking Hrr25 activity in meiosis II fail to sporulate as a consequence of the lack of nuclear division. To test whether restoring nuclear division in *hrr25-as* cells can trigger MP

assembly, we depleted Sgo1 in *CDC20-mAR HRR25* and *CDC20-mAR hrr25-as* cells and followed meiosis by live-cell imaging. Cells depleted of Sgo1 cannot retain centromeric Rec8 during meiosis I, resulting in random chromosome segregation in meiosis II (Katis et al., 2004a; Marston et al., 2004). In both strains, Cnm67 was tagged with RFP to follow SPBs separation and re-duplication as markers of progression through meiosis. Moreover, cells expressed a bacterial tetracyclin repressor (Tet-R)-RFP fusion protein, which localizes diffusely in the nucleus and it serves as a marker for nuclear division **(Figure 12)**. In *CDC20-mAR* P_{CLB2}*-SGO1 HRR25* cells, Mpc70-GFP appeared in metaphase II and it co-localized with the four Cnm67-RFP signals corresponding to the four SPBs characteristic of meiosis II. In *CDC20-mAR* P_{CLB2}*-SGO1 hrr25-as* cells, inhibition of Hrr25 blocked the recruitment of Mpc70-GFP to the SPBs, although nuclear division occurred in this strain due to the depletion of Sgo1. We concluded that Hrr25 affects MP formation directly rather than through its role in promoting cohesin cleavage and, thus, nuclear division.

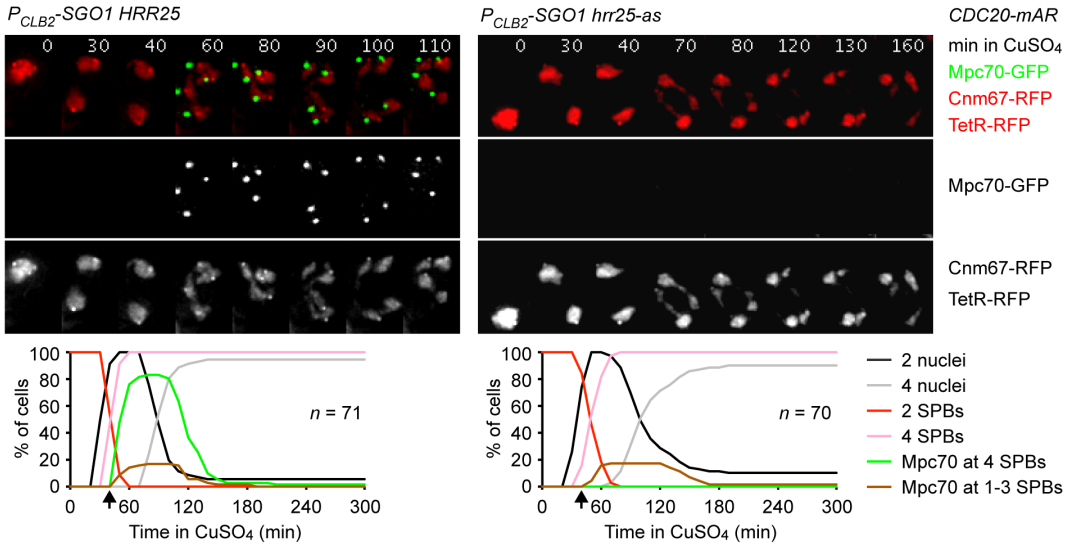


Figure 12. The inactivation of Hrr25 kinase activity at anaphase I in *CDC20-mAR* cells lacking Sgo1 blocks the recruitment of Mpc70-GFP to the SPBs although meiosis II division is restored. Live cell imaging of *CDC20-mAR* P_{CLB2}*-SGO1 HRR25* (Z28237) and *CDC20-mAR* P_{CLB2}*-SGO1 hrr25-as* (Z27474) strains undergoing meiosis. Cells expressing Cnm67-RFP, TetR-RFP, and Mpc70-GFP were arrested in metaphase I by Cdc20 depletion, imaging was started at 7.5 hours in SPM, and cells were released into anaphase by addition of $CuSO_4$ (10 μM) and treated with 1NM-PP1 (5 μM) 40 minutes later. Representative time-lapse series are shown. Below images: graphs displaying percentage of cells with 2 or 4 nuclei, 2 or 4 SPBs and with Mpc70-GFP dots.

2.6 Ama1 is not required either for the recruitment of the MP proteins to the SPBs or for their degradation

The exit from meiosis II is characterized by the destruction of M-phase promoting kinases, such as Ckd1/Clbs and Cdc5. Hrr25 was shown to play an important role in the exit from meiosis II, being required for the APC/C-Cdc20 mediated degradation of Clb1 and for the accumulation of Ama1, which leads, in turn, to the degradation of Ndt80 and Cdc5 (Arguello-Miranda et al., 2017). Ama1 is required for sporulation, more precisely for prospore membrane closure (Diamond et al., 2009). Furthermore, cell lacking Hrr25 activity did not degrade MP proteins, and we speculated that APC/C-Ama1 might be required for their degradation. Therefore we induced *AMA1* and *ama1Δ* cells expressing either Mpc54-GFP, or Mpc70-GFP, or Spo74-GFP, or Spc72, and Cnm67-RFP to enter meiosis. We collected samples every two hours for indirect immunofluorescence staining of α-tubulin and DAPI, and for immunoblot detection of whole cell extracts **(Figure 13)**. As we showed previously, Mpc70 and Spo74 are degraded in wild-type cells at 10 hours after induction of meiosis, which corresponds to the end of divisions. Notably, in *ama1Δ* cells, Mpc70 and Spo74 are also degraded with the same kinetics. Moreover, we observed that Cnm67 accumulates to high levels in *ama1Δ* cells. Next, we filmed MP proteins in the same strains **(Figure 14)**. In control cells, Mpc54, Mpc70, and Spo74 are recruited at the SPBs and removed from the SPBs before cells started to form spores. In *ama1Δ* cells, Mpc70 and Spo74 are also removed with a similar kinetic as the control strains. Mpc54 by contrast, displayed only a partial and slower removal from the SPBs in *ama1Δ* cells, compared to the control strain. We concluded that Ama1 is not required for assembly of the MP, but it seems to function in regulating Cnm67 levels at late time points. Cnm67 is located on the outer plaque of the SPBs, and it represents the interface between the SPB core and the MP, thus we speculated that Cnm67 degradation could be important for MP disassembly. Therefore, Hrr25 mediates MP disassembly in two ways: (i) directly promoting the degradation of MP proteins, and (ii) indirectly, through the activation of Ama1 toward the degradation of Cnm67.

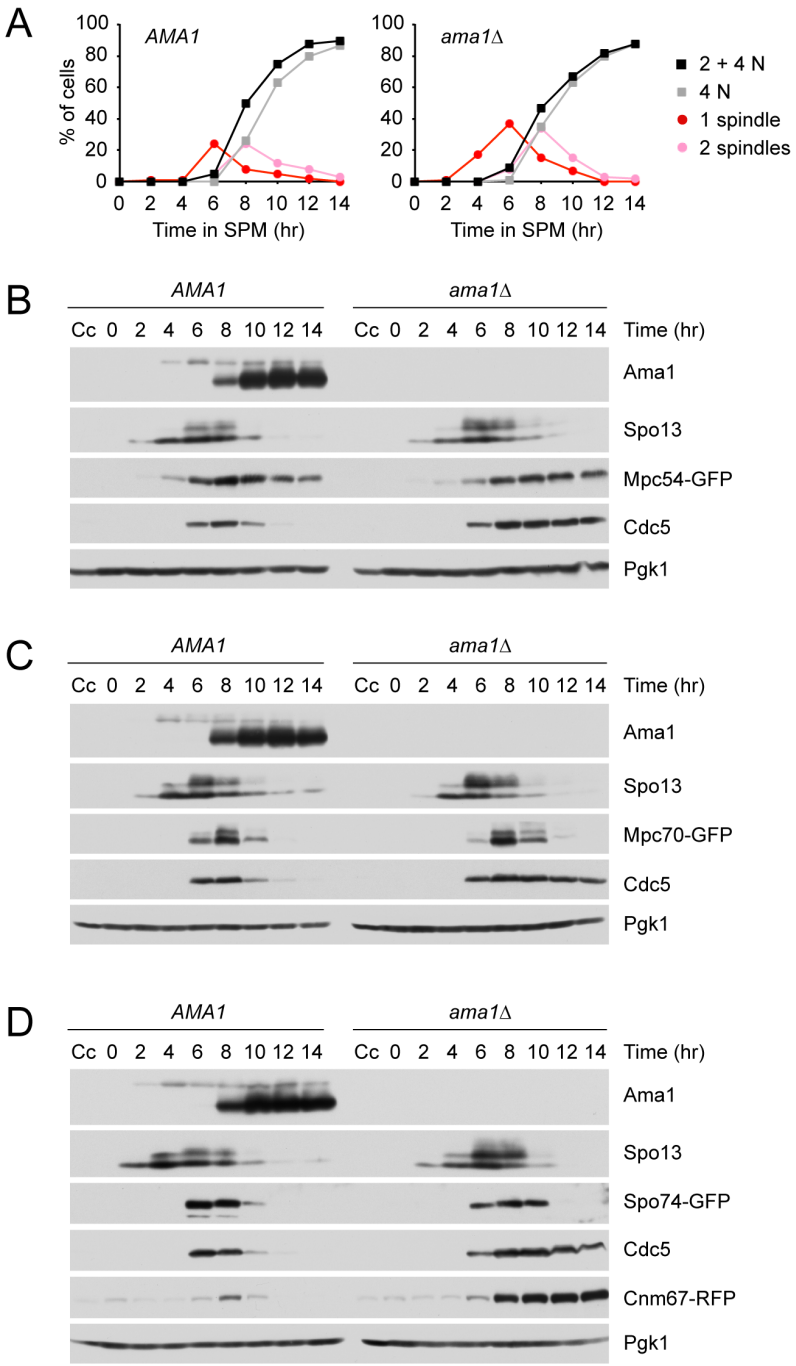


Figure 13. Expression and degradation of Mpc54, Mpc70, and Spo74 occurs normally in *ama1Δ* cells. Meiotic time course of *AMA1* and *ama1Δ* strains containing Mpc54-GFP, Mpc70-GFP, or Spo74-GFP and Cnm67-RFP. (A) Immunofluorescence detection of meiotic spindles (α-tubulin), and nuclei (DAPI) in *AMA1* (Z22000) and *ama1Δ* (Z22001) cells expressing Mpc54-GFP. (B) Immunoblot detection of protein levels. (C) Immunoblot detection of protein levels in *AMA1* (Z21998) and *ama1Δ* (Z21999) cells expressing Mpc70-GFP, and (D) Immunoblot detection of protein levels in *AMA1* (Z29575) and *ama1Δ* (Z29576) cells expressing Spo74-GFP.

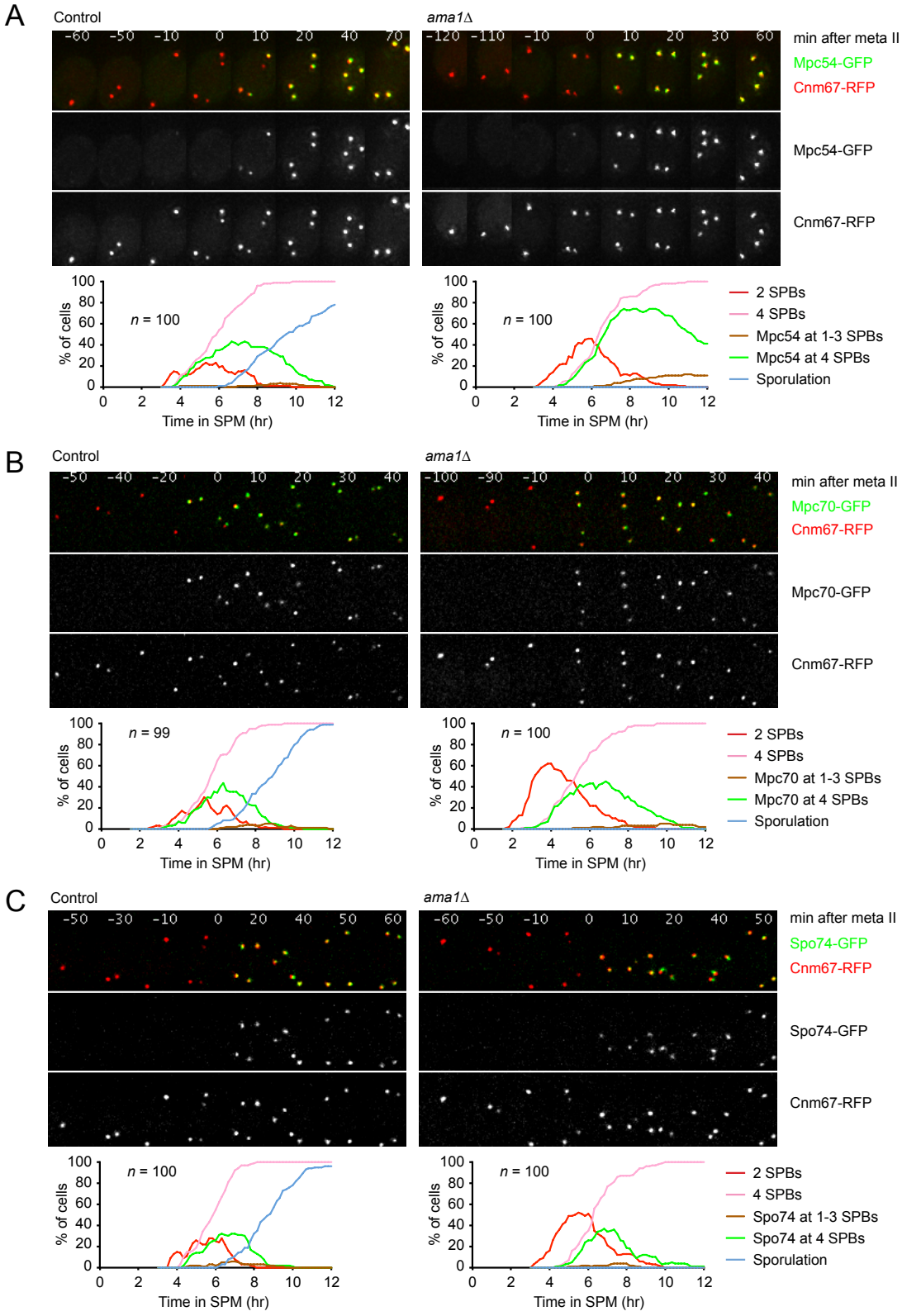


Figure 14. Ama1 is not required for the recruitment of MP proteins from the SPBs, but it has a role in their removal. Live cell imaging of *AMA1* and *ama1Δ* strains undergoing meiosis. (A) *AMA1* (Z22000) and *ama1Δ* (Z22001) strains expressing Cnm67-RFP and Mpc54-GFP were filmed every 10 minutes for 12 hours. Top panel: representative time-laps images of the cells. Bottom: percentage of cells with 2 or 4 Cnm67-RFP signals and with Mpc54-GFP at the SPBs.

