What prevents DNA replication between meiosis I and -II in yeast?

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Abstract

During meiosis, a single round of DNA replication is followed by two consecutive rounds of chromosome segregation. While the suppression of DNA replication between meiosis I and –II is one of the defining features of meiosis, its mechanism has remained unclear. The control of DNA replication has been studied extensively in proliferating cells in which DNA replication during S phase strictly alternates with chromosome segregation at mitosis. The mechanism ensuring that each sequence is replicated only once per cell cycle is based on the dual function of Cdk1: low Cdk1 activity after mitosis allows the establishment of pre-replicative complexes at replication origins (origin licensing). Activation of Cdk1 at the onset of S phase then initiates DNA replication (origin firing) by converting the pre-replicative complex to the post-replicative complex. Since high Cdk1 activity inhibits the reformation of pre-replicative complexes, the next round of DNA replication cannot occur until after Cdk1 has been inactivated during mitosis when replicated chromosomes segregate. However, applying this concept to meiosis would trigger an additional round of DNA replication because Cdk1 activity drops and then re-appears between meiosis I and –II. Two ideas have been proposed to solve this problem: in *Xenopus* eggs, Cdk1 activity is reduced rather than completely destroyed between meiosis I and –II, while in yeast, a Cdk1-related kinase, called Ime2, was thought to prevent origin relicensing at anaphase I. We have tested these ideas by artificially inactivating and then re-activating Cdk1 and Ime2 at anaphase I. Remarkably, DNA replication was not induced even when both kinases were simultaneously inhibited and re-activated at anaphase I. Thus, additional mechanisms must prevent DNA replication between meiosis I and –II.

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1. Introduction

The life cycle of sexually reproducing eukaryotes consists of fertilization, meiosis and mitotic proliferation. During mitosis, the entire genome must be replicated and then equally divided between the two daughter cells. The daughter cells will contain the exact same DNA content as the original parent cell. During meiosis, the genome is replicated as in mitotic cells, but subsequently one round of DNA replication is followed by two rounds of chromosome segregation (meiosis I and II) without an intervening round of DNA replication. Thus, meiosis results in the formation of gametes with a haploid set of chromosomes from diploid germ cells (reviewed in Petronczki et al., 2003b). Without haploidisation, fertilization would duplicate the number of chromosomes in each generation. Additionally, meiosis produces genetic variability in gametes, which is inherited by offspring. This genetic diversity allows our species' and others to survive and adapt to new conditions. Understanding meiosis is of medical importance because errors during meiosis lead to birth defects, mental retardation, and spontaneous abortions in humans (Hassold and Hunt, 2001).

Here I have investigated how re-replication is prevented between meiosis I and II, one hallmark of meiosis, in the budding yeast *Saccharomyces cerevisiae*. To start, I will first introduce the basic principles of mitotic cell cycle control and replication mechanisms in proliferating cells. Then, I will introduce the basic principles of meiosis.

1.1. Overview of the mitotic cell division

Mitosis is a cell division cycle in which one cell divides to produce two new daughter cells with the same genetic material. In mitosis, the entire genome must be replicated and then equally divided between the two daughter cells. The mitotic cell cycle in eukaryotes proceeds through a fixed, circular sequence of four phases: G1 (unreplicated chromosomes) to S (DNA synthesis) to G2 (replicated chromosomes) to M (mitosis) and back to G1 (**Figure 1**) (Morgan, 2007; reviewed in Zachariae and Tyson, 2016). The mitotic cell cycle starts with G1-phase, where cells grow and have unreplicated chromosomes and followed by S-phase, in which DNA replication converts each chromosome into two

identical sister chromatids. To ensure that sister chromatids are correctly segregated into the daughter cells, sister chromatids have to be linked during S-phase, aligned on the spindle at metaphase, and then pulled apart at anaphase (Nasmyth, 2002). During S-phase, sister chromatids are linked through a chromatin-bound protein complex, called the cohesin. As cells enter metaphase, they form the mitotic spindle. This bipolar arrangement of microtubules attaches to sister chromatids through kinetochores, a hierarchical protein complex that links centromeric DNA to spindle microtubules to transmit forces generated by microtubule dynamics (reviewed in Foley and Kapoor, 2013). At anaphase, destruction of sister chromatid cohesion allows the mitotic spindle to segregate sister chromatids towards opposite poles of the cell. At the end of mitosis, the spindle disassembles and the cell undergoes mitotic exit, leading to a re-setting of the cell cycle and entry into the next G1-phase (Sullivan and Morgan, 2007). Mitotic exit is associated with cytokinesis, which generates two separate, genetically identical daughter cells (Lindon, 2008).

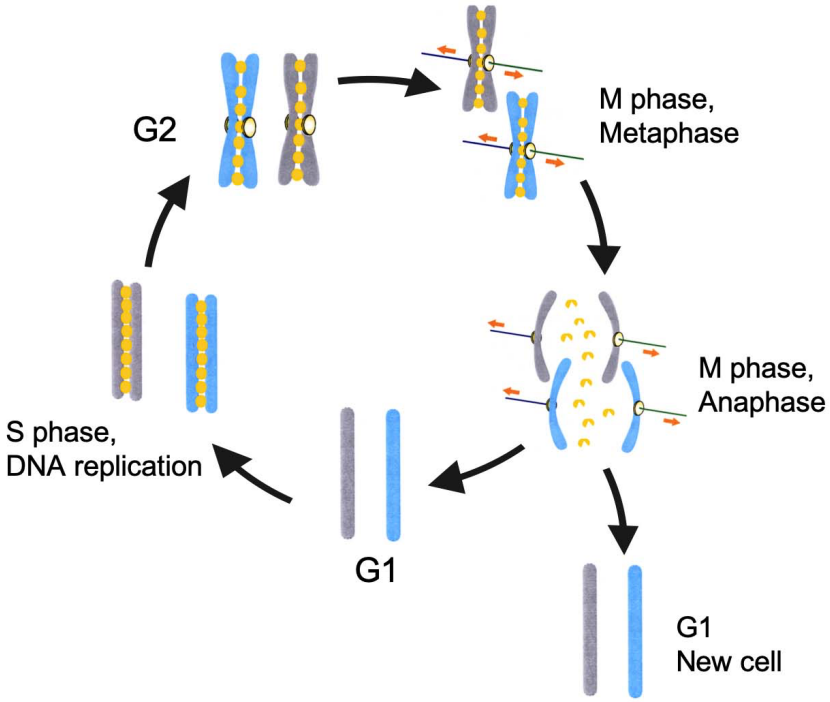


Figure 1. Overview of the mitotic cell cycle
Mitotic cell cycle progression is accomplished through the sequence of events, DNA replication (S phase) and mitosis (M phase) separated temporally by gaps (G1 and G2 phases). The resulting two new cells have identical copies of the original genetic information, and proceed to the next cell cycle. During DNA replication, cohesion (yellow dots) is loaded on chromatin.

1.2. Key regulators of mitotic progression: cyclin-dependent kinase and anaphase-promoting complex

Progression through the cell cycle is controlled by the periodic activation and inactivation of two types of enzymes: cyclin dependent kinases (Cdks) and the anaphase-promoting complex (APC/C), a ubiquitin ligase (Nasmyth et al., 1996; Nigg et al., 2001 and Peters et al., 2006). Cdks are a family of serine/threonine protein kinases, which require the binding of a regulatory cyclin subunit for their activity (Murray et al., 2004). Cyclin/Cdk kinase complexes regulate the activities of various proteins involved in DNA replication, chromosome segregation, and cell division by phosphorylation. In most eukaryotes, different types of Cdk/cyclin complexes are involved in cell division control. The cell cycle in budding yeast *Saccharomyces cerevisiae* is controlled by a single Cdk, which is Cdk1/Cdc28, in combination with several cyclins (Küntzel et al., 1996). In budding yeast, G1 cyclins (Cln1, Cln2 and Cln3) are required for the duplication of spindle pole bodies (SPBs) and the suppression of mechanisms inactivating S-phase Cdk1, such as phosphorylation of Sic1 and Cdh1. DNA replication is induced by Cdk1 bound to B-type cyclins: Clb5 and Clb6. Metaphase is induced by Cdk1 bound to M phase specific cyclins: Clb1, Clb2, Clb3 and Clb4. M-phase cyclins are required for mitotic spindle formation and for the alignment of the sister chromatids on the metaphase spindle (reviewed in Bloom and Cross, 2007). High Cdk1 activity is required for the S and M-phases. However, low Cdk1 activity is required for maintaining a prolonged G1-phase and for exit from mitosis (Wolf et al., 2007). Low Cdk1 activity is primarily achieved through cyclin degradation, which is triggered by the APC/C, the other key cell cycle regulator (Irniger et al., 1995; King et al., 1995). In addition to cyclin degradation, cells have further strategies to keep low kinase activity, such as the synthesis of Cdk inhibitors (CKIs) and inhibitory post-translational modifications on Cdk1. They bind specific Cdk1/cyclin complexes and block their kinase activity (Besson et al., 2008). APC/C catalyzes the formation of poly-ubiquitin chains on its substrates, thereby targeting them for degradation by the *26S* proteasome (Zachariae et al., 1996). APC/C usually targets substrates for degradation containing two sequence motifs: the destruction box (D-box) or the KEN-box (Glotzer et al., 1991; Pfleger and Kirschner, 2000). Ubiquitin-ligation during the cell cycle requires the association of the APC/C with one of two activators, called Cdh1 and Cdc20. Activators

bind the APC/C transiently in a manner that depends on the KEN- or the D-box, resulting in the formation of the active APC/C-Cdc20 or APC/C-Cdh1 holo-enzyme. Therefore, it has been proposed that activators promote ubiquitination by recruiting substrates to the APC/C (Kraft et al., 2005; Schwab et al., 2001; Burton and Solomon, 2000). During the cell cycle, the main function of APC/C-Cdc20 is to trigger the cleavage of sister chromatid cohesion in order to allow the segregation of sister chromatids at the metaphase-to-anaphase transition, and targeting cyclins for degradation in order to initiate exit from mitosis. The main function of APC/C-Cdh1 is to target for degradation cyclins to allow the completion of mitotic exit and to maintain cells in the subsequent G1-phase. The periodic changes of Cdk1 activity during the cell cycle depend on the mutual regulation between the activities of APC/C and Cdk1 (**Figure 2**). APC/C down-regulates Cdk1 activity through cyclin degradation. Cdk1 regulates the activity of the APC/C in an activator-dependent manner: Cdk1 inhibits APC/C-Cdh1 and activates APC/C-Cdc20 (Kramer et al., 2000; Zachariae et al., 1998). S-Cdk1 inhibits APC/C-Cdh1 by phosphorylating Cdh1, which promotes its dissociation from the APC/C (Zachariae et al., 1998). M-Cdk1 activates APC/C-Cdc20 by phosphorylating APC/C subunits, which promotes its binding to Cdc20 (Rudner and Murray, 2000).

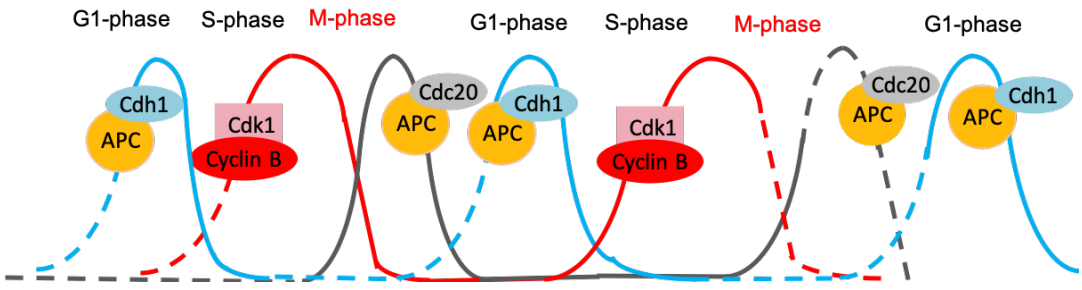


Figure 2. Oscillation of CDK activity in response to the fluctuation of APC/C activity
Cdk1 activates APC/C-Cdc20, which triggers sister chromatid segregation and the destruction of Cdk1. The drop in Cdk1 activity triggers exit from mitosis and leads to the activation of APC/C-Cdh1, which suppresses Cdk1 activity during the following G1-phase. In late G1, Cdk1 appears and inactivates APC/C-Cdh1.