(B) *AMA1* (Z21998) and *ama1Δ* (Z21999) expressing Cnm67-RFP and Mpc70-GFP. (C) *AMA1* (Z29575) and *ama1Δ* (Z29576) expressing Cnm67-RFP and Spo74-GFP.

2.7 The meiotic plaque is not required for the exit from meiosis II

The SPBs are not only a structural component of the mitotic and meiotic spindle apparatus, but they also associate with various cell cycle regulators, including Cdc5 in mitosis and meiosis (Crasta et al., 2008; Shirk et al., 2011), Cdk1 (Maekawa et al., 2003), and several components of the mitotic exit network (MEN) (Bardin and Amon, 2001). This prompted us to ask whether the assembly of the meiotic plaque could represent a signal to initiate the exit from meiosis II. To test this hypothesis, we deleted two of the three MP proteins, namely Mpc54 and Mpc70, and asked whether these cells perform a timely exit from meiosis II **(Figure 15)**. We induced *MPC54 MPC70* and *mpc54Δ mpc70Δ* strains, both expressing Pds1-myc18, to synchronously enter meiosis, and we collected protein extracts every two hours **(Figure 15B)**. Fixed cells were analyzed after staining of spindles, Pds1-myc, and DNA, in order to assess progression through meiosis **(Figure 15A)**. Control cells accumulate Pds1 during prophase, corresponding to t=4 hours in SPM, and they degraded it between t=6 and t=8 hours in SPM, corresponding to the first meiotic division. Rec8 behaves similarly to Pds1, being cleaved upon Pds1 destruction by the APC/C-Cdc20. During meiosis II, Ama1 levels rapidly increase and, consequently, its substrates Ndt80, Cdc5, Clb1 are degraded. *mpc54Δ mpc70Δ* cells progressed normally through meiotic divisions, and they also disassembled meiosis II spindles. Furthermore, the accumulation of Ama1 and the subsequent destruction of its substrates occurred normally in these cells. Thus, we concluded that while MP assembly is necessary for sporulation, it does not represent an essential process for the exit from meiosis II.

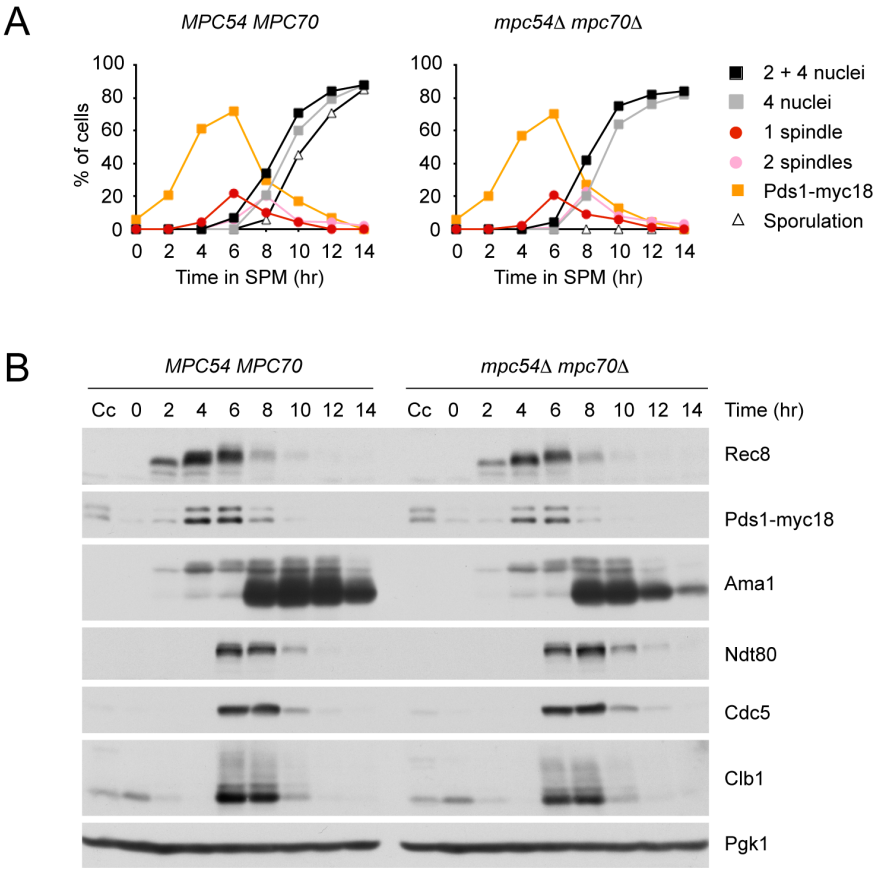


Figure 15. Cells lacking MP proteins perform a timely exit from meiosis II. Meiotic time course of *MPC54 MPC70* (Z2828) and *mpc54Δ mpc70Δ* (Z24653) cells espressing Pds1-myc18. Samples for immunoblot detection of proteins in whole cell extracts and for indirect immunofluorescence staining of spindles (α-tubulin), Pds1-myc18, and nuclei in fixed cells were taken every two hours. (A) The percentage of cells with 1 or 2 meiotic spindles, and 2 or 4 nuclei is shown at each time point. (B) Immunoblot detection of proteins.

2.8 The removal of Spc72 from the SPBs is blocked in cells lacking APC/C activity, due to the stabilization of Clb1 and Spo13

Although Hrr25 is essential for MP formation, it is difficult to understand how it regulates this process, since it is present throughout meiosis, and regulators of its kinase activity have not been described so far. Therefore, we asked whether other activities might regulate MP assembly. First, we tested the roles of the periodic activators of meiotic divisions, namely the APC/C and Cdk1. Because prior to MP assembly, Spc72 is removed from the SPBs, and this does not require Hrr25, we asked whether it requires APC/C-Cdc20 activity. We induced control and P_{SCC1}-*CDC20* cells expressing Spc72-GFP and Cnm67-RFP to enter meiosis and followed meiotic progression by live-cell imaging **(Figure 16)**. Control cells removed Spc72 from the SPBs in anaphase I, whereas P_{SCC1}-*CDC20* cells did not progress into anaphase I. As a result, P_{SCC1}-*CDC20* cells

maintained Spc72 at the SPBs for a long time. We concluded that APC/C activity somehow promotes the removal of Spc72. We speculated that APC/C was unlikely to remove Spc72 directly, since Spc72 occupies the cytoplasmic face of the SPBs, while it is assumed that APC/C-mediated ubiquitination occurs within the nucleus (Shirayama et al., 1998; Zachariae et al., 1996). Therefore, we tested whether APC/C promotes Spc72 removal by destroying one of its canonical substrate, such as cyclins.

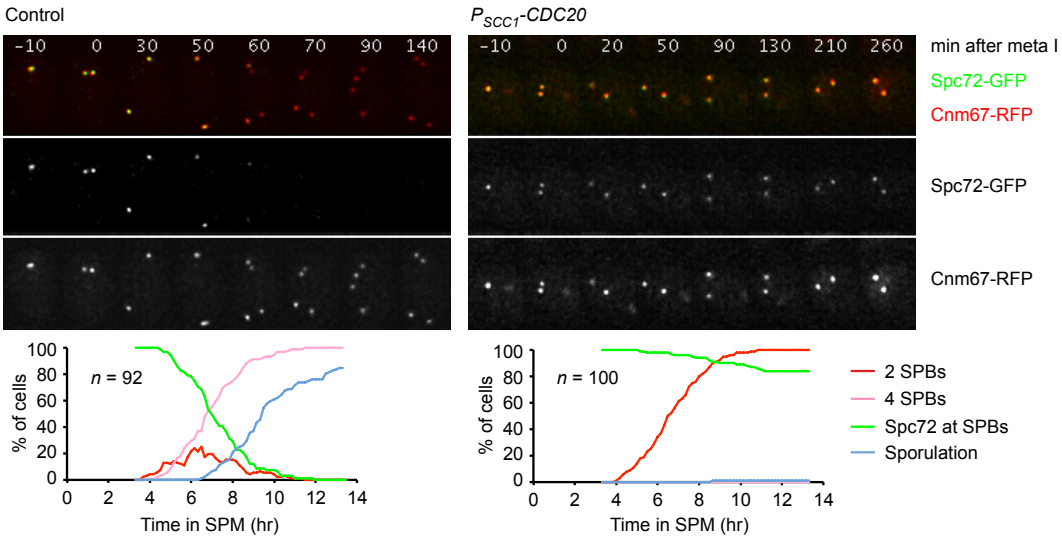


Figure 16. Depletion of Cdc20 blocks the removal of Spc72 from the SPBs. Live cell imaging of *CDC20* and P_{SCC1}-*CDC20* strains undergoing meiosis. *CDC20* (Z31702) and P_{SCC1}-*CDC20* (Z31704) strains expressing Cnm67-RFP and Spc72-GFP were filmed every 10 minutes for 14 hours. Top panel: representative time-laps images of the cells are shown. Bottom: quantification in percentage of cells with 1 or 2 Cnm67-RFP signals and with Spc72-GFP at the SPBs.

A prediction based on this hypothesis is that P_{HSL1}-*CDC20* cells, if deprived of such an APC/C substrate, should make dyad spores. We were aware of an APC/C-Cdc20 substrates that when deleted bypasses the need of Cdc20 to progress into anaphase I, and this was the meiosis-I specific protein Spo13 (Katis et al., 2004b). Next, we asked whether the deletion of any cyclin would have a similar effect. M-phase Cdk is activated by various cyclins in meiosis, namely Clb1, Clb3 and Clb4 (Dahmann and Futcher, 1995). Among these, Cdk1-Clb3 is thought to be active only in meiosis II, while Cdk1-Clb1 and Cdk1-Clb4 are active during both divisions (Carlile and Amon, 2008; Jonak et al., 2017). We induced P_{SCC1}-*CDC20*, P_{SCC1}-*CDC20 spo13Δ*, P_{HSL1}-*CDC20 clb1Δ*, and P_{HSL1}-*CDC20 clb4Δ* strains to enter meiosis, and we scored dyads spores after 24

hours in SPM **(Figure 17)**. As expected, cells depleted of Cdc20 produced very few dyads, whereas P_{SCC1}-*CDC20 spo13Δ* cells produced 77% of dyads. Strikingly, P_{HSL1}-*CDC20 clb1Δ* cells produced dyads up to 37%. By contrast, P_{SCC1}-*CDC20 clb4Δ* cells did not produce more dyad spores than the depletion of Cdc20. These data suggest that while in the absence of Cdc20 sporulation is greatly impaired, the additional deletion of Clb1 or Spo13 releases this block. Thus, we hypothesize that the recruitment of the MP proteins at the SPBs cannot occur in meiosis I, possibly because Clb1-Cdk1 and Spo13 exert an inhibitory function on the removal of Spc72. This would explain why we never observed Spc72 and Mpc70 occupying the SPBs at the same time, and would also confine the formation of the MP to meiosis II, a stage that is subsequent to the degradation of Clb1 and Spo13 by the APC/C-Cdc20.

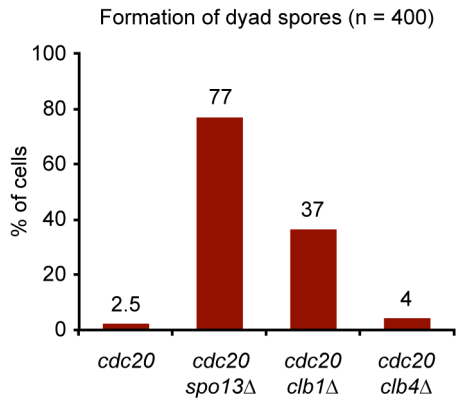


Figure 17. The deletion of Spo13 and of Clb1 allow cells to produce dyad spores in the absence of APC/C-Cdc20. (A) Quantification in percentage of two-spored asci in P_{HSL1}-*CDC20* (Z32767), P_{HSL1}-*CDC20 spo13Δ* (Z32771), P_{HSL1}-*CDC20 clb1Δ* (Z32768), *and* P_{HSL1}-*CDC20 clb4Δ* (Z32769) strains undergoing meiosis. Cells were collected after 24 hours in SPM and dyads were counted in 400 cells per strain.

We have postulated that Spc72 has to be removed from SPBs for the recruitment of the MP proteins to occur. If correct, the deletion of *SPO13* or *CLB1* should restore the removal of Spc72 from the SPBs in cells lacking APC/C activity. We tested this hypothesis by looking at Spc72-GFP on live-cell imaging **(Figure 18)**. In P_{SCC1}-*CDC20 ama1Δ spo13Δ* and P_{HSL1}-*CDC20 ama1Δ clb1Δ* cells, Spc72 is removed from the SPBs shortly after metaphase I **(Figure 18A-B)**. Consistently with the absence of dyads spores, P_{HSL1}-*CDC20 ama1Δ clb4Δ* cells fail to remove Spc72 from the SPBs, indicating that the degradation of Clb4 is not required for the removal of Spc72 from the SPBs **(Figure 18C)**.

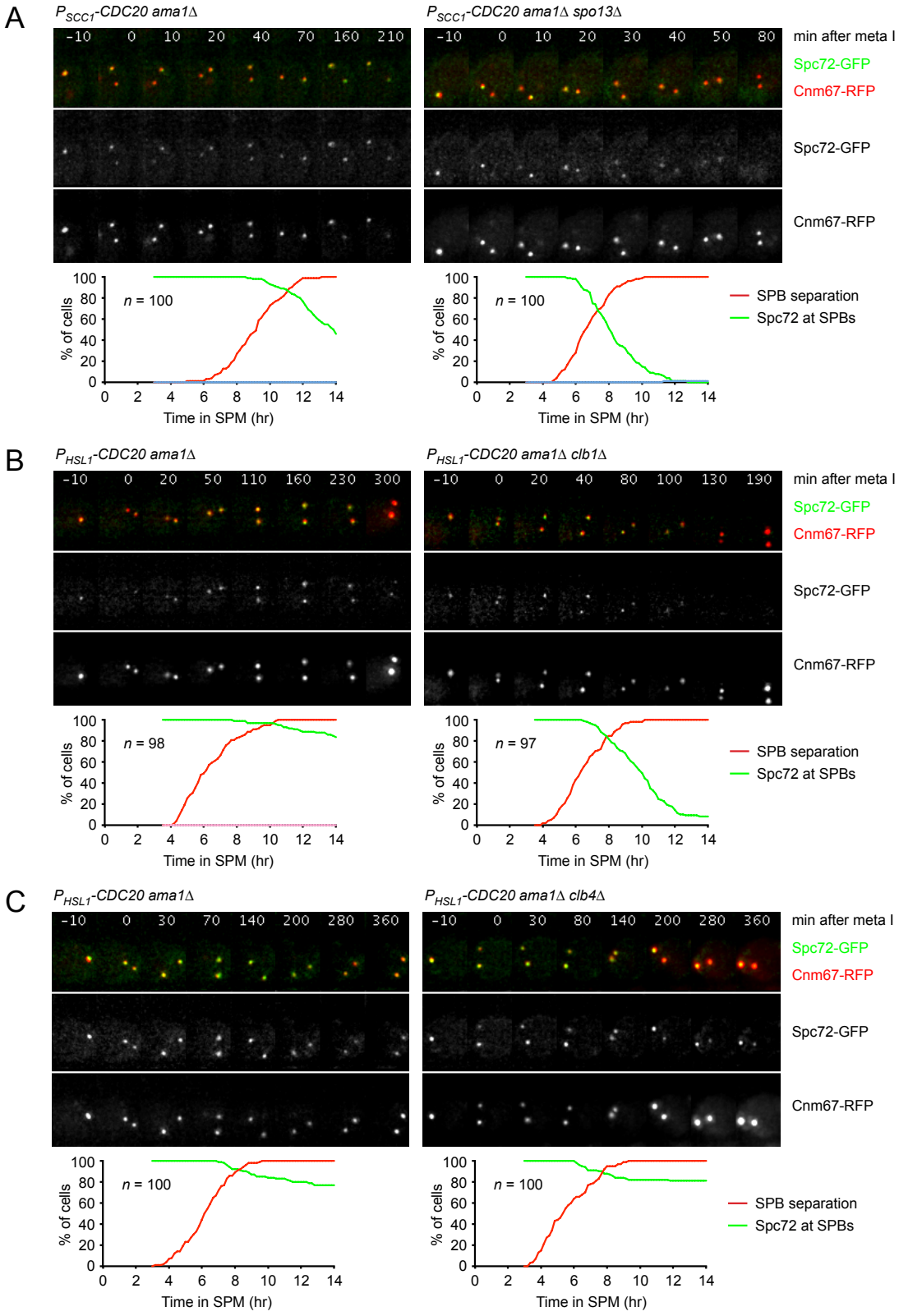


Figure 18. The deletion of Spo13 or Clb1, but not Clb4 causes Spc72 removal from the SPBs in the absence of Cdc20 and Ama1. Live cell imaging of P_{SCC1}-*CDC20 ama1Δ* strains lacking either Spo13, or Clb1, or Clb4 undergoing meiosis. (Legend on the next page)

Since cyclins are the activators of Cdk1, we tested whether inhibiting Cdk1 activity in cells lacking APC/C activity would have a similar affect on Spc72 removal, as the deletion of the *CLB1* cyclin. In other words, we hypothesized that inhibiting Cdk1 activity in metaphase-I arrested cells would be sufficient to elicit Spc72 removal. As above, we induced meiosis in cells lacking two APC/C activators, Cdc20 and Ama1, and carrying an allele of Cdk1 sensitive to kinase inhibition (*cdc28-as1*) (Bishop et al., 2000). We induced P_{HSL1}-*CDC20 ama1Δ CDC28* and P_{HSL1}-*CDC20 ama1Δ cdc28-as1* strains to enter meiosis, and treated cells with 1NM-PP1 (5 μM) in metaphase I (t=8 hours in SPM) **(Figure 19)**. In P_{HSL1}-*CDC20 ama1Δ CDC28* cells, Spc72-GFP persists for long periods of time at SPBs. In the P_{HSL1}-*CDC20 ama1Δ cdc28-as* strain, instead, the inhibition of Cdc28 activity in metaphase I arrested cells, led to the removal of Spc72-GFP from the SPBs, suggesting that the role of APC/C in anaphase I is to lower Cdk1 activity by destroying Clb1, and to degrade Spo13. Since the absence of Spo13 led to the best Spc72 removal in cells lacking APC/C activity, we speculated that loss of Spo13 might also contribute to lower Cdk1 activity. On the other hand, we cannot rule out that the inhibition of Cdk1 activity has an effect on Spo13 regulation.

Figure 18. The deletion of Spo13 or Clb1, but not Clb4 causes Spc72 removal from the SPBs in the absence of Cdc20 and Ama1. Cells were filmed every 10 minutes for 14 hours. All strains contain Cnm67-RFP and Spc72-GFP. (A) P_{SCC1}-*CDC20 ama1Δ* (Z31667), and P_{SCC1}-*CDC20 ama1Δ spo13Δ* (Z31665) cells. (B) P_{HSL1}-*CDC20 ama1Δ* (Z32514) and P_{HSL1}-*CDC20 ama1Δ clb1Δ* (Z32513) cells. (C) P_{HSL1}-*CDC20 ama1Δ* (Z32514) and P_{HSL1}-*CDC20 ama1Δ clb4Δ* (Z33291) cells. Top panels display representative time-lapse series. Below images: quantification in percentage of cells with 1 or 2 Cnm67-RFP signals and with Spc72-GFP at the SPBs.

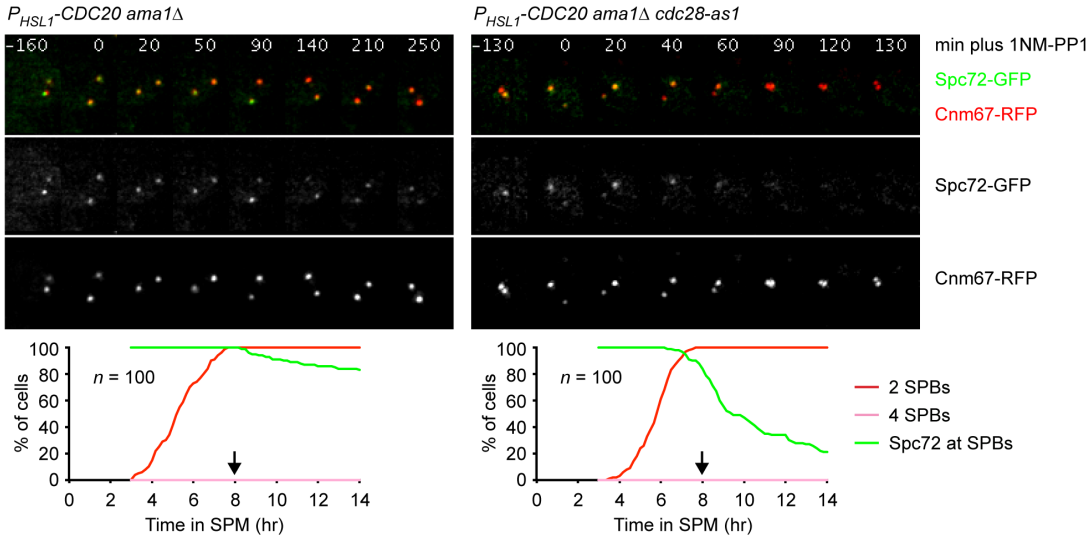


Figure 19. The inhibition of Cdk1 in metaphase I-arrested cells promotes the removal of Spc72 from the SPBs. Live cell imaging of meiosis in P_{HSL1}-*CDC20 ama1Δ* (Z32514) and P_{HSL1}-*CDC20 ama1Δ cdc28-as1* (Z32388) strains expressing Cnm67-RFP and Spc72-GFP. Cells were filmed every 10 minutes for 14 hours. 1NM-PP1 was added at 8 hours in SPM. Top panels display representative time-lapse series. Below images: quantification of cells with 1 or 2 Cnm67-RFP signals and with Spc72-GFP at the SPBs. The arrow indicates the time of addition of 1NM-PP1.

2.9 The removal of Spc72 from the SPBs in the absence of APC/C and Cdk1 activity is not sufficient to enable the recruitment of Mpc70

The finding that cells lacking Cdc20 are able to sporulate when Spo13 or Clb1 are deleted predicts that MP assembly occurs in these cells in meiosis I. To test this, we induced P_{HSL1}-*CDC20 ama1Δ*, P_{HSL1}-*CDC20 ama1Δ clb1Δ*, P_{SCC1}-*CDC20 ama1Δ*, and P_{SCC1}-*CDC20 ama1Δ spo13Δ* cells to enter meiosis, and filmed Mpc70-GFP together with Cnm67-RFP **(Figure 20)**. Consistent with dyad counting, P_{HSL1}-*CDC20 ama1Δ clb1Δ* cells recruited Mpc70 at the SPBs in 40% of cells, whereas P_{SCC1}-*CDC20 ama1Δ spo13Δ* cells recruited Mpc70 at the SPBs in more than 80% of the cells **(Figure 20A-B)**. We sought to test in a similar experiment whether inactivating Cdk1, beyond eliciting Spc72, can lead to the recruitment of Mpc70 at the SPBs. Thus, we induced P_{HSL1}-*CDC20 ama1Δ*, and P_{HSL1}-*CDC20 ama1Δ cdc28-as1* to enter meiosis, and inhibited Cdk1 when cells reached metaphase I (t=8 hours in SPM) **(Figure 20C)**. Notably, inhibition of the kinase did not lead to Mpc70 recruitment, suggesting that although Spc72 has been removed from the SPBs in these cells, the loading of Mpc70 requires, either directly or indirectly, Cdk1 activity.