1.3. DNA replication in mitosis

In a mitotic cell cycle, the chromosomes are duplicated once and only once. Initiation of DNA replication occurs in late G1 and requires the transition from a period of low Cdk activity to a phase with elevated activity. Cdk1 is activated by cyclins that are specialized for different stages of the cell cycle in an ordered cascade of events (reviewed in Bloom and Cross, 2007). G1-CDK (cyclin Cln3 bound to Cdk1) gradually increases in response to growth conditions and, at a specific threshold during mitotic G1, activates the G1/S-CDK (cyclins Cln1/2 bound to Cdk1). Cln's are the yeast G1 cyclins and they trigger the degradation of the Sic1. Sic1 is the S-CDK inhibitor; it blocks the Cdk1-cyclin B activity thereby preventing the G1-to-S-phase transition (Stuart and Wittenberg, 1998). G1/S-CDK drives cells through the mitotic G1-S transition, an irreversible commitment to completing cell division, and is required for activation of S-CDK (S-phase cyclins Clb5/6 bound to Cdk1). DNA replication is characterized by two key events that occur at different stages of the cell cycle. First, an inactive form of the replicative helicase is loaded onto origins of replication during G1 phase, a process called origin licensing. Second, the loaded helicases are activated as cells enter S phase. Helicase activation, known as origin firing, unwinds DNA, providing a template for the replisome to synthesize nascent DNA (Donovan et al., 1997; Seki and Diffley, 2000).

1.3.1. Origin licensing

Origin licensing occurs exclusively during late mitosis and G1, when APC/C activity is high and S-CDK activity is low. Upon exit from mitosis, CDK activity is lowered in two ways: by ubiquitin-mediated degradation of cyclin Clb2 by the 26*S* proteasome (Schwab et al., 1997 and reviewed in Siddique et al., 2013) and by Sic1-mediated inhibition of G1-CDK activity (Schwob et al., 1994). At this stage, the Cdc14 phosphatase also promotes pre-RC assembly by dephosphorylating Cdh1 to promote its association with APC/C (Visintin et al., 1998; Zachariae et al., 1998) and dephosphorylating the transcription factor Swi5 to activate transcription of Sic1 and Cdc6 (Knapp et al., 1996; Visintin et al., 1998). Finally, Cdc14 dephosphorylates Sic1, rendering it from SCF^{Cdc4}-mediated degradation (Visintin et al., 1998). At this low kinase state, the six-subunit origin-recognition complex (ORC) stably binds to DNA sequences

called origins of replication (Liang and Stillman, 1997). Origins of replication are defined as DNA sequences to promote DNA replication initiation. Yeast cells contain ~500 origins of replication. Budding yeast replication origins, or Autonomous Replicating Sequences (ARS's), are ~150 base pairs (bps) in length and include a conserved DNA motif called the ARS consensus sequence (ACS) (reviewed in Leonard and Mechali, 2013). After binding of ORC and Cdc6, Cdt1 proteins are recruited. Cdt1 stably binds to the Mcm2-7 helicase by interacting with multiple subunits, rendering it competent for loading. These three licensing factors together direct the loading of the helicase, the minichromosome maintenance (Mcm2-7) complex, around double-stranded DNA (dsDNA) (reviewed in Yardimci and Walter, 2014). The two Mcm2-7 complexes are loaded sequentially as a head-to-head Mcm2–7 double hexamer that encircles the origin DNA and is ready for bidirectional initiation (**Figure 3**) (Remus et al., 2009; Evrin et al., 2009). This form of the inactive helicase is also referred to as the pre-replicative complex (pre-RC). ORC, Cdc6, Cdt1, and Mcm2-7 are conserved throughout eukaryotes and are all essential for origin licensing (Bell and Kaguni, 2013). Orc1-5, Cdc6, and Mcm2-7 all have either winged-helix motifs or oligonucleotide-binding motifs and belong to the AAA+ family of proteins (Bleichert et al., 2015; N. Li et al., 2015). AAA+ proteins form multiprotein complexes with ATPase sites at the interface of neighboring subunits. ATP hydrolysis by many of the helicase-loading proteins is required for efficient origin licensing and cell viability (Chang et al., 2015; Coster et al., 2014; Frigola et al., 2013; Kang et al., 2014; Randell et al., 2006; Bowers et al., 2004; Schepers and Diffley, 2001). Correct origin licensing is essential for cell viability, and defects in the helicase-loading proteins are associated with congenital disorders, genome instability, and cancer (Arentson et al., 2002; Coulombe et al., 2013; Hossain and Stillman, 2012; Liontos et al., 2007).

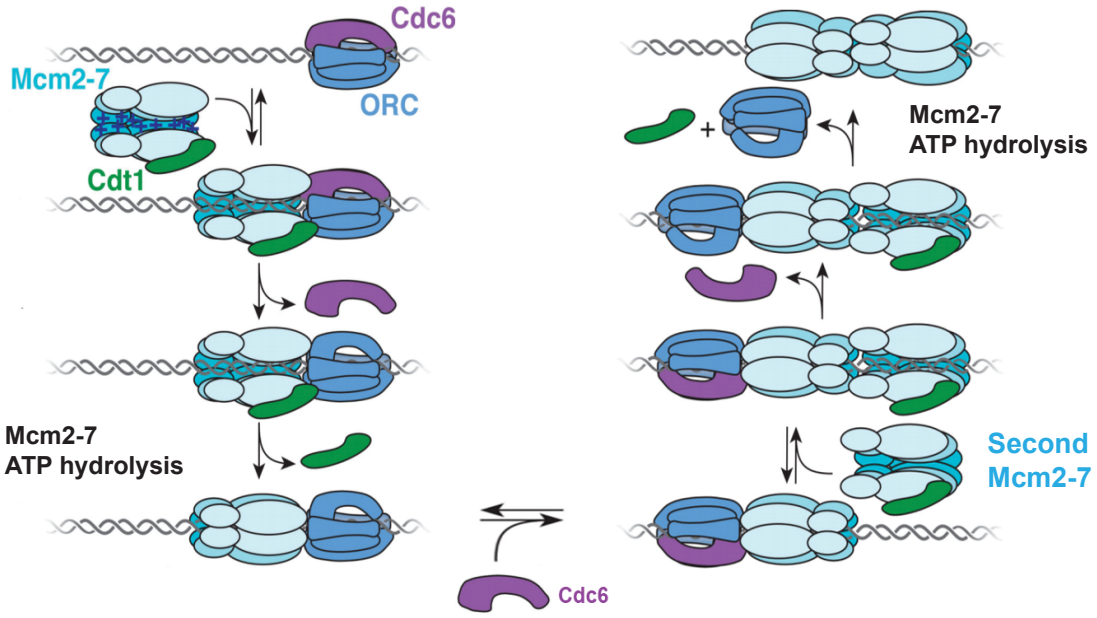


Figure 3. The mechanism of origin licensing
In eukaryotic cells under normal conditions, origin licensing starts with the ORC (dark blue) partially encircling the origin of replication. Cdc6 (purple) then binds to ORC complex to complete a protein ring around the DNA. The ORC-Cdc6 complex interacts with a ring-shaped Mcm2-7 helicase (light blue) which is bound to the Cdt1 protein (green), then it encircles the adjacent DNA. Multiple ATP-hydrolysis steps are required to load the first Mcm2-7 complex around dsDNA These same three proteins are also used to load the second Mcm2-7 complex to form the double-hexamer. (Adapted from Ticau et al., 2017).

1.3.2. Origin firing

Sic1, the CDK inhibitor, is one of the key barriers to origin firing during G1 phase. Therefore, it must be degraded before cells can initiate DNA synthesis (Schwob et al., 1994; Schneider et al., 1996). Simultaneous phosphorylation on multiple CDK consensus sites by Cln-Cdk1 and Clb-Cdk1 targets Sic1 for SCF-mediated polyubiquitination and proteolysis (Feldman et al., 1997; Verma et al., 1997; Koivomagi et al., 2011). Another barrier to origin firing in G1 phase is APC/C-Cdh1 activity, which actively degrades Clb cyclins. During late G1 phase, Cln2-Cdk1 and Clb5-Cdk1 phosphorylate Cdh1 and prevent its association with APC/C (Zachariae et al., 1998; Jaspersen et al., 1999). This allows accumulation of the Clb5-Cdc28 activity essential for origin firing.

Helicase activation, also known as origin firing, is the commitment step of DNA replication initiation. Helicase activation is more complex than helicase loading. To activate the loaded Mcm2-7 helicase, eukaryotic cells use multiple kinases to

phosphorylate Mcm2-7 and other essential replication proteins: (i) The Dbf4-dependent kinase, DDK (Cdc7 kinase and Dbf4 regulatory subunit) (ii) The cyclin-dependent kinase, S-CDK (Cdc28/Cdk1 kinase and the cyclin regulatory subunits Clb5 or Clb6) (reviewed in Bell and Labib, 2016). The first step in helicase activation is DDK phosphorylation of loaded Mcm2-7 complexes. The only essential target of DDK is the Mcm2-7 complex and DDK phosphorylation of Mcm2, Mcm4 and Mcm6 is important for replication initiation (Randell et al., 2010). DDK binds Mcm2-7 and regions within the Mcm4 and Mcm2 N-terminal tails mediate this interaction (Sheu and Stillman 2006; reviewed in Bell and Labib, 2016). DDK phosphorylation drives recruitment of Cdc45 and Sld3 to the Mcm2-7 double hexamer (**Figure 4**). The recruitment of the GINS complex and the completion of CMG-complex formation require S-CDK activity. After the G1-S transition, S-CDK is activated and phosphorylates Sld3 and Sld2, other assembly-factors. These are the two essential CDK targets during replication initiation (Tanaka et al., 2007; Zegerman and Diffley 2007; Yeeles et al., 2015). The phosphorylated forms of these proteins can bind to Dpb11, a third assembly-factor (Tanaka et al., 2007; Zegerman and Diffley, 2007). Binding between Sld3, Sld2, and Dpb11 is required for association of GINS to Mcm2-7-Cdc45 complexes, forming the CMG (Muramatsu et al., 2010). These two factors are tightly associated with Mcm2-7 at replication forks to form the activated helicase known as the Cdc45/Mcm2-7/GINS (CMG) complex (Gambus et al., 2006; Moyer et al., 2006). Finally, Mcm10 binds to the CMG, leading to full activation of the CMG complex and dsDNA unwinding (Looke et al., 2017) (**Figure 4**). Helicase activation has been recently reconstituted with purified proteins (Yeeles et al., 2015), showing that all the essential factors have been identified. Besides the proteins required for dsDNA unwinding, the polymerases are the most important components of the replisome. In eukaryotes, three polymerases travel with the replication fork and each has a distinct role: (i) Pol ε, the leading strand polymerase (ii) Pol α, the primase that synthesizes oligonucleotide primers on ssDNA, making the primer-template junctions on both the leading and lagging strands (iii) Pol δ, the lagging strand polymerase (reviewed in Bell and Labib, 2016).

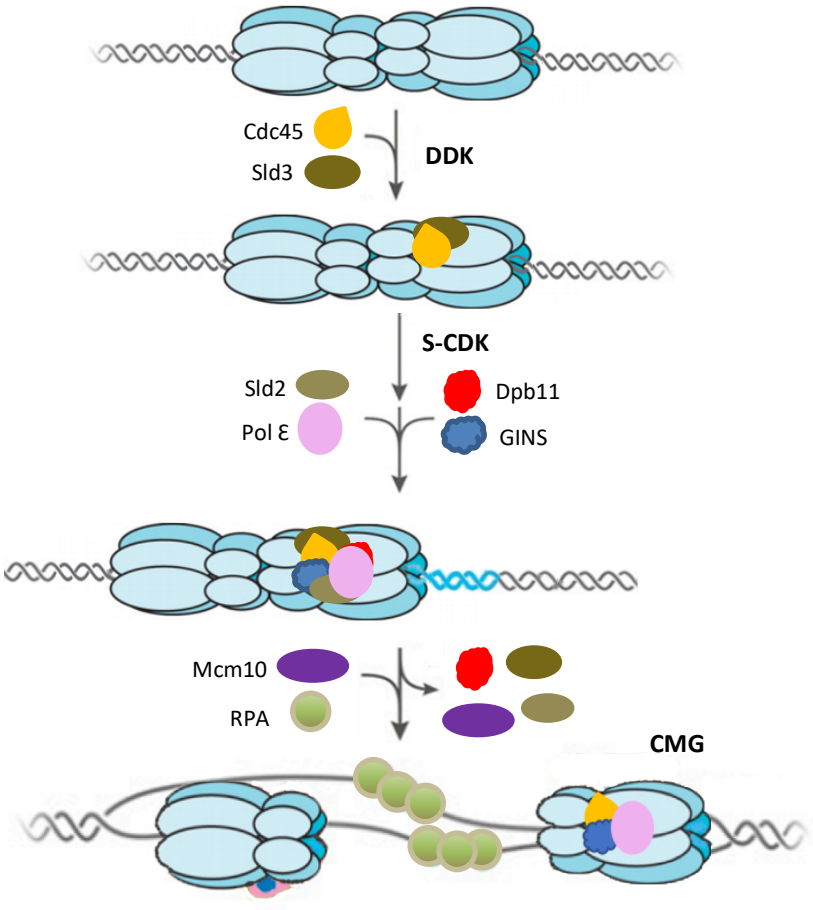


Figure 4. Mechanism of helicase activation
Two kinases, DDK and S-CDK, are required for the association of multiple proteins and activation of the loaded Mcm2-7 helicases. After DDK phosphorylates the Mcm2-7 complex, Sld3 and Cdc45 are able to associate. S-CDK then phosphorylates Sld2 and Sld3, permitting Sld2, Dpb11, GINS, and Pol ε to associate as a complex. Upon subsequent Mcm10 binding, DNA unwinding occurs and RPA associates (Adapted from Bell and Labib, 2016).