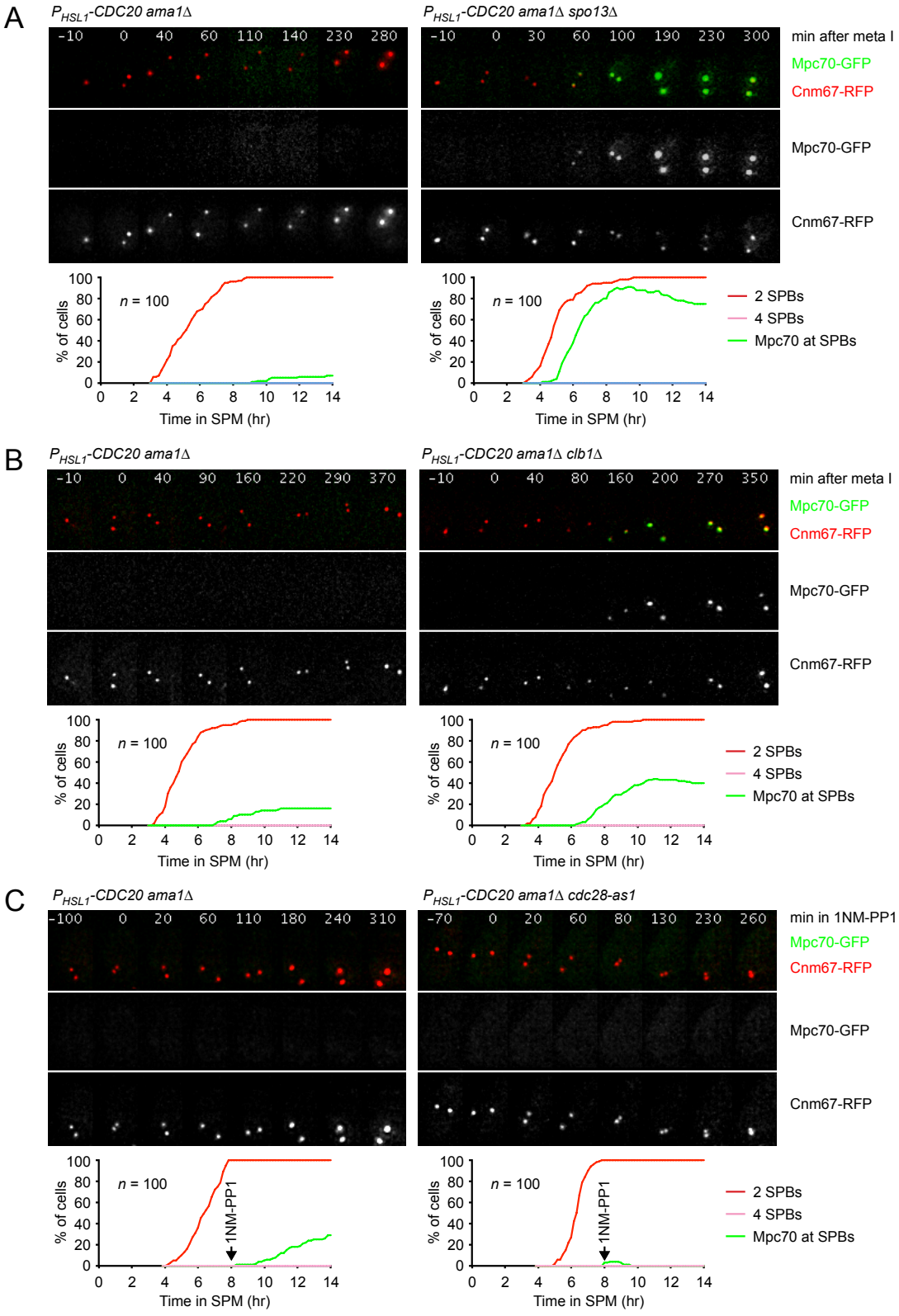


Figure 20. The deletion of Spo13 or Clb1, but not the inhibition of Cdk1, leads to the recruitment of Mpc70 to the SPBs in the absence of Cdc20 and Ama1. Live cell imaging of meiosis. (Legend on the next page)

2.10 The recruitment of Mpc70 at the SPBs requires Hrr25 and Cdk1 activity, while Cdc5 and Ime2 are dispensable.

Once we collected evidences that Spc72 removal requires Cdk1 activity to be low, but that Mpc70 was not loaded onto the SPBs in these conditions, we sought to inactivate the main meiotic kinase activities in meiosis II to identify those required for Mpc70 recruitment at the SPBs. We took advantage of the *CDC20-mAR* system to analyze the consequences of the inhibition of Cdc5, Ime2, and Cdc28 kinases in meiosis II, without perturbing meiosis I **(Figure 21)**. We induced *CDC20-mAR*, and *CDC20-mAR cdc5-as* cells expressing Mpc70-GFP, Cnm67-RFP, and TetR-RFP to enter meiosis, released the cells from metaphase I arrest by adding $CuSO_4$, and added CMK (10 μM) 35 min later. Cells lacking Cdc5 kinase activity in meiosis II duplicate SPBs in meiosis II as wild-type cells, but meiosis II spindles collapse as they start to elongate, so that nuclei cannot divide despite centromeric Rec8 being cleaved in a timely manner (Arguello-Miranda et al., 2017). Consistent with this notion, in our experiment *cdc5-as cells* do not divide nuclei in meiosis II, but neither the loading or the removal of Mpc70-GFP onto the four available SPBs were affected **(Figure 21A)**. We performed a similar experiment with *CDC20-mAR IME2* and *CDC20-mAR ime2-as1* cells, treating cells with 1NA-PP1 (20 μM) 40 min after release. Cells in which Ime2 kinase activity was inhibited in anaphase I, did not divide in meiosis II and fail to sporulate. Nevertheless, they reduplicated SPBs and also accumulate Mpc70 on all four SPBs **(Figure 21B)**. Finally, we analyzed *CDC20-mAR* and *CDC20-mAR cdc28-as1* cells, in this case treating cells with 1NM-PP1 (5 μM) 40 min after metaphase I-release. Cells in which Cdc28 kinase activity was inhibited in anaphase I failed to duplicate the SPBs in meiosis II, thus, a bipolar meiosis II spindle could not form, thereby preventing the second meiotic nuclear division. Importantly, Mpc70-GFP was not recruited to the SPBs, and this defect represented the primary reason why cells did not form spores **(Figure 21C)**.

Figure 20. The deletion of Spo13 or Clb1, but not the inhibition of Cdk1, leads to the recruitment of Mpc70 to the SPBs in the absence of Cdc20 and Ama1. (A) P_{HSL1}-*CDC20 ama1Δ* (Z31778) and P_{HSL1}-*CDC20 ama1Δ spo13Δ* (Z31779), (B) P_{HSL1}-*CDC20 ama1Δ* (Z31778) and P_{HSL1}-*CDC20 ama1Δ clb1Δ* (Z32907), and (C) P_{HSL1}-*CDC20 ama1Δ* (Z31778) and P_{HSL1}-*CDC20 ama1Δ cdc28-as1* (Z32546) strains expressing Cnm67-RFP and Mpc70-GFP were filmed every 10 minutes for 14 hours. Top panels display representative time-lapse images of the cells. Bottom:

quantification of cells with 1 or 2 Cnm67-RFP signals and with Mpc70-GFP at the SPBs. In (C) 1NM-PP1 was added at 8 hours in SPM, as indicated by the arrow.

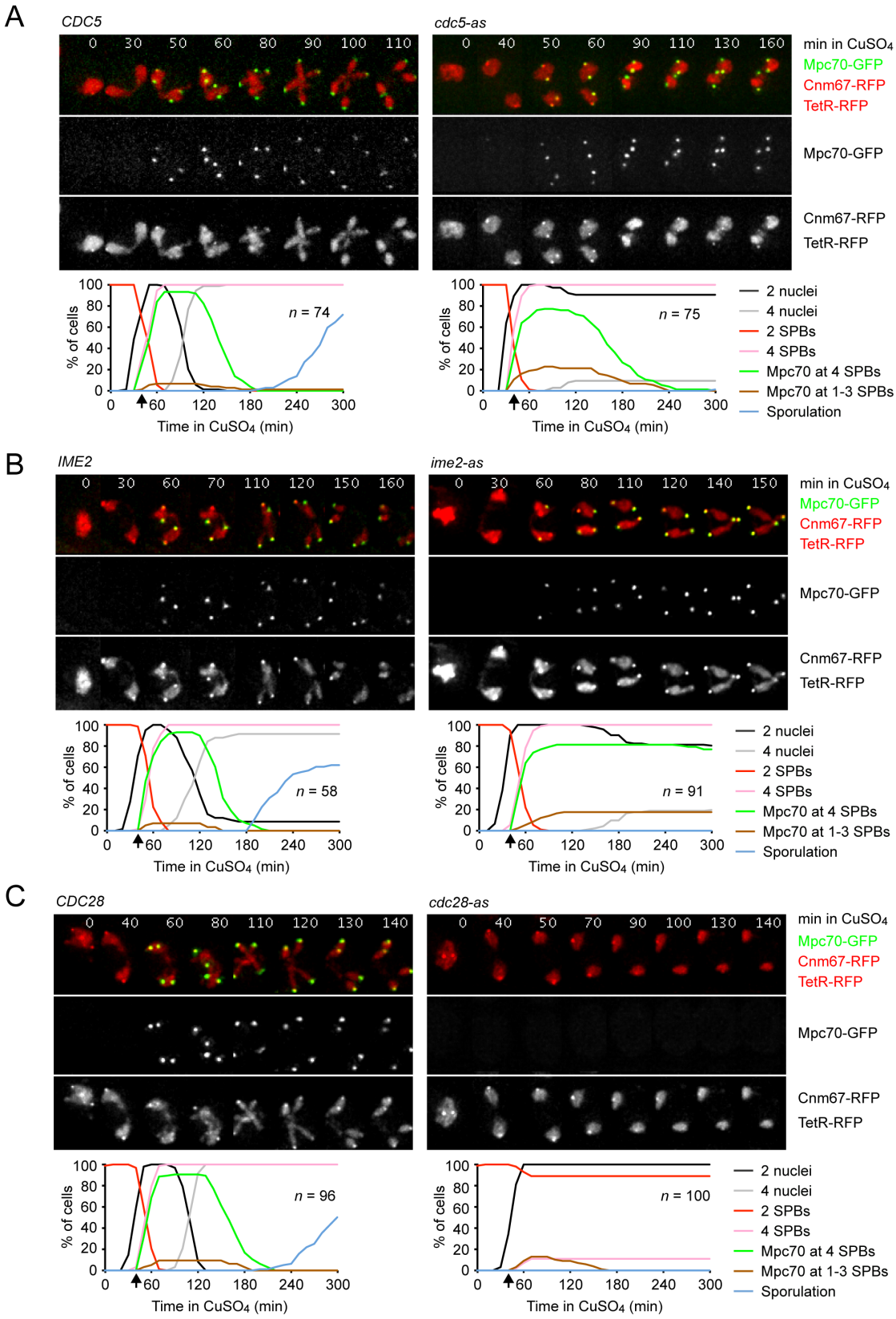


Figure 21. The inactivation of *cdc5-as* or *ime2-as1* at anaphase I in *CDC20-mAR* cells does not affect the recruitment of Mpc70-GFP at the SPBs, while Cdc28 activity is required for Mpc70-GFP localization at the SPBs. (Legend on the next page)

On the basis of the observations presented so far, we postulated that the assembly of the meiotic plaque requires four conditions to be satisfied: (1) the MP proteins have to be expressed, (2) APC/C must be activated to trigger the removal of Spc72 from the cytoplasmic face of the SPBs, through the degradation of Spo13 and Clb1, (3) Hrr25 has to be active, and (4) Cdk1/Clbs activity must be high. In wild-type cells, the requirements are only fulfilled at metaphase II.

2.12 Hrr25 is required for the recruitment of the Mpc70 at the SPBs even in *P_{SCC1}-CDC20 spo13Δ* cells

Do *P_{SCC1}-CDC20 spo13Δ* cells require Hrr25 activity to localize Mpc70 at the SPBs? We have previously shown that Hrr25 activity is required in wild-type cells for the formation of a functional meiotic plaque in meiosis II. Next, we showed that metaphase-arrested cells, lacking the APC/C activators Cdc20 and Ama1, fail to remove Spc72 from the SPBs and to load MP proteins on the cytoplasmic face of SPBs. However, both Spc72 removal and Mpc70 recruitment at the SPBs are restored in the absence of Spo13 or Clb1. To establish which position Hrr25 occupies in the hierarchy of events that lead to gamete differentiation, we tested whether *P_{SCC1}-CDC20 spo13Δ* cells require Hrr25 activity to localize Mpc70 at the SPBs **(Figure 22)**. We filmed *P_{SCC1}-CDC20 spo13Δ HRR25* and *P_{SCC1}-CDC20 spo13Δ hrr25-as1* strains expressing Cnm67-RFP and Mpc70-GFP, adding 1NM-PP1 (5 μM) three hours after induction of meiosis. As we expected, *P_{SCC1}-CDC20 spo13Δ HRR25* cells accumulated Mpc70 at both available SPBs. By contrast, cells lacking Hrr25 kinase activity fail to localize Mpc70 to the SPBs, suggesting that even if MP formation was induced in meiosis I, Hrr25 is still required for the formation of the MP.

Figure 21. The inactivation of *cdc5-as* or *ime2-as1* at anaphase I in *CDC20-mAR* cells do not affect the recruitment of Mpc70-GFP at the SPBs, while Cdc28 activity is required for Mpc70-GFP localization at the SPBs. (A) Live cell imaging of *CDC20-mAR* (Z24053) and *CDC20-mAR cdc5-as* (Z31053) strains undergoing meiosis. Cells expressing Cnm67-RFP, TetR-RFP, and Mpc70-GFP were arrested in metaphase I by Cdc20 depletion, imaging was started at 7.5 hours in SPM, and cells were released into anaphase by addition of $CuSO_4$, and treated with CMK (10 μM) 35 minutes later. (B) Live cell imaging of *CDC20-mAR* (Z24053) and *CDC20-mAR ime2-as* (Z31398). Cells were treated with 1NA-PP1 (20 μM) 40 minutes after release. (C) Live cell imaging of *CDC20-mAR* (Z24053) and *CDC20-mAR cdc28-as1* (Z31259) strains undergoing meiosis. Cells were treated with 1NM-PP1 (5 μM) 40 minutes after release. Upper panels show representative time-lapse series. Graphs display the percentage of cells with 2 or 4 nuclei, 2 or 4 SPBs and with Mpc70-GFP at the SPBs.

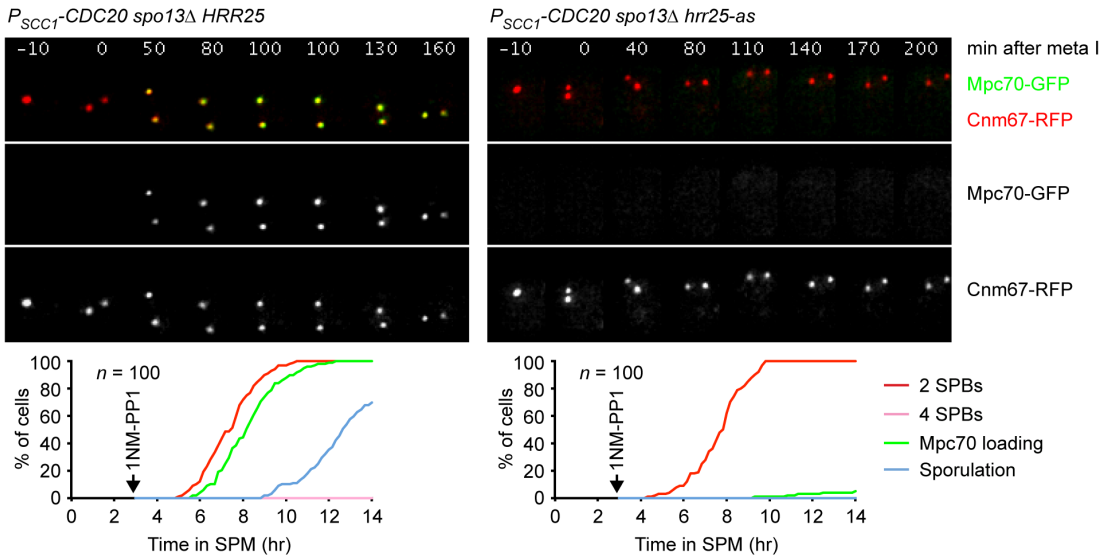


Figure 22. Hrr25 is required for Mpc70 recruitment at the SPBs even when it occurs at meiosis I. Live-cell imaging of P_{SCC1}-*CDC20 spo13Δ HRR25* and P_{SCC1}-*CDC20 spo13Δ hrr25-as1* strains undergoing meiosis. P_{SCC1}-*CDC20 spo13Δ HRR25* (Z32751) and P_{SCC1}-*CDC20 spo13Δ hrr25-as1* (Z32750) strains expressing Cnm67-RFP and Mpc70-GFP were filmed every 10 minutes for 14 hours. Top panels show representative time-lapse images of the cells. Below images: percentage of cells with 1 or 2 Cnm67-RFP signals and with Mpc70-GFP at the SPBs. The arrow indicates the time of addition of 1NM-PP1.

These data show that Hrr25 is constitutively required for MP assembly, either when it is confined to meiosis II by the antagonistic activities of APC/C and Cdk1, or when it is experimentally induced in meiosis I through the elimination of Spo13 or Clb1.

2.13 Activating Hrr25 only in meiosis II restores nuclear division, exit from meiosis, and MP assembly

The data collected so far led us to construct a model for the assembly of the meiotic plaque. First, it requires four conditions to be satisfied: (1) the MP proteins have to be expressed, (2) Cdk1/Clbs activity must be high, (3) APC/C must be activated to trigger the removal of Spc72 from the cytoplasmic face of the SPBs and (4) Hrr25 has to be active. These 4 conditions are all satisfied, in wild-type cells, only in meiosis II. If they are sufficient, we could induce meiosis in the absence of Hrr25 activity, reactivate the kinase only after meiosis II, and we would expect spore formation to be restored since: (1) MP proteins expression does not require Hrr25, (2) without Hrr25 activity Ama1 is not active enough to destroy Clb1, thereby Cdk1 activity stays high, (3) Spc72 removal does not depend on Hrr25. We induced *CDC20-mAR hrr25-as Pds1myc18* cells

to progress into and out of the metaphase I-arrest in the presence of 1NM-PP1 (2 μM), producing mononucleate cells with two spindles **(Figure 23)**. Entry into M-phase, Pds1 destruction, meiosis I spindle disassembly, and meiosis II spindle formation occurred normally in the absence of Hrr25 activity, although nuclear division did not take place. As a result, cells were mononucleate and had two meiosis II spindles. Next, we washed-away the inhibitor by filtration and resuspended the culture in fresh media, with or without inhibitor. As judged by immunofluorescence staining of tubulin and DNA, cells that were resuspended in the presence of 1NM-PP1 (2 μM) remained mostly mononucleate and retained meiosis II spindles until the end of the time course. By contrast, cells that were washed and resuspended in media without inhibitor made a tetrapolar division and disassembled spindles, suggesting that Hrr25 activity was restored and sufficient to promote nuclear division and exit from meiosis II. Furthermore, we used TEM to test whether MP formation is restored in these conditions, and observed that in more than half of the SPBs, a normal-looking meiotic plaque was present. We concluded that the meiosis II-specific processes that depend on Hrr25, do not require Hrr25 activity in meiosis I. Furthermore, we showed that once Clb1 and Spo13 have been destroyed, it is possible to delay MP formation by inhibiting Hrr25. MP formation is induced, however, when Hrr25 is reactivated.

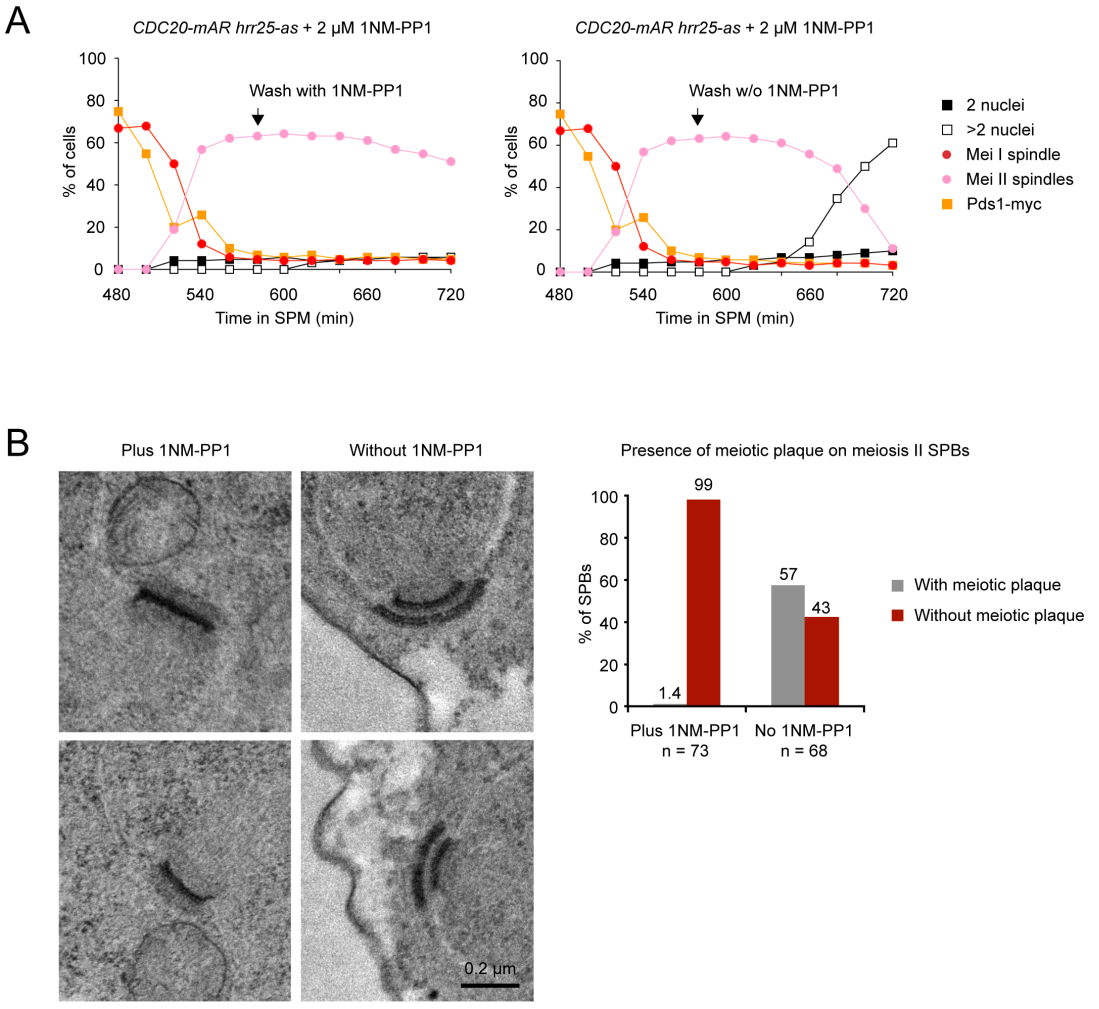


Figure 23. Wash-out of 1NM-PP1 from *hrr25-as* cells at anaphase II restores the formation of MP. P_{CLB2}-*CDC20* P_{CUP1}-*CDC20 hrr25-as1* cells containing *Pds1-myc18* (Z21852) were treated with 1NM-PP1 (2 µM) after three hours in SPM, arrested in metaphase I by Cdc20 depletion, released into anaphase by addition of $CuSO_4$ and washed from the inhibitor in anaphase II. Half of the culture was resuspended in SPM with inhibitor and the other half was resuspended in SPM without inhibitor. (A) Immunoflurescence samples were taken every 20 minutes, stained by indirect immunofluorescence to detect α-tubulin, Pds1-myc18, and DNA. (B) A sample at time point 660 minutes in SPM was processed for electron microscopy. Representative pictures of spindle pole bodies and histograms showing the percentage of spindle pole bodies with (grey) or without (red) a meiotic plaque.

3. Discussion

3.1 Meiosis and gamete differentiation are likely to be coordinated

More than 40 years ago, researchers observed that gamete differentiation in yeast starts precisely at metaphase II (Moens and Rapport, 1971). This observation was soon followed by the discovery of the structural components of a metaphase II-specific plaque on the cytoplasmic face of the SPBs (Bajgier et al., 2001; Knop and Strasser, 2000). Such plaque serves as a docking platform on top of which membrane precursors fuse together to form a novel membrane compartment that encapsulates each of the 4 haploid nuclei arising from meiotic divisions. Meiotic divisions and gamete differentiation are likely to be coupled, since starting sporulation at metaphase II ensures that nuclei are packaged only once genome haploidization has occurred. Gametogenesis is regulated in yeast and other eukaryotes by a transcriptional program that produces mRNAs encoding both cell cycle regulators, and differentiation factors, prior to the first meiotic division. How are the activities of cell cycle regulators and differentiation factors regulated so that differentiation starts only at metaphase II? We propose that there is a crosstalk between the cell cycle machinery and the differentiation program, and we provide an hypothetical mechanism for this crosstalk, that could be tested in other organisms. The most characterized system to address the regulation of gamete differentiation is *Drosophila* male spermatogenesis. In flies, 16 spermatogonia differentiate at the same time and they are in communication with somatic cells. In the spermatogonia, the switch from mitosis to meiosis is a critical stage, regulated by various signaling pathways and by different cell types. The two meiotic divisions are then followed by gamete differentiation. Even though differentiation is not dependent on the progression through the divisions, it has been proposed that meiotic divisions and gamete differentiation are somehow coordinated, due to the isolation of mutants that are arrested in meiosis and do not perform neither nuclear divisions, nor spermatid differentiation (Lin et al., 1996). Nevertheless, the control point at which the meiotic cell cycle and the spermatid differentiation program become independent was not found to date. We show here that in yeast, the fundamental cell cycle regulators that

orchestrate meiotic divisions, such as Cdk1, the APC/C, and Hrr25, also play a key role in gamete differentiation. Based on our results, we formulated the first hypothesis of how the antagonistic activities of APC/C and Cdk1 restrict the initiation of sporulation to meiosis II. Furthermore, we uncovered a role for Hrr25 in this process, although Hrr25 is required for sporulation at various stages, and not only at the onset.