1.4. Mechanisms to prevent re-replication

To prevent over-replication and genome instability, replication origins must fire only a single time per cell cycle. Replicating any part of the DNA more than once per cell cycle is known as DNA re-replication. DNA re-replication at even a few origins can result in DNA damage or cell death (Archambault et al., 2005). Additionally, re-replicated DNA can be integrated into the chromosome and passed to the daughter cells, promoting genome instability (Green et al., 2010). To prevent DNA re-replication, mitotic cells use oscillations of CDK activity to temporally separate helicase loading (licensing) and helicase activation (firing) during the cell cycle. Helicase loading only occurs in G1 phase when CDK levels

are low to ensure that no origin of replication can reload Mcm2-7 at an origin that has initiated replication (Arias and Walter, 2007). The increased CDK levels present during S, G2, and M phases are required for activating CMG assembly and helicase activation. Furthermore, high CDK activity prevents helicase loading through multiple mechanisms (**Figure 5**). Cdks trigger disassembly of the pre-RC by phosphorylating its components, thereby ensuring that origins cannot be relicensed until Cdks are inactivated at the end of the cell cycle (Bell and Dutta, 2002; Blow and Dutta, 2005; Nguyen et al., 2001). This regulation ensures that no origin can initiate replication more than once per cell cycle. CDK phosphorylates Mcm3, ORC, and Cdc6 to promote nuclear export of non-chromatin bound Mcm2-7 complexes, inhibit an interaction between ORC and Cdt1, and promote proteolytic degradation of Cdc6, respectively (Labib et al., 1999; Liang and Stillman, 1997; Nguyen et al., 2001). Cdt1 stably binds to free Mcm2-7, causing it to be exported from the nucleus along with the helicase (Tanaka and Diffley, 2002). The inhibition of ORC activity is due to phosphorylation of both Orc2 and Orc6 (Chen and Bell, 2011; Nguyen et al., 2001). In addition, Cdk1 containing Clb5 cyclin binds directly to Orc6, further inhibiting its helicase-loading function (Wilmes et al., 2004). Bypassing these CDK-dependent mechanisms leads to DNA re-replication by eliminating the inhibition of helicase loading outside of G1 phase (Archambault et al., 2005; Green and Li, 2005; Nguyen et al., 2001). These bypass alleles are made by fusing a nuclear localization signal to an Mcm2-7 subunit, mutating the CDK-target sites on Orc2 and Orc6, and inducing overexpression of a non-degradable Cdc6 allele (Nguyen et al., 2001). Rereplication induced by these manipulations is, however, uneven and incomplete.

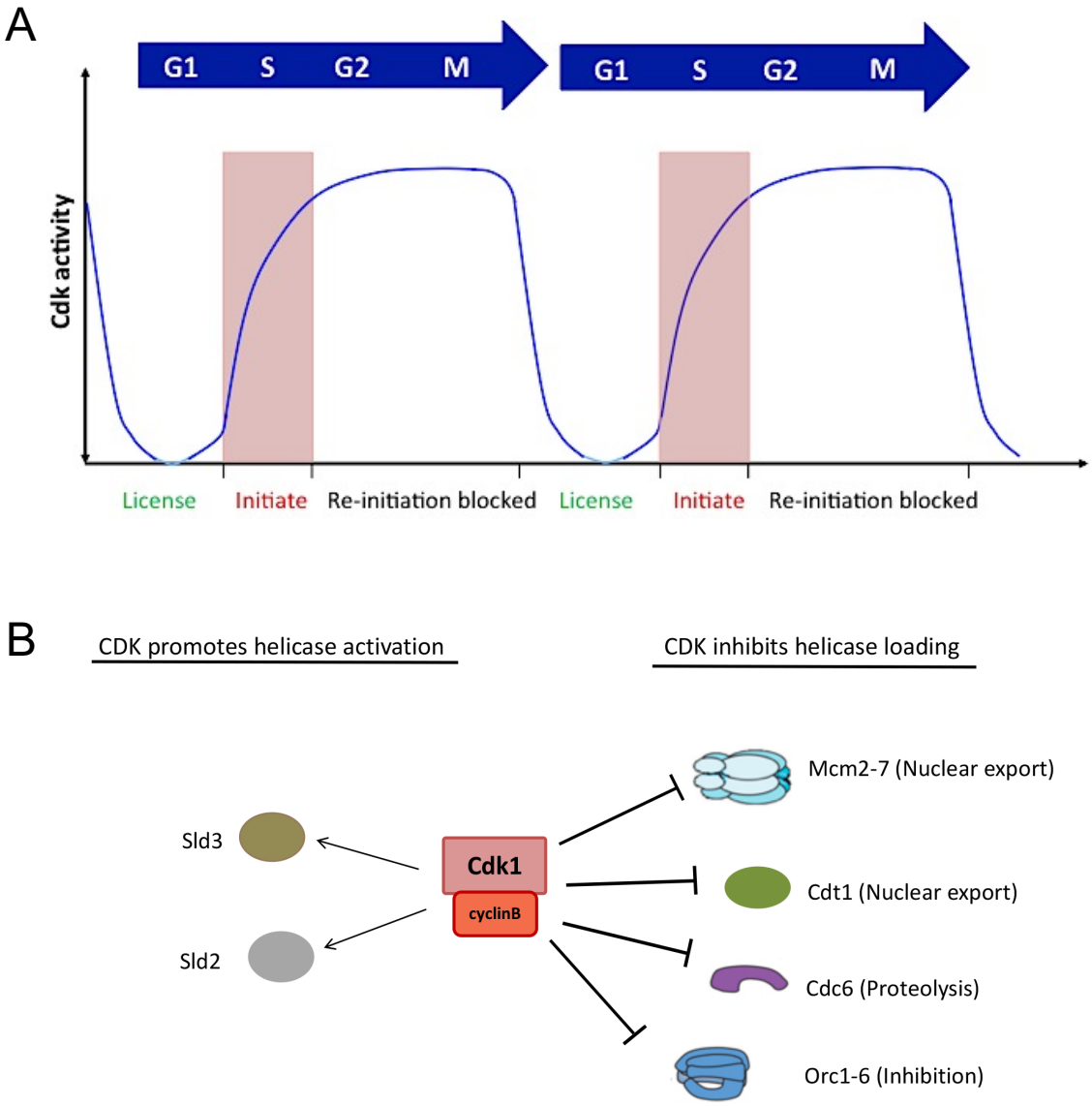


Figure 5: Oscillations of CDK activity temporally separate helicase loading and activation
A. G1 phase is permissive for helicase loading but not helicase activation due to the absence of CDK activity. Upon CDK activity increasing, helicase activation can take place but not helicase loading. This state stays until CDK activity decreases at the next G1 phase. **B.** Molecular mechanisms for CDK promoting Mcm2-7 activation while simultaneously inhibiting Mcm2-7 loading (adapted from Phizicky et al., 2018).

1.5. Meiosis: generating haploid gametes from diploid cells

During meiosis, a single round of chromosome duplication is followed by two rounds of chromosome segregation (meiosis I and II), resulting in four haploid gametes, which take the form of spores in the case of yeast, eggs for female

metazoans, or sperm for male metazoans. Without genome haploidization, fertilization would result in zygotes whose chromosome number doubles in each generation and accurate chromosome segregation would be impossible. To achieve the hallmark reduction in ploidy, the meiotic program differs from the mitotic program in four important aspects (Kerr et al., 2012, reviewed in Petronczki et al., 2003).

First, meiotic cells use a recombination pathway during prophase to physically link homologous chromosomes. Reciprocal recombination (crossovers) between homologous non-sister chromatids creates the chiasmata that link maternal and paternal chromosomes and produce bivalent chromosomes. Recombination permits the exchange of DNA segments between paternal and maternal chromosomes, generating genetic diversity. Homologs are held together by sister chromatid cohesion along chromosome arms distal to crossovers. In meiosis, a specific form of the cohesin complex mediates the establishment of sister chromatid cohesion during DNA replication. In budding yeast, meiotic cohesin differs from mitotic cohesin by the replacement of the kleisin subunit Scc1 with a meiosis-specific variant, called Rec8 (Klein et al., 1999). Cohesin containing Rec8 is essential for the processing of double-strand breaks in recombination (Klein et al., 1999) and lack of Rec8 results in recombination failure (Watanabe and Nurse, 1999b).

Second, during MI, meiotic cells regulate the kinetochores of sister chromatids such that they attach to microtubules emanating from the same pole and segregate to the same centrosome (Hauf and Watanabe, 2004). This monopolar attachment, or mono-orientation, ensures that maternal and paternal centromeres of bivalents are pulled to opposite directions. In yeast, monopolar attachment requires the loading of the monopolin complex onto kinetochores (Petronczki et al., 2003; Tóth et al., 2000).

Third, centromeric cohesin is protected during MI, and this protection is required to keep sister chromatids together so that they can be segregated correctly during MII. Cohesin containing Rec8 is also essential for the resistance of centromeric cohesin to cleavage by separase in anaphase I (Tóth et al., 2000).

Fourth, meiosis I is not followed by DNA replication but by a second round of chromosome segregation. Therefore, the meiotic program must prevent a round of DNA replication between two successive chromosome segregation events. This fourth point is the focus of my thesis work. Because no intervening DNA

replication takes place between both meiotic divisions, it is essential that cells use cohesion loaded during pre-meiotic S-phase to mediate not only meiosis I, but also meiosis II. The specialized program of chromosome segregation in meiosis I is followed by a second round of chromosome segregation, meiosis II, where sister chromatids segregate. The remaining centromeric cohesion resists the pulling forces of the meiosis II spindle before separation of sister chromatids. At the onset of anaphase II, separase becomes active and cleaves Rec8 and triggers the separation of sister chromatids to opposite poles (Petronczki et al., 2003).

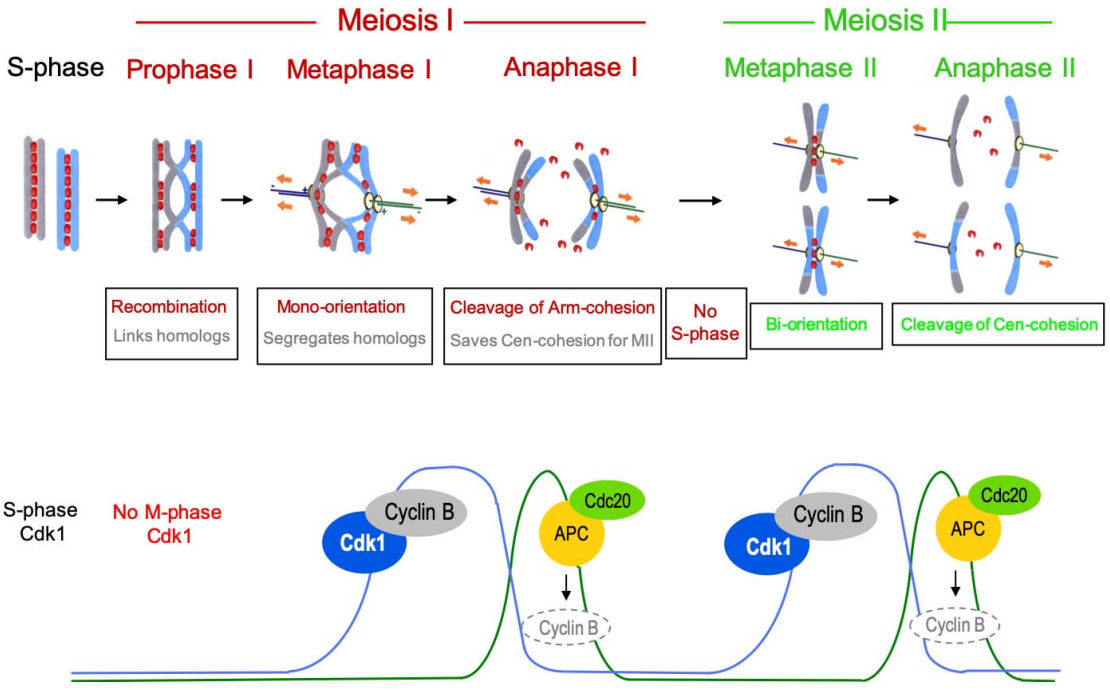


Figure 6. Overview of meiosis
During pre-meiotic DNA replication, cohesions (red dots), containing the meiosis-specific Rec8 subunit, is loaded on chromatin. In prophase I, reciprocal recombination between homologous non-sister chromatids creates chiasmata, which link maternal and paternal chromosomes through cohesion on chromosomal arms. In metaphase I, monopolar attachment of sister kinetochores ensures that maternal and paternal centromeres are pulled to opposite spindle poles. Cleavage of Rec8 on chromosome arms at the onset of anaphase I triggers segregation of homologs. Cohesion around centromeres is protected from cleavage in meiosis I, which is required for bipolar attachment of sister kinetochores in metaphase II. Cleavage of centromeric cohesion finally triggers segregation of sister chromatids and the generation of haploid gametes (Okaz et al., 2010). **Bottom part.** Cdk1 and APC/C-Cdc20 activity in meiosis.