3.2 Hrr25 coordinates various meiosis II events

Budding yeast Hrr25 is part of a large family of serine/threonine kinases, the so-called Casein Kinase 1 (CK1) family. Vertebrates possess various CK1 isoforms, and Hrr25 is related to vertebrate CK1δ. Hrr25 is the only soluble CK1 kinase in yeast, whereas the others (Yck1, Yck2 and Yck3) are localized at the plasma membrane (Wang et al., 1992; Wang et al., 1996). Hrr25 is involved in DNA repair (Hoekstra et al., 1991), ribosome biogenesis (Schafer et al., 2006), vesicle trafficking, autophagy (Pfaffenwimmer et al., 2014; Wang et al., 2015), and microtubule assembly (Peng et al., 2015). Hrr25 has a number of important functions in meiosis. First, it is a subunit of the monopolin complex (Petronczki et al., 2006). Second, it phosphorylates, together with Cdc7, the cohesin's kleisin subunit Rec8, which is required for cohesin cleavage (Katis et al., 2010). Recently, Arguello et al. have shown that Hrr25 is required also in meiosis II, to phosphorylate centromeric Rec8, to assemble the meiotic plaque, and, finally, to orchestrate the exit from meiosis II (Arguello-Miranda et al., 2017). Therefore, Hrr25 is the first common regulator of meiosis II events. To date, there have been no reports of Hrr25 activity being regulated in the classical sense through modification by enzymes or interaction with regulatory subunits. This raises the question of how are some functions of Hrr25 activated only in meiosis II, or suppressed in meiosis I, or both? Possibly, its relationship with the monopolin complex could serve as model for Hrr25 regulation. In meiosis I, Hrr25 binds to and phosphorylates Mam1, namely the monoplin subunit that retains the complex on kinetochores (Corbett and Harrison, 2012; Petronczki et al., 2006). Mam1 might work in recruiting Hrr25 to kinetochores to create a functional monopolin complex. Furthermore, in meiosis I, Hrr25 binds to Rec8, suggesting that either monopolin and Rec8 cooperate to retain Hrr25 on the chromosomes, or that different populations of Hrr25 molecules

exist in the cell. In meiosis II, we showed that Hrr25 not only phosphorylates Rec8, but also removes Sgo1-PP2A from centromeres. Moreover, it induces the destruction of M-phase kinases, and finally it mediates the recruitment of the MP proteins to the SPBs. We found that Hrr25 binds to the MP proteins before they get localized to the SPBs, and we speculated that Hrr25 might bind MP proteins in order to make them competent for recruitment to the SPBs. Another possibility is that Hrr25 is required for assembling the MP proteins into a soluble complex in the cytoplasm, and it is recruited at the SPBs *via* unknown mechanisms that do not depend on Hrr25. Hrr25 was shown to have other cytoplasmic substrates, such as a receptor protein for the cytosol-to-vacuole targeting pathway of autophagy, named Atg19 (Pfaffenwimmer et al., 2014). Therefore, we cannot exclude that MP formation is regulated by a cytoplasmic pool of Hrr25. Hrr25 being a kinase, we speculated that it phosphorylates the MP proteins, but we observed that Mpc70 and Spo74 appear to be post-translationally modified, possibly phosphorylated, in both the presence and the absence of Hrr25 activity, suggesting that MP proteins are substrate of multiple kinases (Knop and Strasser, 2000). In order to gain new insights into the importance of the interaction between Hrr25 and the MP proteins, we aim to disrupt this interaction.

3.3 Antagonistic activities of APC/C and Cdk1/Clbs synchronize metaphase II with gamete differentiation

How is MP assembly confined to metaphase II in a wild-type meiosis? While Hrr25 links meiotic nuclear divisions to gamete differentiation, it does not provide an explanation for how gamete differentiation starts only at metaphase II. We speculated that the periodic activators of meiotic divisions, namely the APC/C and Cdk1, might carry out this task. Based on our observations, we postulated that the MP is assembled only on SPBs that are not occupied by Spc72. First, we showed that there is a correlation between the removal of Spc72 and the subsequent assembly of the MP: in cells lacking APC/C activity, Spc72 is stably localized at the SPBs, and MP formation does not occur. Spc72 removal does not require APC/C activity per se, since when APC/C-Cdc20 substrates Clb1 or Spo13 are deleted, cells not only remove Spc72 from the SPBs, but also form dyads, suggesting that all MP proteins are properly assembled in the MP.

In cells that progress normally through meiosis, and undergo two rounds of SPBs duplication, the MP proteins first appear on the two newest SPBs, while the two oldest SPBs, where Spc72 is localized, are delayed for a few minutes in the recruitment of MP proteins. This indicates that Spc72 and MP proteins are mutually exclusive for the binding to the cytoplasmic face of the SPBs. It has been suggested that Spc72 occupies the same Nud1 binding sites as the MP proteins, thereby directing MP assembly preferentially to the newer SPBs (Gordon et al., 2006). Gordon et al. also presented arguments against this model. For instance, a strain devoid of Spc72 on the outer plaque does not produce spores. The elimination of Spc72 from the outer plaque was achieved, in their work, by fusing Spc72 to the half-bridge protein Kar1. Therefore, this strategy was based upon moving Spc72 from one binding site to another, while it might be better to completely remove Spc72 from the SPBs, in order to understand its contribution to MP assembly. According to our hypothesis, once cells have removed from the SPBs either Spc72, or another unknown activity that depends on Spc72, MP assembly is "licensed". What is needed to subsequently "fire" MP assembly is high Cdk1-Clbs activity. Indeed, in cells that lack APC/C activators, when we inhibit Cdk1 activity, Spc72 is removed from the SPBs, but Mpc70 fail to localize, due to the need of Cdk1 activity for the loading of MP proteins onto the SPBs. The temporal separation of Spc72 removal and MP assembly is reminiscent of DNA replication control, in which each replication round is divided in two non-overlapping phases. During mitotic G1 phase, low Cdk1 activity allows the loading of the replicative helicase Mcm2-7 onto origins of replication (known as "licensing" of the replication origins). Upon S-phase entry, Cdk1 bound to S-phase cyclins phosphorylates the proteins that promote helicase activation (known as "firing" of the origins) (Dahmann et al., 1995; Kelly and Brown, 2000). Furthermore, S-phase Cdk1/Clbs and M-phase Cdk1/Clbs inhibit helicase loading during S, G2, and M-phase (Blow and Dutta, 2005). Having two mutually exclusive states, replication control system ensures that chromosomal DNA is replicated exactly once per cell cycle. Remarkably, oscillations of Cdk1 activity also ensure that SPBs duplication occurs once per cell cycle (Jaspersen et al., 2004). In this context, Cdk1 is required to directly phosphorylate the SPB core subunit Spc42. This modification is important for Spc42 assembly into the

SPB, but is not required for SPBs duplication. Additionally, Cdk1 phosphorylates Mps1, which in turn phosphorylates Spc42 on other residues, thereby promoting Spc42 further assembly, and driving SPBs duplication (Castillo et al., 2002; Jaspersen et al., 2004). In a similar fashion, the periodic activation of Cdk1-Clbs and APC/C is fundamentally important to confine spore formation to the right time of meiosis. We showed support of this hypothesis by delaying MP assembly until after the degradation of Pds1 in meiosis II, by reactivating Hrr25 in cells lacking Hrr25 activity during the meiotic divisions. This led to the resumption of a tetrapolar nuclear division, of the formation of the MP, and finally to the formation of spores.

3.3 How does the spo13Δ mutant make 2 haploid spores instead of 4

In the absence of Spo13, diploid yeast cells undergo a single meiotic division, on a single spindle axis, during which sister chromatids often separate (Klapholz and Esposito, 1980). The *spo13Δ* phenotype is very complex, but can be summarized as follows: (a) cells undergo one division segregating either homologous chromosomes or sister chromatids, (b) Pds1 degradation occurs only once per meiosis, (c) the second reduplication of SPBs does not occur, thereby cells assemble only one spindle, and (d) the Spindle Assembly Checkpoint (SAC) delays anaphase I (Hugerat and Simchen, 1993; Katis et al., 2004; Shonn et al., 2002). Although the lack of Spo13 causes various problems in meiosis, Spo13 is a relatively small, meiosis-I specific, protein that lacks functional domains. Thus, it is likely that Spo13 interacts with other proteins, possibly acting as a regulatory subunit for them. For example, Spo13 was found to interact with Cdc5 to modify the monopolin subunit Lrs4 in metaphase I (Matos et al., 2008). It is remarkable that a null mutant of SPO13 starts to sporulate in a meiosis I-like state. Cells lacking Spo13 recruit Mpc70 at the SPBs with wild-type kinetics, and prior to APC/C activation, suggesting that the coordination between the periodic activation of APC/C and Cdk1/Clbs activities and spore differentiation is defective in these cells. Furthermore, we showed that cells arrested in metaphase-I due to the lack of APC/C activity do not sporulate, but deletion of either *SPO13* or *CLB1*, causes the loss of Spc72 and the formation of the MP. We speculated that Spo13, together with Clb1, constitutes the inhibitory activity that prevents Spc72 removal from the SPBs,

by a yet unknown mechanism, and the consequent MP formation in meiosis I. Should this be the case, stabilizing these two proteins would be sufficient to delay Spc72 removal and MP assembly in otherwise wild-type cells. We cannot exclude that there are other APC/C substrates that must be degraded in anaphase I to allow Spc72 removal and promote MP assembly, therefore we are implementing a strategy to (a) stabilize Spo13 and Clb1, and test whether it is sufficient to delay Spc72 removal, and the subsequent MP formation, and (b) enable cells to enter meiosis in the absence of APC/C activators and Spc72, and test whether MP assembly occurs as soon as Cdk1-Clbs activity raises in metaphase I.

3.4 On the removal of the MP

PSM growth was often taken as a paradigm for *de novo* membrane formation, and it was described extensively from a morphologic perspective (Heywood and Magee, 1976). Even before descriptions of the process based on mutant phenotypes became available, the SPBs were recognized as one of the essential structure for PSM formation (Davidow et al., 1980). In meiosis II, the SPBs make up a novel plaque composed of the meiosis-specific proteins Mpc54, Mpc70 and Spo74. In the absence of any of the three proteins, the membrane precursors of the PSM do not efficiently localize at the SPBs and, those that manage, do not initiate the formation of a continuous membrane (Knop and Strasser, 2000). There is another cellular machinery that is required for PSM formation, namely the leading edge complex (LEP). Only one of the three LEP components identified to date, Ssp1, was shown to be required for sporulation, although all three components co-localize on the edge of the growing PSM. Cells lacking Ssp1 form PSMs with a very narrow opening, which can engulf very little cytoplasm, but is nevertheless able to close. An interesting yet unclear aspect of PSM formation is its closure, which is obviously important for the survival of the gamete. As previously reported, we observed that MP proteins accumulate upon entry into meiosis, and then are degraded during sporulation. Furthermore, we reported that cells lacking Hrr25 activity accumulate Mpc70 and Spo74 to high levels, suggesting that the system that creates the MP also mediates its removal. Very little is known about the relevance of the degradation of the MP proteins, but we speculated that the

removal of the MP contributes to creating a continuous PSM, which in turn guarantees a closed spore wall. We hypothesized that Hrr25 activity is required for both the assembly and the removal of the MP. We showed that Hrr25 works in metaphase II to recruit the MP proteins to the SPBs, and we showed that cells lacking Hrr25 activity fail to activate Ama1 and this leads to the accumulation of Cnm67. Although Ama1 is not required for either the degradation of MP proteins or their removal from the SPBs, wild-type cells degrade Cnm67 upon exit from meiosis, while cells lacking Ama1 do not, suggesting that the degradation of Cnm67 is relevant for PSM closure, and that PSM closure indirectly requires Hrr25 activity. Working in two distinct ways, Hrr25 regulates PSM formation and closure, thereby keeping the two processes in register. Interestingly, we also showed that cells lacking Ime2 activity in meiosis II fail to remove Mpc70 from the SPBs and to sporulate, corroborating the idea that the removal of MP proteins from the SPBs is important for sporulation. Nevertheless, we cannot exclude that Ime2 has also other functions during sporulation: Berchowitz et al. showed that Ime2 controls the translation of various genes involved in spore differentiation, such as Spo20, Gip1 and Sps1 (Berchowitz et al., 2013). To better understand the relationship between PSM growth and MP removal, it would be therefore interesting to test whether cells lacking Ime2 activity in meiosis II can form PSMs around the nuclei although MP proteins are stabilized at the SPBs.

3.5 Is yeast sporulation a relevant model for gamete differentiation in eukaryotes?

Gametogenesis consists of the production of gametes from diploid progenitor cells, and it requires: (a) the expression of genes that drive the special form of cell division required for genome haploidization, and (b) a morphogenetic process that produces highly specialized cells. In yeast the possibility to enter gametogenesis is restricted to diploid *MATa/MATalpha* cells containing mitochondria, and it is triggered by nutrient limitation (van Werven and Amon, 2011). The master regulator of meiosis in *Saccharomyces cerevisiae* is *IME1*, it encodes a transcription factor that activates the transcription of early meiotic genes, including Ndt80, which is, in turn, required for the induction of genes regulating meiotic divisions and gamete differentiation (Mandel et al.,

1994; Pak and Segall, 2002; Smith et al., 1990). Fission yeast and budding yeast diverged hundreds of millions of years ago, but their entry into sporulation is similar. As budding yeast, also *Schizosaccharomyces pombe* has a transcription factor that induces the expression of genes important or meiosis and sporulation, named Ste11, whose expression is induced by a nitrogen starvation response (Sugimoto et al., 1991). Gamete differentiation in yeast is more similar to spermiogenesis, since in both cases the meiotic divisions result in 4 haploid cells for every diploid cell. While different cues induce gametogenesis in male animals and yeast, a common feature is the transcriptional program that regulates gametogenesis. The transcription factor MYBL1 in mice can be viewed as the functional homologue of Ime1 and Ste11 (Bolcun-Filas et al., 2011). While most of the mRNAs are promptly translated in yeast, they are subject to translational control in spermiogenesis. In both cases, it is unclear how the activities of cell cycle regulators and differentiation factors are regulated so that differentiation starts only after meiotic divisions started. To provide a potential solution to the problem, we aimed to understand the mechanism by which gamete differentiation is confined to the second meiotic division in budding yeast. We found that the molecular mechanism that coordinates meiotic divisions and sporulation relies on the mutual regulation of highly conserved molecules, such as Cdk/Clbs and the APC/C. We suggested that a role for APC/C and Cdk1 in gamete differentiation might be conserved in other species. The study of the role of such fundamental cell cycle regulators in mouse sperm cells is difficult, due to the fact that both Cdc20 and Cdk1 are essential for embryonic development (Manchado et al., 2010; Satyanarayana et al., 2008). In *Drosophila* instead, female-sterile mutations in the APC/C activator *fzy* cause both meiosis I and meiosis II arrest in Drosophila oocytes (Swan and Schupbach, 2007), but its role in spermiogenesis was not addressed to date. We show here that spore formation is initiated in yeast by a two-step process, in which APC/C-Cdc20 "licenses" SPBs for MP assembly at anaphase I by mediating the degradation of Spo13 and Clb1. This, in turn, activates the mechanism that removes Spc72 from the cytoplasmic face of the SPBs. MP assembly is then "fired" when Cdk1-Clb activity reappears in metaphase II. MP assembly also requires Hrr25 activity, which coordinates subsequent PSM closure with the second meiotic division and spindle

disassembly. We still need to address some interesting questions, such as: (a) is Spc72 itself the inhibitor of MP formation? (b) what is the molecular mechanism of Spc72 removal from the SPBs and degradation? (c) how does Spo13 regulate Cdk1/Clb1 activity? We think that the model describing confinement of sporulation to meiosis II, more than being readily applicable to other species, could be a paradigm of how meiosis II events are inhibited in meiosis I, or activated in meiosis II, or both.

4. Material and methods

4.1. Yeast strains

All experiments were performed with diploid *Saccaromyces cerevisiae* strains of the fast-sporulating SK1 genetic background (*ho::LYS2 lys2 ade2Δ::hisG trp1::hisG leu2::hisG his3Δ::hisG ura3*). Diploid strains were obtained by mating of the correspondent haploids. Mutations in diploid strains are homozygous, unless noted otherwise. Genotypes of strains used in this work are listed in **Table 1**. The following alleles have been described previously: *MPC70-eGFP*, *MPC54-eGFP*, *mpc54Δ::KanMX4*, and *mpc70Δ::KanMX4* (Knop and Strasser, 2000), *SPC72-eGFP* (Pereira et al., 1999), *hrr25-as1* and *HRR25-HA3*, (Petronczki et al., 2006), P_{CLB2}-*SGO1* (Katis et al., 2010), *CNM67-tdTomato*, and *TetR-tdTomato* (Matos et al., 2008), P_{CUP1}-*CDC20* (Arguello-Miranda et al., 2017), P_{CLB2}-*CDC20* (Lee and Amon, 2003), P_{SCC1}-*CDC20* (Clyne et al., 2003) *cdc28-as1* and *ime2-as* (Benjamin et al., 2003), and *cdc5-as* (Snead et al., 2007).

4.2. Construction of plasmids and yeast strains

To perform live-cell imaging of Rec8, YIplac128 carrying SK1 *REC8* (-333 to +2212), C-terminally tagged with mNeonGreen (Shaner et al., 2013) was integrated into the promoter of the *rec8Δ::KanMX4* locus as described (Buonomo et al., 2000). To visualize Spo74 in live-cell imaging experiments, PCR-generated cassettes were used for C-terminal tagging of Spo74 with sfGFP (Pedelacq et al., 2006). Tagged proteins are fully functional as judged from normal proliferation and sporulation of homozygous diploids. To suppress Cdc20 expression in meiotic cells, the endogenous *CDC20* promoter was replaced by the mitosis-specific promoter of *HSL1*.

4.3. Meiotic time course experiments

Synchronous sporulation of SK1 strains was carried out as previously described (Oelschlaegel et al., 2005). All steps of the procedure were carried out at 30 °C. Fresh zygotes produced by the mating of the corresponding haploids were streaked on glycerol plates (YPG) to select single colonies within 36-48 hours. The single colonies were transferred to yeast extract peptone dextrose

(YPD) plates and grown in a small (2 cm^2) patch. After 24-28 hours, the patch was plated as a homogeneous lawn on YPD plates. The selected diploids were then inoculated in 250 ml of liquid YEPA medium (YP plus 2% K-acetate) to an OD_{600} of 0.3. The cultures were shaken at 200 rpm for 12-17 hours in an orbital shaker. At the end of this period, the OD_{600} reached 1.8-2 and cells arrested in G1, with less than 15% of 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 3.5. At the indicated time points, samples were collected for live-cell imaging analysis, trichloroacetic acid (TCA) protein extracts, immunofluorescence, or transmission electron microscopy. To inhibit Hrr25 or Cdc28 activity in *hrr25-as* cells or *cdc28-as* cells, 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 (Bishop et al., 2000). To inhibit Ime2 activity in *ime2-as* cells, 1NA-PP1 (Cayman Chemicals) was added to a final concentration of 20 μM from a stock solution of 20 mM in DMSO, stored at -20°C until use (Bishop et al., 2000). To inhibit Cdc5 activity, CMK (AccendaTech) was added to a final concentration of 20 μM from a stock solution of 20 mM in DMSO, stored at -20°C until use (Snead et al., 2007).

4.4. Indirect immunofluorescence microscopy of fixed cells

Indirect immunofluorescence was performed with cells fixed overnight at 4 °C in 3.7% formaldehyde as described (Salah and Nasmyth, 2000). Briefly, 900 μl of cells were fixed by adding 100 μl of 35% formaldehyde overnight at 4 °C. Samples were then washed three times with 1 ml of 0.1 M potassium phosphate buffer pH 6.4, one time with 1 ml spheroplasting buffer (0.1 M potassium phosphate buffer pH 7.4, 1.2 M sorbitol, 0.5 mM $MgCl_2$) and resuspended in 200 μl of spheroplasting buffer. 5 μl of a freshly prepared 10% solution of β–mercaptoethanol were added to each sample, which was then incubated at 30 °C with shaking for 10 min. To obtain spheroplasts, 10 μl of zymolase solution (Zymolyase 100T from Amsbio, 1 mg/ml in spheroplasting buffer) were added to the samples. After 10 minutes incubation, cells were checked by phase-contrast microscopy for cell wall removal. Digestion was stopped by addition of 1 ml of cold spheroplasting buffer. After gentle centrifugation, the

spheroplasts were resuspended in 200 µl of spheroplasting buffer. 5 µl of spheroplasts per sample were deposited on a polylysine-coated 15-well slide. Spheroplasts were allowed to stick to the slide for 10 min, the excessive volume of liquid was removed and the cells were dehydrated by incubating the slides for 3 minutes in methanol and 10 seconds in acetone, both at -20 °C. The slides were rehydrated by incubating with 5 µl of filtered 1X PBS (50 mM NaCl phosphate pH 7.4, 0.15 M NaCl, 0.1% sodium azide) per well, and then blocked with PBS containing 1% bovine serum albumin (PBS-BSA). Primary antibodies were incubated for two hours at room temperature in a humid chamber. Slides were washed six times with PBS-BSA for 5 minutes. Secondary antibodies were incubated for two hours in a humid chamber, in the dark, and after six washes with PBS-BSA, the wells were covered with mounting media containing 4',6-diamidino-2-phenylindole (DAPI) to stain DNA, and a coverslip was applied, carefully avoiding the formation of air bubbles. The following primary antibodies were used: monoclonal mouse anti-Myc (1:5, 9E10), monoclonal rat anti-tubulin (1:250, Serotec YOL 1/34), rabbit anti-GFP (Zachariae Laboratory). Affinity purified, preabsorbed secondary antibodies conjugated to CY3 (1:200, Abcam), CY5 (1:200, Abcam), Alexa488 (1:200, Chemicon) were used for detection. Cells were observed on an Axioskop 2 epifluorescence microscope with a 100x plan-apochromat 1.40 NA oil immersion objective lens (Carl Zeiss). Pictures were taken with a Retiga Exi CCD camera controlled by QCapture 2.9.12 software (QImaging) and processed with Adobe Photoshop. For quantifications, at least 100 cells per time point were counted.

4.5. Live-cell imaging of meiosis

4.5.2. Experimental setup

Imaging of living cells undergoing meiosis was performed as previously described (Matos et al., 2008). Cultures were induced to enter meiosis as described above. 40 µl of cells were resuspended in 260 µl of SPM contained in an 8-well slide (µ-Slide 8 Well, ibiTreat: #1.5 polymer coverslip, tissue culture treated, sterilized, Ibidi, Cat. No. 80126) coated with Concanavalin A (Sigma C5275, 0.5 mg/ml in PBS) to give a final density of roughly 20 cells per field of view. Imaging was performed on a DeltaVision Elite system controlled by the SoftWoRx5.0 software (Applied Precision). Optical components of the system

included an Olympus IX71 microscope, an InsightSSI solid-state illumination system, an Olympus UPlanSApo 100x/1.4NA/oil objective, Delta Vision filter sets, and a Photometrics CoolSnap HQ_2 CCD camera. Images were acquired in the green and the red channels every 10 minutes for 10 hours using a 12% neutral density filter (TrueLight Additional ND filter, ND090-37 12%T, Lumencor) and exposure times of 50-300 ms. To attenuate the excitation light intensity, %T was set to 10 for the green channel and to 32 for the red channel. For each time point, 8 Z-sections (1 µm apart) were acquired, deconvolved, and projected to a single 2D-image (SoftWoRx 5.0 maximum intensity projection).

4.5.2. Data Presentation and Analysis

Images were processed with ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/). For quantifications, cells in 6-12 fields of view were individually followed through meiosis (50-100 cells in total). Percentages of meiotic events were then calculated for each time point using Microsoft Excel. The resulting graphs show percentages of meiotic events over time. To display normalized data, a cellular event taking place in both control and mutant strain (e.g., duplication of Cnm67-RFP labeled spindle pole bodies) was chosen as a reference and set to t = 0 in each cell. The parameters of 100 cells were aligned and the percentages of other events (e.g., localization of Mpc70-GFP at the spindle pole bodies) were calculated at 10 min intervals before and after the reference event. To produce images representative of the observed phenotype, a cell was cut out from the original image files. Stacks of the merge and the single channels were combined using the "Stack Combiner" plugin. The time scale was added to the stacks with the "Time stamper" tool, then the "Montage" tool was used to fuse the stacks into a single image.

4.6. Preparation of meiotic cells for Electron Microscopy

For examination of spindle pole bodies by electron microscopy, cells were prepared essentially as described (Byers and Goetsch, 1991). 2 ml of sporulating cells were harvested by centrifugation, incubated for 5 min in pretreatment solution (0.2 M Tris pH 9, 20 mM EDTA, 1 M NaCl, 10 mM Dithiothreitol (DTT)), washed twice with 0.7 M sorbitol, and fixed overnight at 4°C in 3%

glutarldehyde (EM-quality, 25% aqueous solution, Science Services GmbH) in sodium-cacodylate buffer (0.2 M pH 7.4, Electron Microscopy Sciences). After washing with Phosphate-citrate buffer (0.17 M K_2HPO_4, 30 mM sodium citrate (weighed out) to yield pH 5.8), cells were resuspended in the same buffer containing 10 mM DTT and incubated at 30°C for 10 min with shaking. To digest the cell wall, zymolase (Zymolyase 100T from Amsbio, 5 mg/ml in Phosphate-citrate buffer) was added to the samples, at a final concentration of 0.2 mg/ml. Spheroplasting was stopped after 30 minutes incubation by the addition of 1 ml of Phosphate-citrate buffer and by putting the samples on ice. Cells were collected by gentle centrifugation and washed two times with 0.1 M sodium acetate, pH 6.1. The following steps were carried out under a fume hood: cells were transferred to a 2% osmium tetroxide post-fixation solution generated by mixing equal volumes (250 µl per sample) of 0.1 M sodium acetate and a 4% osmium tetroxide stock solution (Science Services GmbH), for 15 minutes. After rinsing them three times with distilled water, the cell pellets were overlayed with 1% aqueous uranyl acetate and incubated for 60 minutes in the dark. During uranyl acetate incubation the Spurr resin was prepared according to manufacturer instructions (Low viscosity embedding media Spurr's kit, Electron Microscopy Sciences). After two washes in distilled water, cells were dehydrated by transferring them (5 minutes each step) through an ethanol series: 1x in 15% EtOH, 1x in 50% EtOH, 1x in 75% EtOH, 2x in 95% EtOH, 2x in 100% EtOH. Embedding of cells in the resin was carried out by gradually substituting the ethanol with the resin. First, the cell pellets were resuspended in 1 ml of a 2:1 (v/v) solution of ethanol and Spurr resin. Infiltration of the resin was carried out for two hours. To let ethanol evaporate, a hole was punched into the cap of the tube and a piece of dialysis membrane was placed at the closure. Tubes were rotated on a slowly rotating platform during the whole embedding procedure. Cells were pelleted at 3500 rpm for 5 minutes, the resin was removed by pipetting, and replaced with a 1:1 (v/v) solution of ethanol and spurr resin. Ethanol was left to evaporate over night. The next day, this mixture was replaced by 1 ml of Spurr resin, and after two exchanges of 60 minutes each, cells were pelleted at 3000 rpm for 10 minutes, the excess of resin was removed, and the pellets were gently re-suspended in the remaining resin, and transferred into Beem® embedding capsules (size 00,

Ted Pedella, Inc.). To pellet the cells, the capsules were placed into 2 ml Eppendorf tubes and centrifuged at 3000 rpm for 20 min. Finally, the samples were hardened at 70 °C for 24 hours in an oven. The resin block was extracted by cutting the capsule with a scalpel. Ultrathin sections (50 µm) were cut with an ultramicrotome (EM UC6, Leica) by Marianne Braun (EM laboratory, Max Planck Institute of Neurobiology, Martinsried). The sections were mounted on Copper-Slotgrids coated with Formvar (Electron Microscopy Sciences) and observed with an JEM-1230 (JEOL) transmission electron microscope with a voltage of 80 kV. Images were acquired using an Orius SC1000 digital camera (Gatan) and the accompanying Software (DigitalMicrograph™).