1.6. Pre-meiotic DNA replication

DNA replication in pre-meiotic S-phase and pre-mitotic S-phase are controlled by similar mechanisms. In *Saccharomyces cerevisiae*, premitotic as well as premeiotic DNA replication is triggered by S-phase cyclin dependent kinases (CDKs), which are composed of the two B-type cyclins Clb5 and Clb6, and Cdc28 (Cdk1) (Dirick et al., 1998; Stuart and Wittenberg, 1998). However, despite the usage of the same machinery, there are also considerable differences. In all organisms analyzed so far, pre-meiotic S-phase is substantially longer than pre-mitotic S-phase and some replication factors are exclusively required for pre-meiotic DNA replication (Cha et al., 2000; Davis et al., 2001). Longer S phase and additional factors might be necessary because meiotic recombination, homolog pairing, and formation of the synaptonemal complex (SC) require pre-meiotic S-phase events (Borde et al., 2000; Smith et al., 2001). This requirement could be explained by the fact that cohesin has an essential role in these processes (Molnar et al., 1995). In budding yeast, the switch between growth phase and meiosis is controlled by nutritional signals. Under nitrogen and carbon starvation conditions, diploid budding yeast cells undergo meiosis (Kassir et al., 2003). At the onset of meiosis, nutritional signaling pathways converge on the transcriptional regulation of Ime1. Ime1 is a transcription factor that drives entry into meiosis by promoting transcription of meiosis-specific genes. One essential target of Ime1 is Ime2, a serine/threonine protein kinase that is only expressed during meiosis. Ime2 is evolutionarily related to Cdk1 and they share many substrates. However, they have different consensus motifs. The preferential phosphorylation sites for Cdk1 and Ime2 are S/T-P-x-K/R and R-P-x-S/T, respectively (Holt et al., 2007; Moore et al., 2007). Most of the shared substrates are phosphorylated with similar efficiencies but phosphates added by Cdk1 or Ime2 can be differently accessible to phosphatases. Moreover, Ime2 is not itself a cyclin-dependent kinase since it does not require cyclin binding. In fact, functions as a monomer (Holt et al., 2007). Early expression of *IME2* is required for the degradation of Sic1 at the G1/S transition (Dirick et al., 1998; Stuart and Wittenberg, 1998). Sic1 inhibits specifically Cdk1 associated with B-type cyclins (Schwob et al., 1994). Thus, removal of Sic1 is an essential process, allowing Clb5/6-Cdk1 to become active. In the mitotic cell cycle, Sic1 is phosphorylated by Cdk1 associated with the G1 cyclins Cln1 and Cln2 (Verma et al., 1997). In meiosis, Ime2 takes over this task (Dirick et al., 1998). Ime2

directly phosphorylates Sic1, thereby promoting its recognition by the F-box protein Cdc4, a substrate binding factor of the ubiquitin ligase SCF (Sedgwick et al., 2006; Sawarynski et al., 2007; Cardozo and Pagano, 2004). Later in meiosis, Ime2 has a key role in turning on middle meiotic genes by phosphorylating both the Ndt80 and Sum1 transcriptional regulators (Sopko et al., 2002; Moore et al., 2007; Shin et al., 2010). Ime2 induces the removal of Sum1 and promotes the efficient expression of middle meiotic genes by Ndt80, the global regulator of middle meiotic genes (Benjamin et al., 2003). Ime2 is present throughout meiosis with its multiple roles at different stages of meiosis (reviewed in Irniger, 2011).
Meiotic DNA replication initiation works very similarly in meiosis and mitosis, and it has been described previously in detail. Briefly, S-CDK promotes assembly of the replisome on loaded Mcm2-7 complexes, and these machines copy each chromosome to make two sister chromatids (Bell and Labib, 2016). These chromatids are joined together by cohesin, and completion of replication is monitored by the DNA damage checkpoint and intra-S phase checkpoint.

1.7. Mechanisms for preventing re-replication between meiosis I and II

The mechanism by which DNA replication is suppressed at the meiosis I-to meiosis II transition in budding yeast is poorly understood. In meiosis, the lack of a DNA-replication phase between meiosis I and meiosis II, requires the establishment of specific conditions. This is a difficult task because both chromosome segregation and DNA replication are similarly regulated by oscillations of CDK activity. During the MI-MII transition, CDK activity must decrease upon exit from MI and then increase upon entry into MII (Carlile and Amon, 2008). This oscillation of CDK activity is required for the disassembly of the MI spindle, re-duplication of the spindle pole bodies, and assembly of the MII spindles (Buonomo et al., 2003; Marston et al., 2003). However, an oscillation of CDK activity is also sufficient for replication of the entire genome in mitotic cells. This idea was first tested in experiments which showed that inactivation of Clb kinases by expression of the specific Clb–Cdk1 inhibitor, Sic1, is sufficient to induce a pre-replicative state at origins of replication in cells blocked in G2/M phase. Moreover, re-activation of Clb–Cdk1 kinases induces a complete second round of DNA replication in majority of the cells (Dahmann et al., 1995). As a result, dual function of Cdk1 can explain why origins can fire only once during the cell cycle but this does not apply for MI-MII transition.

Thus, there must be meiosis-specific mechanisms to uncouple replication and segregation. How the meiotic machinery meets these two incompatible conditions has been addressed in yeast and frogs (Iwabuchi et al., 2000; Holt et al. 2007).

Work in frog oocytes has shown that an intermediate level of CDK activity is retained between meiosis I and meiosis II and it is sufficient to reset the chromosome segregation program for MII while still being sufficient to inhibit Mcm2-7 loading. This intermediate level is achieved by two mechanisms, increased synthesis and partial inhibition of cyclin B destruction (Furuno et al., 1994; Iwabuchi et al., 2000). Indeed, it has been shown that experimentally inducing a complete oscillation of Cdk1 activity causes DNA re-replication in *Xenopus* oocytes (Iwabuchi et al., 2000). Based on some evidences, budding yeast is also thought to only partially inactivate CDK during the MI-MII transition. First, the APC/C-Cdh1, which promotes the complete degradation of cyclins at the end of mitosis, is not activated between MI and MII (Holt et al., 2007). Second, the Cdc14 phosphatase that reverses phosphorylation of CDK substrates has limited activity during the MI-MII transition (Attner and Amon, 2012; Buonomo et al., 2003; Kamieniecki et al., 2005). At the end of mitosis and MII, Cdc14 is released by two networks: the Cdc14 Early Anaphase Release Network (FEAR), which causes transient Cdc14 release into the nucleus, and the Mitotic Exit Network (MEN), which causes persistent Cdc14 release into the entire cell (Stegmeier and Amon, 2004). Between MI and MII, the MEN is inactive and Cdc14 is only released by the much weaker FEAR network, indicative of the need to keep some CDK-substrates phosphorylated.

Previous work in yeast showed that an auxiliary kinase activity present during the meiotic divisions could inhibit origin licensing even in the absence of CDK activity and in the presence of active Cdc14. Recently, such a kinase has been described to exist, Ime2. Ime2 is known to be active during the MI-MII transition (Benjamin et al., 2003; Berchowitz et al., 2013; Krylov et al., 2003). Ime2 has the ability to phosphorylate a large fraction of the Cdc28/Cdk1 substrates. Interestingly, the resulting phosphoproteins are resistant to the Cdk1-counteracting phosphatase, Cdc14 (Holt et al., 2007). This activity has been proposed to be capable of phosphorylating pre-RC components while permitting the spindle cycle to continue (Holt et al., 2007).

1.8. Aims of the study

It is not clear how budding yeast cells prevent DNA replication between meiosis I and II. Previous studies showed that either Cdk1 (Iwabuchi et al., 2000) or Ime2 (Holt et al., 2007) would be responsible for preventing origin licensing at this stage of meiosis. However, it was not clear which of these kinases was maintaining this inhibition during the MI-MII transition. Here, we showed that Cdk1 and Ime2 work together to inhibit pre-RC loading during the MI-MII transition, providing strong evidence for the cooperative-inhibition model. After artificial inhibition of kinases, we tested the reactivation of kinases in order to induce re-replication. However, DNA replication was not induced. We further investigated the functions of other kinases, DDK, Cdc5, and Swe1, and also phosphatase, Cdc14, on re-replication.

1.9. Contribution

I carried out all the experiments described in this thesis.

Katarzyna Jonak constructed the plasmids described in section 4.2 and developed *cdc20-3-mAR* system.

2. Results

During meiosis, the Cdk1-oscillation presents a unique problem at the MI-MII transition. After MI has been completed, Cdk1 activity decreases, and then increases again upon entry into MII. This oscillation is required for the spindle disassembly and reassembly. However, the DNA replication program must remain inhibited between MI and MII to achieve the genome haploidization, the hallmark of meiosis. An oscillation of Cdk1 activity is sufficient for re-replication of the entire genome in mitotic cells (Dahmann et al., 1995). However, it is unclear how meiotic cells reset the chromosome segregation program while keeping DNA replication inhibited. As mentioned in the introduction, two models have been proposed to explain how meiotic cells uncouple DNA replication and chromosome segregation during the MI-MII transition. First, the Cdk1-balance model suggests that partially inactivating Cdk1 is sufficient to reset the chromosome segregation program while still inhibiting Mcm2-7 loading and replication initiation (Iwabuchi et al., 2000). Second, the alternative-kinase model suggests that a second kinase inhibits Mcm2-7 loading during the MI-MII transition, allowing the oscillation of Cdk1 activity to reset the chromosome segregation program without resetting the DNA replication program. Ime2, a conserved meiosis-specific kinase, has been proposed to fulfill this role in yeast (Holt et al., 2007). Previous studies found that Ime2 is active during both meiotic divisions (Berchowitz et al., 2013) and that kinase can also promote Mcm2-7 nuclear export upon completion of meiotic S phase (Holt et al., 2007). Here, we have analyzed the effects of both kinases on DNA replication.

2.1. Cdk1 and Ime2 activity is required for preventing Pre-RC formation between MI and MII

In wild-type cells, Mcm2-7 and Cdt1 proteins are present at constant levels throughout meiosis. However, they disappear from the nucleus shortly after DNA replication (Phizicky et al., 2018). We aimed to test whether Cdk1 and Ime2 kinase activity prevents nuclear entries of Mcm2-7 and Cdt1. To do so, we analyzed the localization of the pre-RC components in cells where Cdk1 and Ime2 kinase activity was inhibited. For this purpose, we used analog-sensitive alleles of the kinases, which can be inhibited by ATP analogs (Bishop et al., 2000).

Analog-sensitive (as) kinase technology is a chemical-genetic technique that allows for the rapid and highly specific inhibition of individually engineered kinases. The technique consists of two components. First, a kinase containing a space-creating mutation in the ATP-binding pocket is engineered. To make an analog-sensitive kinase, a residue at a structurally conserved position in the kinase active site, termed the gatekeeper (Liu et al., 1999), is mutated from the natural amino acid to a residue bearing a smaller side chain (glycine or alanine). Second, an ATP-competitive small molecule inhibitor complements the shape of the mutant ATP pocket and inactivates the kinase (Bishop et al., 2000; Lopez et al., 2014). In the case of Ime2-as, addition of the ATP analog 1NA-PP1 inhibits its kinase activity (Benjamin et al., 2003) and 1NM-PP1 inhibits Cdk1-as (also called Cdc28-as in *S.cerevisiae*) (Bishop et al., 2000). To analyze Mcm7 nuclear localization with high temporal resolution, we observed meiosis in individual living cells containing an allele of Mcm7 carrying a C-terminal mCherry tag and the spindle pole body (SPB) component Spc42 bearing a C-terminal GFP tag **(Figure 7)**. Spindle pole bodies duplicate in meiosis during S-phase. Due to their close proximity, they appear as a single dot-like signal until entry into metaphase I (Jaspersen and Winey, 2004). SPB separation is the landmark of metaphase I, therefore Spc42-GFP served as a marker of entry into metaphase I. Control cells and cells containing *cdc28-as*, *ime2-as*, or both were arrested at metaphase I by the meiotic depletion of Cdc20 in order to manipulate the functions of kinases. A stable metaphase I arrest is achieved by placing the APC/C activator Cdc20 under the control of the mitosis-specific promoters, such as *SCC1*, *CLB2*, and *HSL1*. During meiosis, the mitotic promoter is repressed, which leads to the depletion of the Cdc20 protein. We induced the cultures to enter meiosis and added 1NM-PP1 and 1NA-PP1 when the majority of cells were in metaphase I (t=7 hours). In all strains, Mcm7-mCherry is present at the beginning of meiosis and is visible as a diffused nuclear signal. Upon the end of S-phase, the nuclear signal of Mcm7 disappears. When inhibitors were added to the cultures, we observed the re-accumulation of Mcm7 in the nucleus only upon simultaneous inhibition of Cdc28-as and Ime2-as cells **(Figure 7)**. In cells carrying only one of the mutant kinase alleles, Mcm7 did not re-entered the nucleus. This is reflected by the accumulation curves of Mcm7-mCherry on the graphs, where all cells were aligned to the separation of spindle pole bodies (metaphase I). These graphs

show that Mcm2-7 starts to reappear in the nucleus 1.5 hours after inhibition. Furthermore, we asked whether another subunit of the Mcm complex, Mcm2, is bound to the DNA upon Ime2 and Cdc28 kinase activity inhibition. We analysed Ha-tagged version of Mcm2 from synchronous meiotic cultures of *P_{SCC1}-CDC20 cdc28-as ime2-as* **(Figure 8)**. Immunofluorescence microscopy was used to visualize SPBs and chromatin-associated proteins on chromosome spreads. Consistent with the live-cell imaging results, Mcm2 was present on the chromatin at the beginning of meiosis and then disappeared upon SPB separation. We added inhibitors when the majority of cells were in metaphase I. Approximately one hour after kinase inhibition, Mcm2 appeared to be bound to the chromatin. We concluded that both kinases are required for suppressing the association of Mcm2-7 with chromatin at metaphase I.