4.7. SDS-PAGE analysis of protein extracts

To analyze protein levels, extracts prepared by trichloroacetic acid (TCA) precipitation were separated in SDS polyacrylamide gels, followed by immunoblot detection of proteins. For each sample, cells from 10 ml of meiotic culture (OD_{600} ~3.5) were collected by centrifugation, resuspended in 1 ml of 10% TCA, and frozen in liquid nitrogen. Samples were thawed on ice and resuspended in 500 µl of 10% TCA. Cells were broken by vigorous shaking with zirconia beads (0.5 mm diameter) (Roth, 11079105z) for 30 min at 4 °C. After low-speed centrifugation (10 min, 3000 rpm), the resulting pellet was resuspended in reducing 2xSDS sample buffer (62.5 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS, 0.01% bromophenol blue, 30 mM β-mecaptoethanol), neutralized with half-volume of 1 M Tris base, and heated to 95 °C for 10 minutes. Samples were centrifuged at 14000 rpm for 10 minutes and the protein concentration in the supernatant was determined with a colorimetric Bio-Rad Protein Assay. Absorbance was measured at 595 nm with an Ultrospec 3100pro UV/Visible Spectrophotometer (Amersham Bioscience). After calculating protein concentration, 60 - 100 µg of total protein were loaded on SDS-8% polyacrylamide gels.

4.8. Co-immunoprecipitation assay

To analyze protein-protein interactions, extracts for immunoprecipitations were prepared essentially as described previously (Oelschlaegel et al., 2005). In brief, 0.2 M PMSF in DMSO was diluted 1:100 into 30 ml of culture. Cells were

washed with cold water containing 2 mM PMSF and processed for immunoprecipitation. Cells were resuspended in B150 buffer (50 mM HEPES-KOH pH 7.4, 150 mM KOA, 20 mM ßglycerophosphate, 5 mM magnesium acetate, 0.1% Triton X-100, 10% glycerol) containing protease inhibitors (Complete Roche, 5mM Pefabloc, 2 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml pepstatin, and 2 mg/ml leupeptin). Cell breakage was performed using zirconia beads. Samples were placed in a vibrax and vigorously shaken 5 times for 4 min with cooling in water-ice in-between. The lysate was centrifuged at 20,000 rpm for 30 minutes. The resulting supernatant was cleared by incubation with 200 µl of protein A-agarose beads (Roche) for 30 minutes. The beads were removed and the extracts were incubated with primary antibodies for 1 hour, in water containing ice. Protein-A agarose beads previously incubated with B150 buffer containing 10% BSA (40 µl) were added and incubated for 30 minutes with gentle rotating motion to capture the antibodies. Beads were washed with 1 ml aliquots of the following buffers: 3x B150, 2x B200 and 1x B70. Buffers B70 and B200 are similar to buffer B150, with the exception that they contain 70 or 200 mM KOAc, respectively. Immunoprecipitates were analyzed through immunoblotting.

4.9. Western blotting and immunodetection of proteins

For immunodetection of proteins, semidry western blotting (0.45 mA/cm^2) was used to transfer proteins on PVDF membrane (Immobilon P, Millipore). Membranes were blocked for 1 hour in PBS containing 0.1% Tween 20 and 4% non-fat milk powder (PBS-T), and incubated with primary antibody for 1 hour at room temperature. After three washes of 10 minutes in PBS-T, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase, for two hours at room temperature. After three washes of 10 minutes in PBS containing 0.1 % Tween 20, the membrane was incubated with a luminol-based chemiluminescent substrate for the detection of horseradish peroxidase (ECL detection system, GE Healthcare) and developed in a Kodak X-omat machine. Mouse monoclonal antibodies 9E10 (1:100, Zachariae lab) were used for the detection Myc tagged proteins, Pgk1 (1:40000, Invitrogen), GFP tagged proteins (1:1000, Sigma), and RFP tagged proteins (1:2000, Zachariae lab). Rabbit polyclonal antibodies were used for the detection of Ama1 (1:2000

(Oelschlaegel et al., 2005)), Cdc5 (1:5000 (Matos et al., 2008)), Ndt80 (1:10000, a gift from Kirsten Benjamin), Spo13 (1:5000, Zachariae Lab.), Rec8 (1:5000 (Katis et al., 2010)). Goat polyclonal antibodies were used for the detection of Clb1 (1:300, Santa Cruz sc-7647). Rat monoclonal antibodies were used for the detection of HA tagged Hrr25 in co-immunoprecipitation experiments (1:1000, Sigma).

4.10. Abbreviations

APC/C – anaphase-promoting complex/cyclosome
as – analog sensitive
ATP – adenosine triphosphate
BSA – bovine serum albumin
CDK – cyclin-dependent kinase
CMK – pyrrolopyrimidine chloromethylketone
DAPI – 4', 6'-diamino-2-phenylindole
DMSO – dimethyl sulfoxide
MP – meiotic plaque
NA – numerical aperture
OD – optical density
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PSM – prospore membrane
SPM – sporulation medium
YEPA – yeast peptone medium plus 2% potassium acetate
YPD – yeast peptone dextrose medium

Table 1. *Saccharomyces cerevisiae* SK1 strains used in this study

Figure	Strain[1]	Genotype[2]
5A,B	Z23834	*cdc20::P_{CLB2}-CDC20::HphMX4 trp1::P_{CUP1}-CDC20::TRP1 rec8Δ::KanMX4::REC8-mNeonGreen::LEU2 ura3::HIS3p-scarlet-tub1::URA3 hrr25Δ::KanMX4::HRR25::HIS3*
5A,B	Z23833	*cdc20::P_{CLB2}-CDC20::HphMX4 trp1::P_{CUP1}-CDC20::TRP1 rec8Δ::KanMX4::REC8-mNeonGreen::LEU2 ura3::HIS3p-scarlet-tub1::URA3 hrr25Δ::KanMX4::hrr25-as1::HIS3*
6	Z23794	*HTB1/HTB1-eGFP::KanMX4 hrr25Δ::KanMX4::HRR25::HIS3 [pRS426-P_{TEF2}-mRFP-spo20$^{51-91}$]*
6	Z23795	*HTB1/HTB1-eGFP::KanMX4 hrr25Δ::KanMX4::hrr25-as1::HIS3 [pRS426-P_{TEF2}-mRFP-spo20$^{51-91}$]*
7A, 9A	Z21830	*MPC54/MPC54-eGFP::KanMX4 CNM67-3mCherry::NatMX4 hrr25Δ::KanMX4::HRR25::HIS3*
7A, 9A	Z21831	*MPC54/MPC54-eGFP::KanMX4 CNM67-3mCherry::NatMX4 hrr25Δ::KanMX4::hrr25-as1::HIS3*
7B, 9B	Z21840	*MPC70/MPC70-eGFP::KanMX4 CNM67-3mCherry::NatMX4 hrr25Δ::KanMX4::HRR25::HIS3*
7B, 9B	Z21841	*MPC70/MPC70-eGFP::KanMX4 CNM67-3mCherry::NatMX4 hrr25Δ::KanMX4::hrr25-as1::HIS3*
7C, 9C	Z28716	*SPO74/SPO74-GFP::KlTRP1 CNM67-tdTomato::NatMX4 hrr25Δ::KanMX4::HRR25::HIS3*
7C, 9C	Z28717	*SPO74/SPO74-GFP::KlTRP1 CNM67-tdTomato::NatMX4 hrr25Δ::KanMX4::hrr25-as::HIS3*
8A,B	Z23218	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 hrr25Δ::KanMX4::HRR25::HIS3*
8A,B	Z23219	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 hrr25Δ::KanMX4::hrr25-as::HIS3*
8A,B	Z29809	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 mpc54Δ::AurMX4 mpc70Δ::BleMX4*
10A,B	Z22117	*SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4 hrr25Δ::KanMX4::HRR25::HIS3*
10A,B	Z22118	*SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4 hrr25Δ::KanMX4::hrr25-as::HIS3*
11A	Z6344	*MPC54-eGFP::KanMX4*
11A	Z24006	*MPC54-eGFP::KanMX4 HRR25-Ha3::HIS3MX6*
11B	Z28238	*MPC70-eGFP::KanMX4*
11B	Z25585	*MPC70-eGFP::KanMX4 HRR25-Ha3::HIS3MX6*
11C	Z30966	*SPO74-sfGFP::KlTRP1*
11C	Z30967	*SPO74-sfGFP::KlTRP1 HRR25-Ha3::HIS3MX6*
12	Z28237	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4 leu2/leu2::P_{URA3}-tetR-tdTomato::LEU2 hrr25Δ::KanMX4::HRR25::HIS3 sgo1:: P_{CLB2}-SGO1-Ha3::NatMX4*

12	Z27474	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4 leu2/leu2::P_{URA3}-tetR-tdTomato::LEU2 hrr25Δ::KanMX4::hrr25-as::HIS3 sgo1::P_{CLB2}-SGO1-Ha3::NatMX4*
13A,B, 14A	Z22000	*MPC54/MPC54-eGFP::KanMX4 CNM67-tdTomato::NatMX4*
13A,B, 14A	Z22001	*ama1Δ::CaURA3 MAP54/MPC54-eGFP::KanMX4 CNM67-tdTomato::NatMX4*
13C, 14B	Z21998	*MPC70/MPC70-eGFP::KanMX4 CNM67-tdTomato::NatMX4*
13C, 14B	Z21999	*ama1Δ::CaURA3 MPC70/MPC70-eGFP::KanMX4 CNM67-tdTomato::NatMX4*
13D, 14C	Z29575	*SPO74/SPO74-sfGFP::KlTRP1 CNM67-tdTomato::NatMX4*
13D, 14C	Z29576	*ama1Δ::CaURA3 SPO74/SPO74-sfGFP::KlTRP1CNM67-tdTomato::NatMX4*
15	Z2828	*PDS1-myc18::KlTRP1*
15	Z24653	*PDS1-myc18::KlTRP1 mpc54Δ::KanMX4 mpc70Δ::KanMX4*
16	Z31702	*SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4 PDS1-tdTomato::KlTRP1*
16	Z31704	*cdc20::P_{SCC1}-CDC20::HphMX4 SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4 PDS1-tdTomato::KlTRP1*
17	Z32767	*cdc20::P_{HSL1}-CDC20:: HphMX4 PDS1-myc18::KlTRP1*
17	Z32771	*cdc20::P_{HSL1}-CDC20::HphMX4 spo13Δ::HIS3MX6 PDS1-myc18::KlTRP1*
17	Z32768	*cdc20::P_{HSL1}-CDC20::HphMX4 clb1Δ::BleMX4 PDS1-myc18::KlTRP1*
17	Z32769	*cdc20::P_{HSL1}-CDC20::KanMX4 clb4Δ::KanMX4 PDS1-myc18::KlTRP1*
18A	Z31666	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4 PDS1-tdTomato::KlTRP1*
18A	Z31664	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 spo13Δ::BleMX4 SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4 PDS1-tdTomato::KlTRP1*
18B, C	Z32514	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 SPC72-eGFP::KanMX4CNM67-tdTomato::NatMX4*
18B	Z32513	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 clb1Δ::BleMX4 SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4*
18C	Z33291	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 clb4Δ::NatMX4SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4*
19	Z32514	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4*
19	Z32388	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 cdc28-as SPC72-eGFP::KanMX4 CNM67-tdTomato::NatMX4*

20A	Z31778	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 PDS1-tdTomato::KlTRP1CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4*
20A	Z31779	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 spo13Δ::BleMX4 PDS1-tdTomato::KlTRP1 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4*
20B, C	Z32908	*cdc20::P_{HSL1}-CDC20::HphMX4 ama1Δ::CaURA3 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4*
20B	Z32907	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 clb1Δ::BleMX4CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4*
20C	Z32546	*cdc20::P_{SCC1}-CDC20::HphMX4 ama1Δ::CaURA3 cdc28-asCNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4*
21A-C	Z24053	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4 leu2/leu2::P_{URA3}-tetR-tdTomato::LEU2*
21A	Z31053	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 cdc5-as::HphMX4 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4 leu2/leu2::P_{URA3}-tetR-tdTomato::LEU2*
21B	Z31398	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4 leu2::P_{URA3}-tetR-tdTomato::LEU2*
21C	Z31259	*cdc20::P_{CLB2}-CDC20::HphMX4 ura3::P_{CUP1}-CDC20::URA3 cdc28-as CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4 leu2/leu2::P_{URA3}-tetR-tdTomato::LEU2*
22	Z32751	*cdc20::P_{SCC1}-CDC20::HphMX4 hrr25Δ::KanMX4::HRR25::HIS3 spo13Δ::BleMX4 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4*
22	Z32750	*cdc20::P_{SCC1}-CDC20::HphMX4 hrr25Δ::KanMX4::hrr25-as::HIS3 spo13Δ::BleMX4 CNM67-tdTomato::NatMX4 MPC70/MPC70-eGFP::KanMX4*
23	Z21582	*cdc20::P_{CLB2}-CDC20::KanMX6 trp1::P_{CUP1}-CDC20::TRP1 PDS1-myc18::HIS3MX6 hrr25Δ::KanMX4::hrr25-as1::HIS3*

[1]Strains are listed for each figure from left to right and/or top to bottom. [2]All SK1 strains are diploid with the background *MATa/MATα ho::LYS2 lys2 ade2Δ::hisG trp1Δ::hisG leu2Δ::hisG his3Δ::hisG ura3*. Mutations are homozygous unless stated otherwise.

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