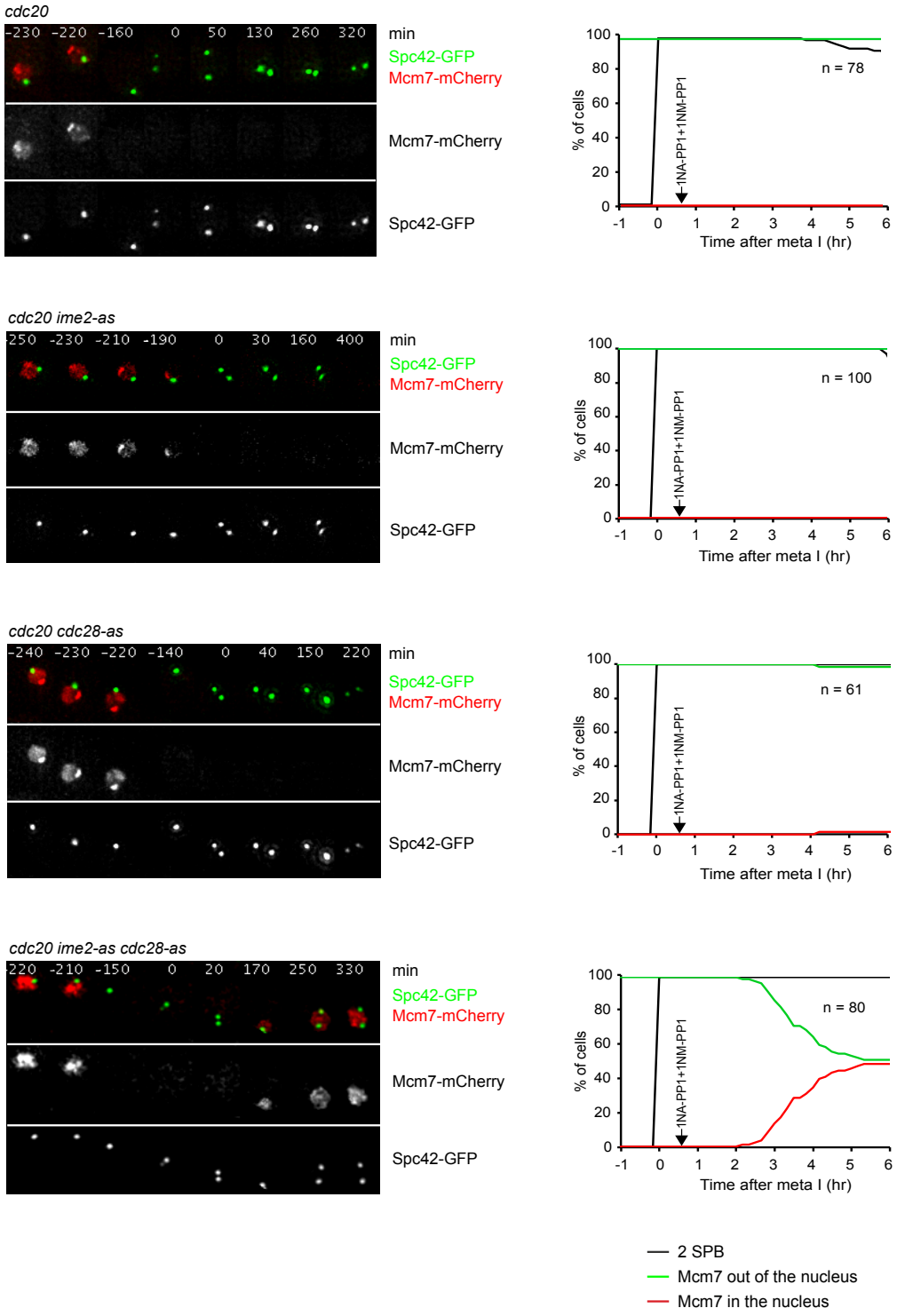


Figure 7. Inactivation of both Cdc28-as and Ime2-as in metaphase I results in the reaccumulation of Mcm7. Live-cell imaging of P_{SCC1}-*Cdc20* with *IME2 CDC28* (Z22116), *cdc28-as* (Z22288), *ime2-as* (Z22289) and *ime2-as cdc28-as* (Z22451) strains expressing Spc42-GFP and Mcm7-MCherry. Cells were treated with 1NA-PP1 (20 μM) and 1NM-PP1 (5 μM) at 7 hr in SPM. Images were acquired every 10 minutes. Left, representative time-lapse series are shown. Right, graphs displaying percentage of cells with 2 SPBs and with nuclear Mcm7-mCherry after metaphase I are shown.

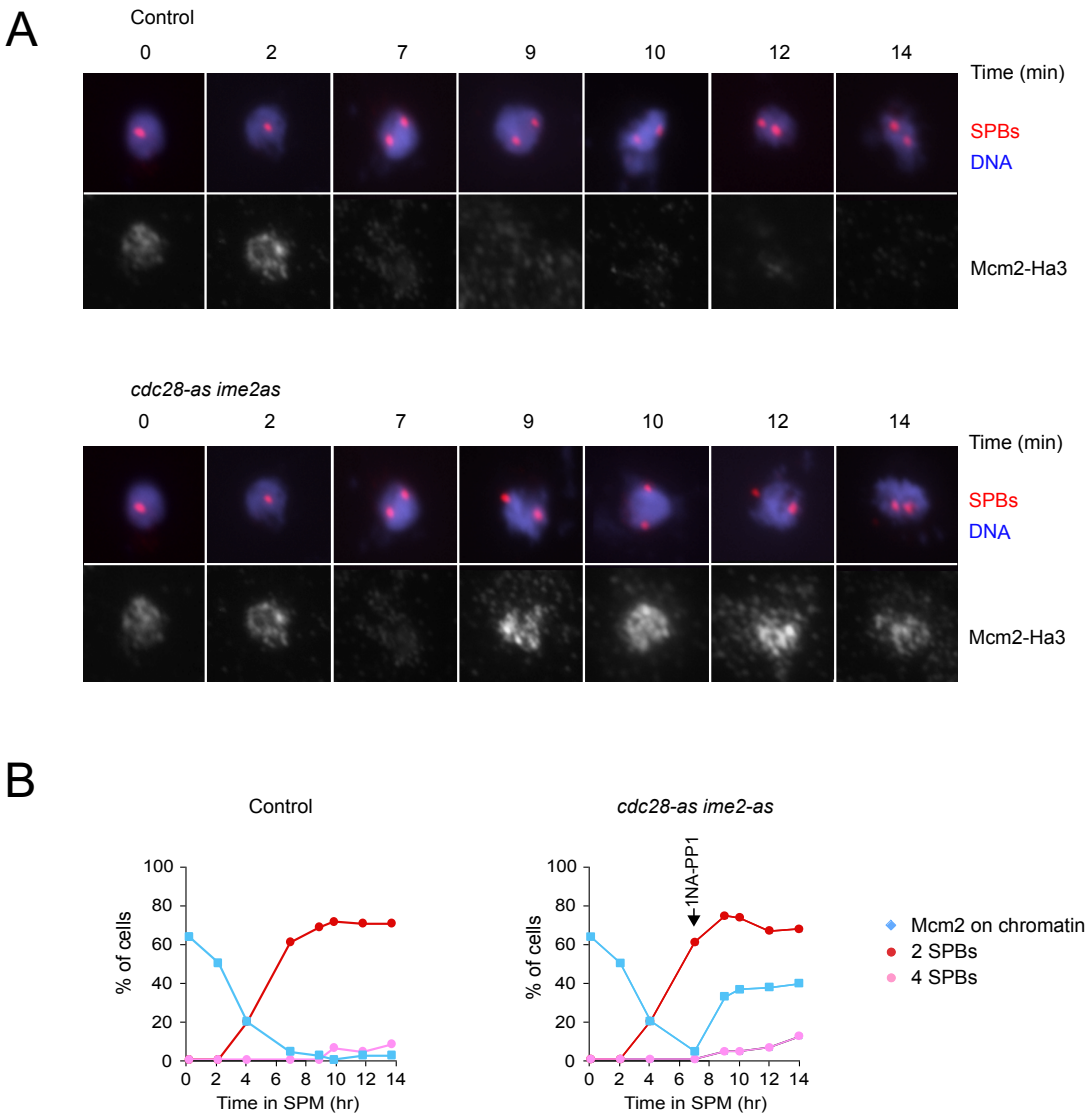


Figure 8. Inactivation of Cdc28-as and Ime2-as in metaphase I in APC off cells causes the association of Mcm2 on the chromatin. *P_{SCC1}-CDC20 ime2-as cdc28-as2* (Z30489) cells were treated with 1NA-PP1 (20 μM) at 7 hr in SPM. **(A)** Representative pictures of the spread nuclei, stained with anti-Ha and anti-Tub4 (SPB) antibodies. **(B)** Quantification of the spread nuclei (n=100). Graphs represent the percentage of cells with Mcm2 bound to chromatin at every time point.

Next, we addressed whether Cdc28 and Ime2 are involved in the regulation of other pre-RC components, such as Cdt1 and Cdc6. We imaged wild-type, *cdc28-as*, *ime2-as* and *cdc28-as ime2-as* strains arrested at metaphase I, expressing RFP-tagged SPB (Cnm67-tdTomato) to follow meiotic progression, and the pre-RC component Cdt1 tagged with the green fluorescent protein neonGreen **(Figure 9)**. Strains were induced to synchronously enter meiosis and we started imaging at six hours. Inhibition of Ime2-as or Cdc28-as alone did not have any effect on Cdt1 accumulation. Similarly to the MCM complex, Cdt1 appeared as a diffused nuclear signal only when we inhibited both Ime2-as and Cdc28-as. Shortly after inhibition, the signal of Cdt1 appeared in ~ 50 % of the cells. To analyze the effect of kinase inhibition on Cdc6, we synchronously induced meiosis in wild-type, *ime2-as*, *cdc28-as* and *ime2as cdc28as* cells, arrested at metaphase I **(Figure 10)**. These strains contained the endogenous Cdc6 protein tagged with nine PK epitopes (PK9) at its C-terminus. Samples were taken every two hours for indirect immunofluorescence staining on fixed cells of α-tubulin to visualize spindles, and DNA staining to monitor nuclear division. In addition, samples were taken for the preparation of whole-cell extracts, which were analyzed by SDS-PAGE followed by western blotting. Samples were also taken for flow cytometric analysis of cellular DNA content. We considered the appearance of meiosis I spindle as the landmark event for the high-kinase state of metaphase I. Furthermore, we used the Ndt80 phosphorylation status and the disassembly of metaphase I spindles as read-outs of efficient Ime2 and Cdc28 kinase inhibition, respectively. The modification of Ndt80 is dependent upon the activity of Ime2. Ndt80 is a meiosis-specific transcription factor and activates transcription of a large set of genes required for both meiotic nuclear divisions and spore formation (Hepworth et al., 1998). In our synchronized cultures, wild-type cells started to assemble meiosis I spindles around six hours after induction of meiosis. Seven hours after induction of meiosis, we added 1NA-PP1 and 1NM-PP1 to inhibit kinase activities.

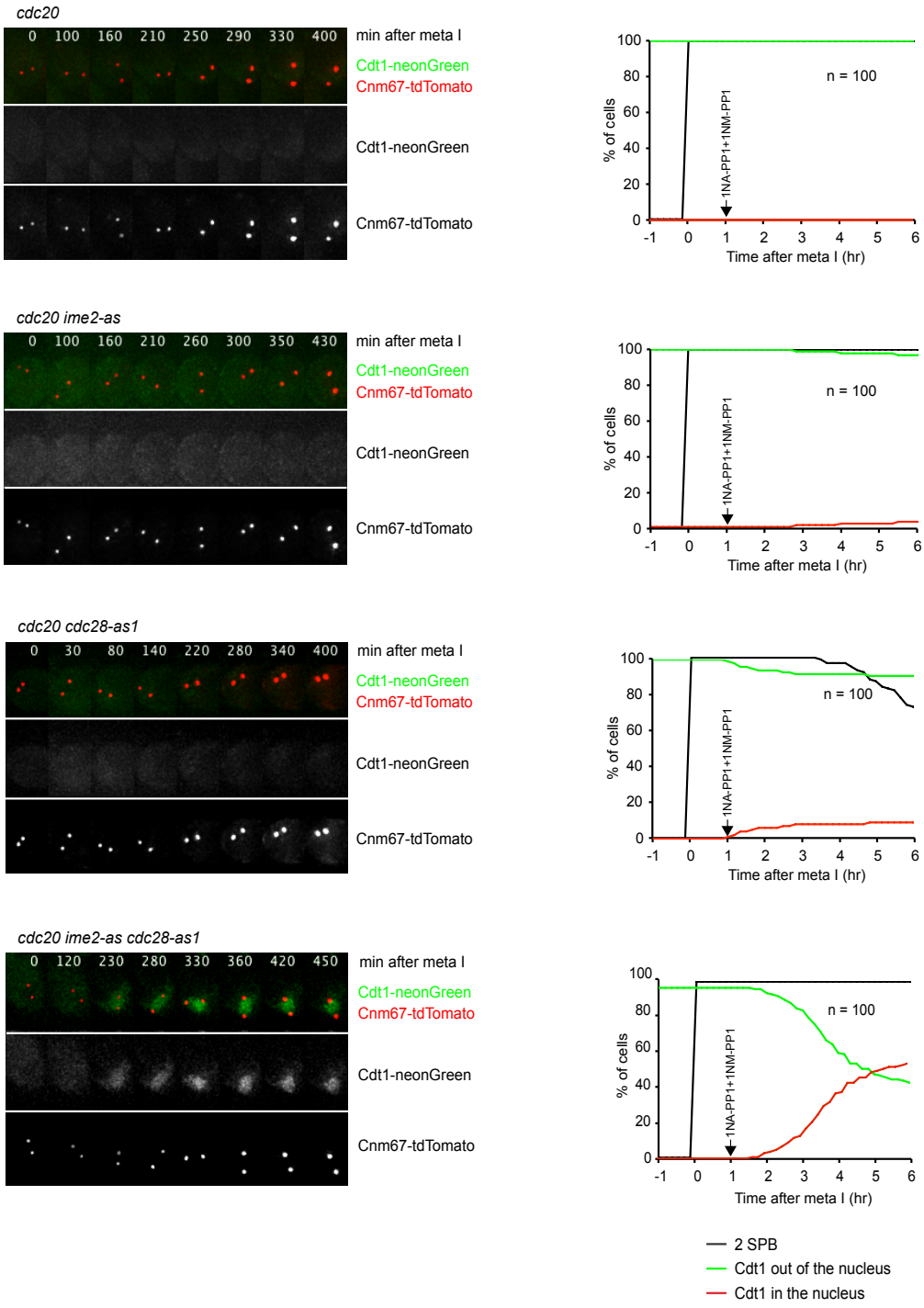
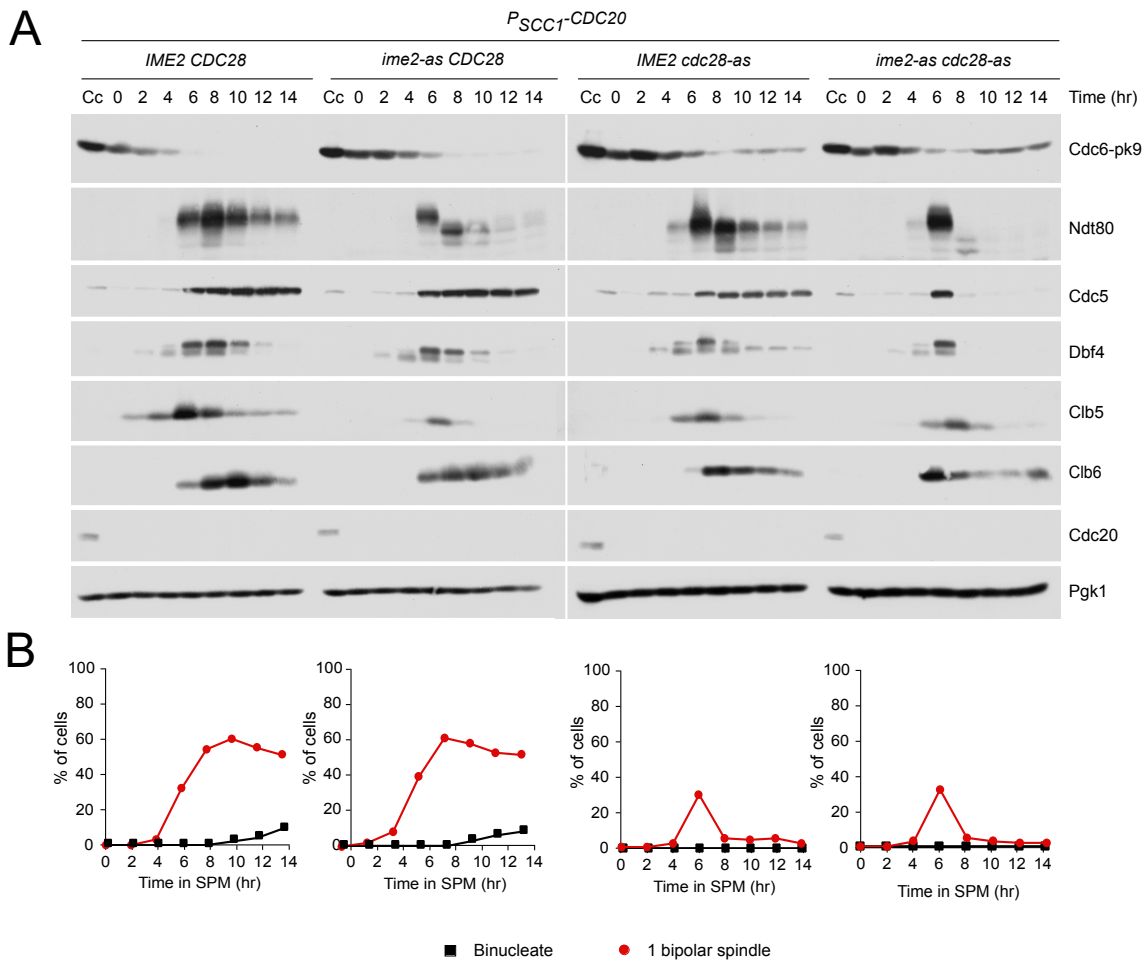


Figure 9. Simultaneous inhibition of Cdc28-as1 and Ime2-as results in the accumulation of Cdt1. P_{SCC1}-*Cdc20* with *IME2* and *CDC28* (Z29667), *pSCC1-Cdc20 ime2-as* (Z29665), P_{SCC1}-*Cdc20 cdc28-as1* (Z29668) and P_{SCC1}-*Cdc20 ime2-as cdc28-as1* (Z29666) were treated with 1NA-PP1 (20 μM) and 1NM-PP1 (5 μM) at 7 hr in SPM. Cdt1 was tagged with neonGreen and Cnm67 with tdTomato. Left, representative time-lapse series are shown. Right, graphs display percentage of cells with 2 SPBs and with nuclear Cdt1-neonGreen after metaphase I. Inhibition of both kinases at the same time resulted in Cdt1 accumulating in the nucleus ~1 hour after inhibition.

Immunofluorescence countings clearly showed that Cdc28 was inhibited since bipolar spindle countings were zero after the addition of inhibitors (**Figure 10B**). We observed that Ndt80, Cdc5, Clb5 and Dbf4 were fully degraded in the *ime2-as cdc28-as* strain one hour after inhibition suggesting that cells were in the low kinase state. The Cdc6 protein is present in cells during pre-meiotic G1 phase, but becames undetectable in MI when Cdk1 is highly active. However, Cdc6 protein levels raised again in *ime2-as cdc28-as* cells upon the addition of the inhibitors (**Figure 10A**). According to flow cytometry results, all strains completed pre-meiotic DNA replication with similar kinetics within four hours of meiotic induction (**Figure 10C**). We did not observe re-replication after inhibition of kinases, even though cells re-accumulated the pre-RC components Cdc6, Mcm2-7 and Cdt1.



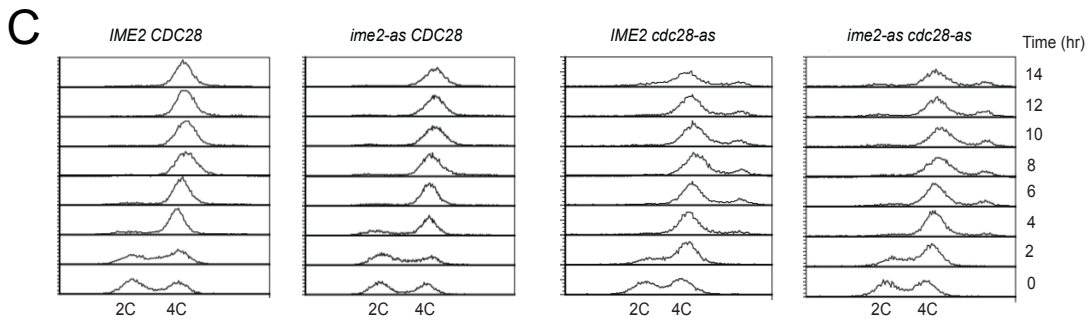


Figure 10. Inhibition of both Ime2-as and Cdc28-as causes re-accumulation of Cdc6. P_{SCC1}-*CDC20* cells with *IME2 CDC28* (Z23749), *ime2-as* (Z23751), *cdc28-as1* (Z23750), or *ime2-as cdc28-as1* (Z23752) were induced to enter meiosis and samples were withdrawn every 2 hr. Cells were treated with 1NA-PP1 (20 μM) and 1NM-PP1 (5 μM) at 7 hr in SPM. **(A)** Immunoblot detection of protein levels along the time course. Cc stands for cycling cells and indicates a sample from proliferating cells. **(B)** Immunofluorescence detection of bipolar spindles (α-tubulin) and of the number of nuclei (DAPI) **(C)** Flow cytometric analysis of cellular DNA content. P_{SCC1}-*CDC20* strains arrest in metaphase I, as seen from the accumulation of proteins and the absence of nuclear division.

2.2. Inhibition of both Ime2-as and Cdc28-as1 causes Sic1 and Cdh1 activation

Budding yeast has three APC/C co-activators: Cdc20, Cdh1 and the meiosis-specific co-activator, Ama1 (Okaz et al., 2012). Here, we tested whether inhibiting Cdk1 and Ime2 activity in metaphase-I arrested cells would have an effect on the Cdc28 inhibitor Sic1, and on Cdh1. We induced meiosis in cells lacking the APC/C activator Cdc20, and carrying either an allele of *cdc28-as1* or *ime2-as* or both **(Figure 11 A, B)**. We collected samples every two hours. Immunofluorescence staining of α-tubulin was used to visualize the spindles in fixed cells. Protein levels were analyzed by immunoblotting of whole cell extracts. We imaged *CDC28 IME2* and *cdc28-as ime2-as* strains, expressing neonGreen-tagged Sic1 to visualize Sic1 accumulation in the nucleus, and RFP-tagged SPB (Cnm67-tdTomato) **(Figure 11C)**. The appearance of meiosis I- spindles can be observed six hours after induction of meiosis in all strains. We treated cells with 1NM-PP1 or/and 1NA-PP1 when the majority of the cells were at metaphase I (t=7 hours in SPM). The inhibition of kinases resulted in the activation of Cdh1 and reaccumulation of Sic1 (**Figure 11A, 11C**). Cdh1 is active when it is dephosphorylated and it interacts with the APC/C (Zachariae et al., 1998). This may explain the degradation of various APC/C substrates, such as Dbf4, Cdc5, and Clb5. Sic1 is a stoichiometric inhibitor of Cdk1 in budding yeast and transcription of *SIC1* starts when cells are in the low kinase state (Schwob et al.,

1994; Knapp et al., 1996). Sic1 keeps a temporal window in G1 free of Clb5,6-Cdk1 activity, which is essential for origin licensing (Lengronne and Schwob, 2002). We concluded that the inhibition of Ime2 and Cdk1 kinase activity leads to a state similar to G1 phase and creates favorable conditions for the licensing of replication origins.

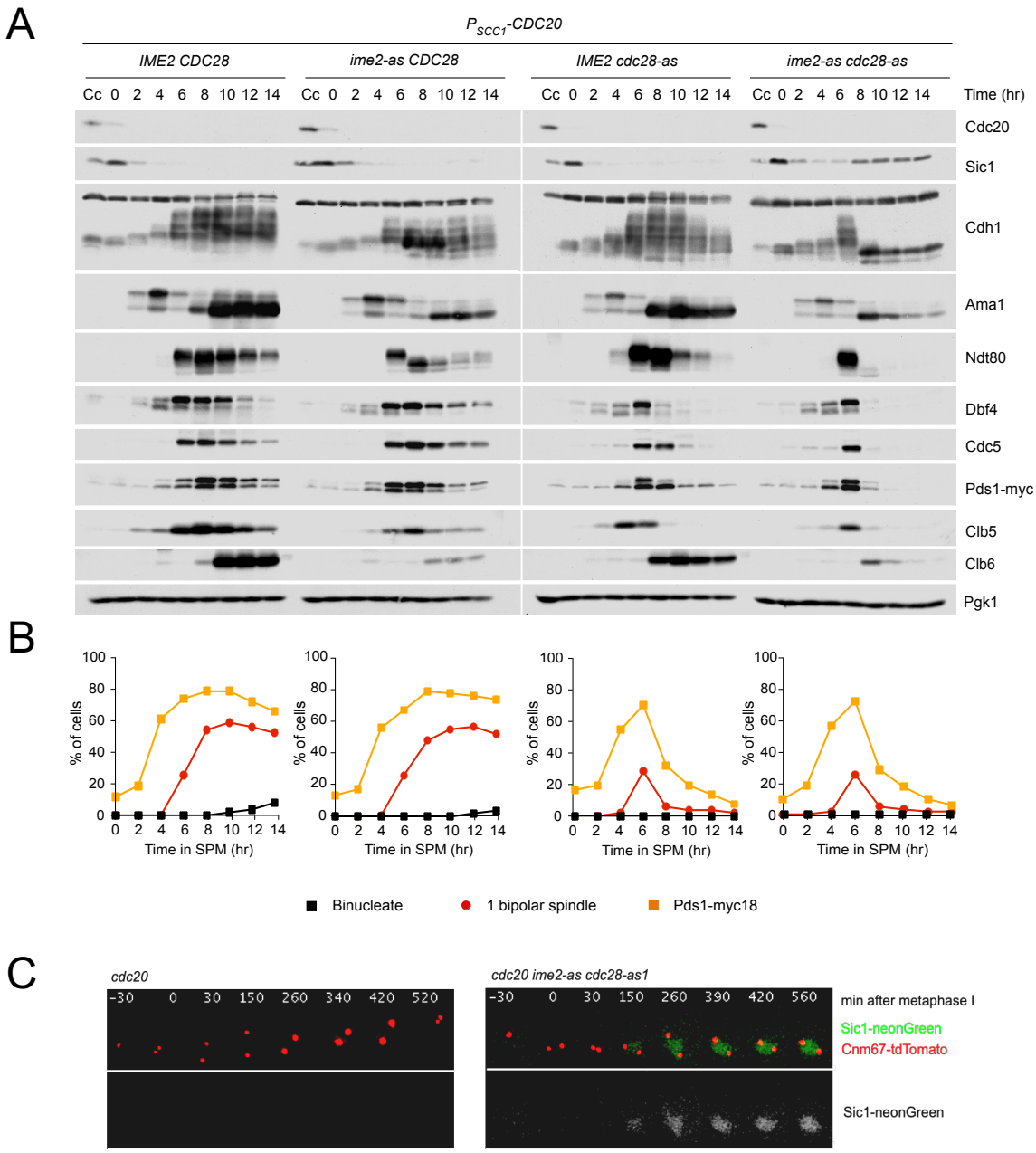


Figure 11. Inhibition of both Ime2-as and Cdc28-as causes accumulation of Sic1 and reactivation of Cdh1. (Legend on the next page)

2.3. DDK activity beyond anaphase I does not cause re-replication

Dbf4-dependent Cdc7 kinase (DDK) is known to be essential for the initiation of DNA replication (Sclafani, 2000). The kinase is activated upon binding of the catalytic Cdc7 subunit to the unstable Dbf4 protein. It associates with replication origins where it phosphorylates components of the pre-replicative complex, including the MCM helicase. During anaphase I, Dbf4 is marked for degradation by the APC/C- Cdc20. Unlike other APC/C substrates, Dbf4 does not reappear in meiosis II (Matos et al., 2008). This might be a perfect mechanism to prevent re-replication between MI and-MII. Thus, we wanted to keep Cdc7 active throughout meiosis and we used a non-degradable form of Dbf4 which has mutations in three D box motifs (*dbf4-3mDB-Ha3)* for this purpose. To explore how non-degradable Dbf4 affects Cdc7 during meiosis, we observed meiosis in living cells containing Cdc7 protein tagged with mCherry and a SPB component, Spc42, tagged with *GFP*. 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 all cells, expression of Cdc7-mCherry was detectable as a diffused nuclear signal until anaphase I (**Figure 12A**). In control cells, Cdc7-mCherry disappeared in anaphase I. In *dbf4-3mDB* cells, Cdc7 stayed in the nucleus until the end of meiosis, ~80 min longer (**Figure 12A, bottom panel**). Next, we wanted to analyze the DNA content by flow cytometry (**Figure 12B**). These data revealed similar kinetics between *DBF4* and *dbf4-3mDB* strains. We concluded that Cdc7 present active beyond anaphase I with non-degradable Dbf4 does not cause re-replication.

Figure 11. Inhibition of both Ime2-as and Cdc28-as causes the accumulation of Sic1 and the reactivation of Cdh1. P_{SCC1}-*CDC20* cells with *IME2 CDC28* (Z22789), *ime2-as* (Z22788), *cdc28-as* (Z22786), or *ime2-as cdc28-as* (Z22787) were induced to enter meiosis and samples were withdrawn every 2 hours. Cells were treated with 1NA-PP1 (20 μM) and 1NM-PP1 (5 μM) at 7 hr in SPM. (**A)** Immunoblot detection of protein levels along the time course. **(B)** Immunofluorescence detection of securin (Pds1-myc), bipolar spindles (α-tubulin) and of the number of nuclei (DAPI) were quantified. **(C)** Representative time-lapse series are displayed. P_{SCC1}-*CDC20* with *IME2 CDC28* (Z23972) and P_{SCC1}-*Cdc20 ime2-as cdc28-as* (Z23971) were treated with 1NA-PP1 (20 μM) and 1NM-PP1 (5 μM) at 7 hr in SPM. Imaging was started at 6 hr with 10 min per frame. Sic1 was tagged with neonGreen and Cnm67 with tdTomato.

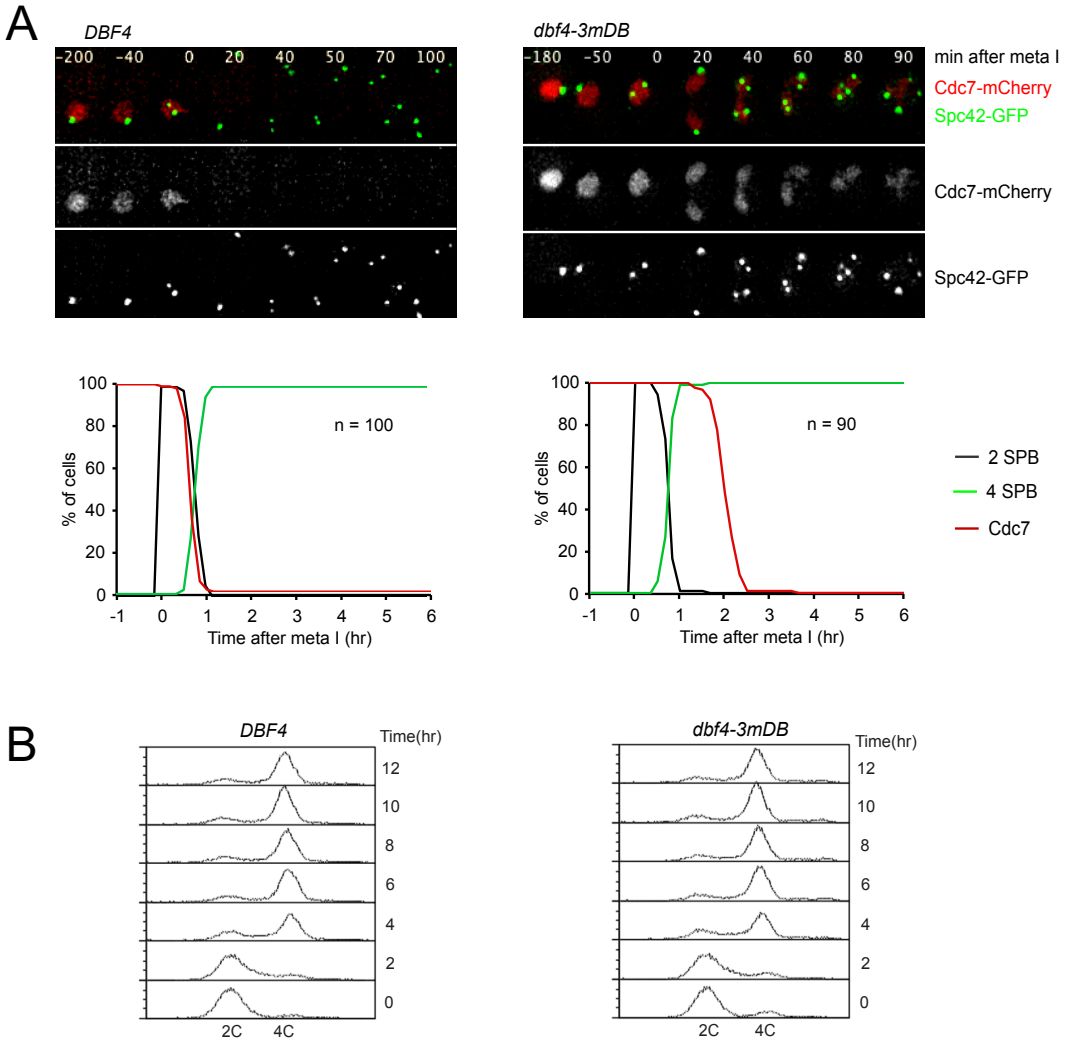


Figure 12. Dbf4-mDB recruits Cdc7 into nuclei. *dbf4-3mDB* mutant cells keep Cdc7 active after meiosis I. Isogenic control (Z34580) and *dbf4-3mDB* (Z34579) cells were induced to enter meiosis. Filming was started at 3 hr in SPM with 10 min per frame. Cdc7 was tagged with mCherry and Spc42 with GFP. **(A)** Representative time-lapse series are displayed on the top while the countings displayed on the bottom are synchronized to the formation of two SPBs. **(B)** Flow cytometric analysis of cellular DNA content.

2.4. Reactivation of Cdk1

Cdk1 is the master regulator of mitosis and meiosis with hundreds of potential substrates even in a unicellular eukaryote like budding yeast (Holt et al., 2009). In *Saccharomyces cerevisiae*, Clb5 and Clb6-associated kinases are important for promoting DNA synthesis since they phosphorylate a number of DNA replication factors (Ubersax et al., 2003). Cdk1-dependent phosphorylation of Sld2 and Sld3 are the key events known to be essential for the initiation of DNA replication in budding yeast (Zegerman et al., 2007; Tanaka et al., 2007). In our experimental setup, we inhibited kinases and observed the re-accumulation of the replication components. Here, we aimed to test the reactivation of the kinases in order to create oscillations in their activity similar to proliferating cells. It is well known that kinases are needed for origin firing (Labib, 2010). To this end, we used different strategies.

2.4.1. Strategy to inhibit and reactivate kinase: Cdc28-as2

For inhibition of Cdc28 experiments, we used the *cdc28-as1* allele [F88G], which is specifically inhibited by the ATP analog 1NM-PP1. Cell-permeable inhibitors that are selective for individual protein kinases allow the direct investigation of the kinases. This chemical-genetic approach holds several advantages over temperature-sensitive alleles. These inhibitors act quickly and reversibly (Bishop et al., 1999; 2000). First, we tested the restoration of Cdk1 activity by removal of the 1NM-PP1 by filtration for the *cdc28-as1*. As judged by immunofluorescence staining of tubulin, cells that were washed and resuspended in fresh medium without inhibitor did not assemble spindles efficiently, suggesting that Cdc28 activity was not restored (data not shown). To generate another analog-sensitive version of Cdc28, we exchanged phenylalanine in the ATP-binding cleft to alanine. We performed PCR-mediated site directed mutagenesis using genomic DNA from SK1 cells (which have a wild type *CDC28* gene) as a template to introduce the F88A mutation. We then transformed this mutant *cdc28-as2* allele into *cdc28-4* temperature-sensitive cells and select positive clones by selection for growth at elevated temperatures. Cells carrying this *cdc28-as2* mutation grew similar to cells containing a wild-type version of *CDC28*, indicating that Cdc28 function is not affected by the mutation *per se* (data not shown). However, they grew extremely slowly on medium containing the ATP analogues 1NA-PP1 or

1NM-PP1. To compare the inactivation and reactivation kinetics of *cdc28-as1* and *cdc28-as2*, we observed meiosis in individual living cells containing tubulin tagged with RFP and the meiosis-specific transcription factor, Ndt80, tagged with GFP (**Figure 13**). We also deleted *AMA1* in order to prevent the activation of APC/C mediated degradation system. We filmed P_{HSL1}-*CDC20 ama1*Δ, P_{HSL1}-*CDC20 ama1*Δ *cdc28-as1* and P_{HSL1}-*CDC20 ama1*Δ *cdc28-as2* strains. We added 1NA-PP1 seven hours after induction of meiosis and washed-away the inhibitor by filtration and resuspended the culture in fresh media, with or without inhibitor. As judged by live-cell imaging of tubulin, cells that were resuspended in the presence of 1NA-PP1 failed to assemble spindles until the end of the time course. By contrast, *cdc28-as2* cells that were washed and resuspended in medium without inhibitor assembled spindles very efficiently, suggesting that Cdc28 activity was restored (**Figure 13**). Ndt80-GFP was an internal control to count only meiotic cells. According to the countings of the live-cell imaging experiment, 60% of *cdc28-as2* cells accumulated spindles while only 25 % of *cdc28-as1* cells accumulated spindles (**Figure 13A and 13B, bottom panels**). We concluded that reactivation of Cdk1 activity by removal of the analogue is more efficient in *cdc28-as2* cells.

2.4.2. Depletion of Sic1 and Cdh1 restores Cdk1 activity

As already mentioned, inhibition of kinases resulted in the activation of Cdh1 and the re-accumulation of Sic1 (**Figure 11**). Even though we washed away the inhibitors, Sic1 degradation and Cdh1 phosphorylation were not fully efficient (data not shown). Cdk1 activation is necessary for origin firing during S phase and active Sic1 and Cdh1 are potential inhibitors of re-replication. B-type cyclins are marker for degradation by APC/C-Cdh1 (Visintin et al., 1997), and any remaining Cdk1 inhibitors (Sic1) can inhibit Cdk1 (Schwob E. et al.,1994). Thus, we depleted Sic1 and Cdh1 by changing their promoters to mitotic promoters *CLN1* and *HSL1*, respectively. First, we induced wild-type *SIC1* and P_{CLN1}-*SIC1* cells to enter meiosis (**Figure 14**). Accumulation of Ndt80 and Cdc5 occurred with similar kinetics in control and P_{CLN1}-*SIC1* cells. Immunofluorescence microscopy showed that even though the number of spindles and tetra-nucleate cells were lower in Sic1-depleted cells, the timings of these events were comparable (**Figure 14B**). Additionally, spore viability of P_{CLN1}-*SIC1* is 70 % while it is 100 % for control cells. We concluded that *CLN1* promoter can be used to deplete Sic1 in meiosis.

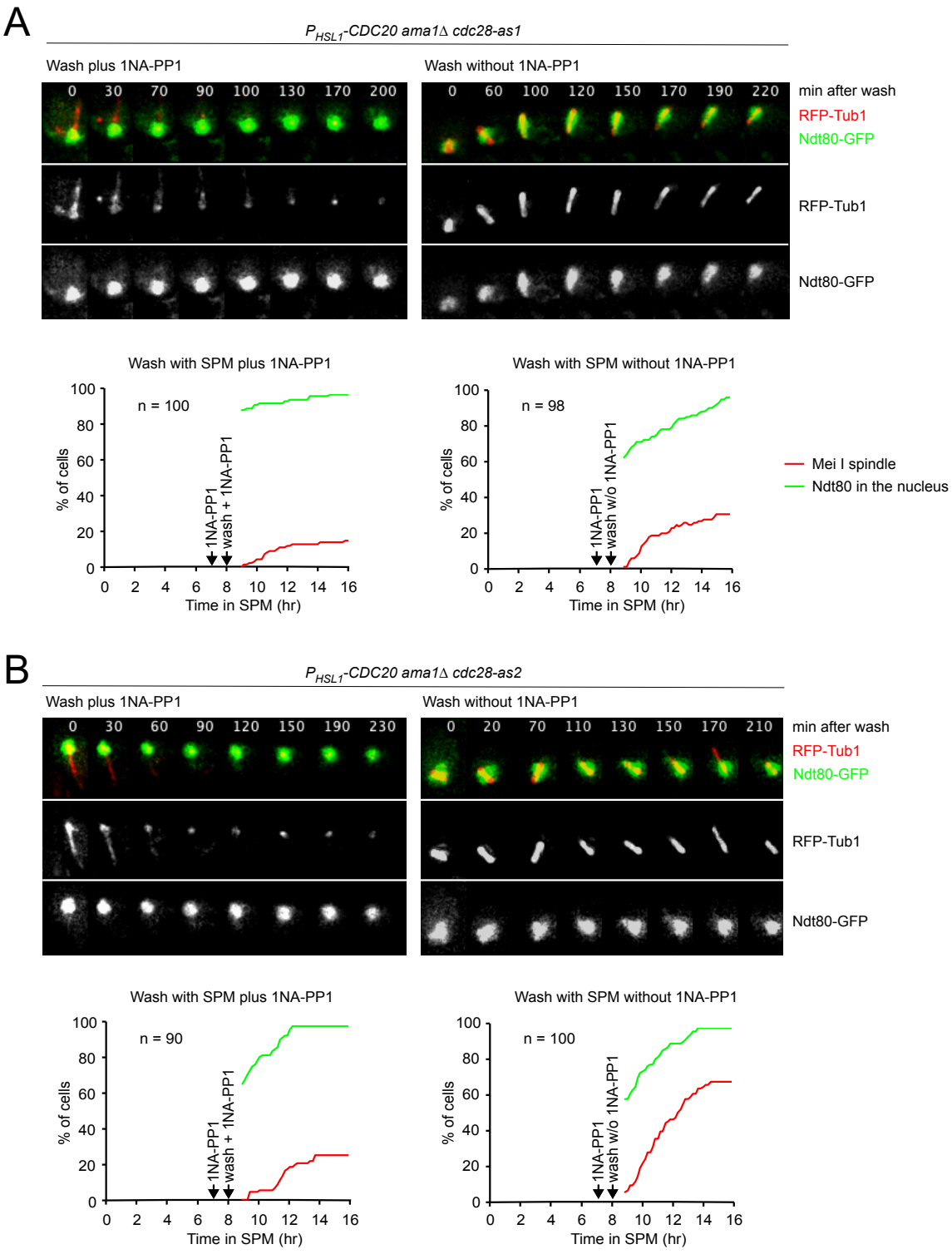


Figure 13. Washout comparison for *cdc28-as1* and *cdc28-as2*. (A) *P_{HSL1}-CDC20 ama1Δ cdc28-as1* (Z34131) and **(B)** *P_{HSL1}-CDC20 ama1Δ cdc28-as2* cells (Z32606) were treated with 1NA-PP1 (10 μM) at 7 hr in SPM. At 8 hr in SPM, cells were washed by filtration with conditioned SPM containing (10 μM, control) or lacking (washout) 1NA-PP1. RFP-Tub1 and Ndt80-GFP were imaged every 10 min starting from 9 hr in SPM. Representative time-lapse series are shown. Graphs display percentages of cells with nuclear Ndt80-GFP and spindles after washout.

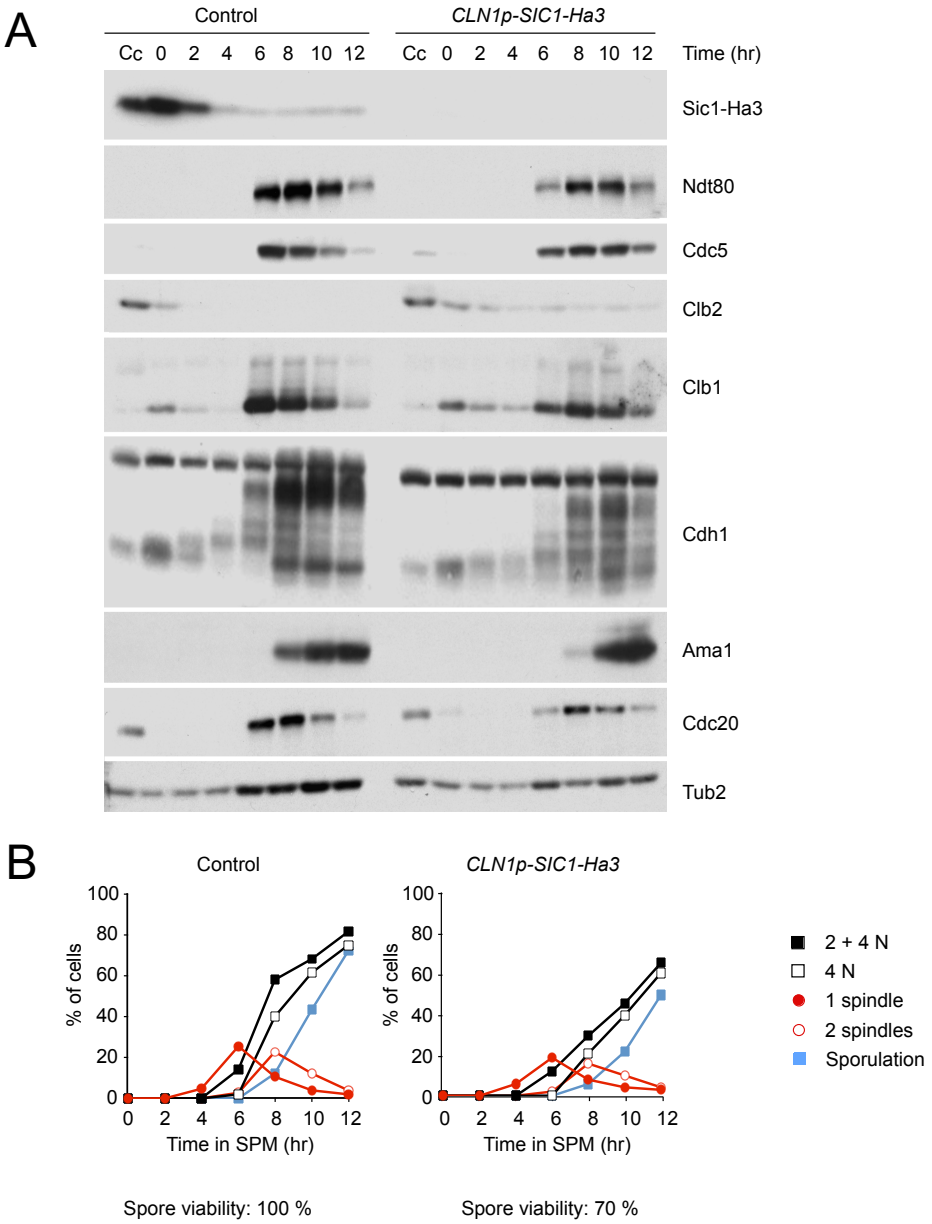


Figure 14. Depletion of Sic1 under CLN1 promoter. P_{CLN1}-*SIC1* cells (Z29132) and wild type control cells (Z29179) were induced to enter meiosis and samples were withdrawn every 2 hr. **(A)** Immunoblot detection of protein levels along the time course. **(B)** Immuno-fluorescence detection of bipolar spindles (α-tubulin), the number of nuclei (DAPI), and spore counting.

Similarly, we depleted Cdh1 in meiosis by replacing its promoter with the mitotic *HSL1* promoter (P_{HSL1}-*CDH1*). This prompted us to ask whether the depletion of Sic1 and Cdh1 could help to initiate re-replication together with inactivation and reactivation of the kinases Cdc28 and Ime2. We induced meiosis in cells lacking three APC/C activators, Cdc20, Ama1, and Cdh1 (called *APC*off) in order to prevent the activation of degradation mechanism. We induced *APCoff* P_{CLN1}-*SIC1 dbf4-3mDB ime2-as cdc28-as2 cells* to enter meiosis and collected samples every two hours. Immunofluorescence staining of α-tubulin was used to visualize the spindles and Cdc14 was used to display its nucleoplasmic/nucleolar localization in fixed cells **(Figure 15B)**. The Cdc14 phosphatase activity is tightly regulated by its cellular localization. Cdc14 is released from the nucleolus in early anaphase and recaptured in late anaphase during both meiosis I and II (Marston et al., 2003). Once released, it directly targets Cdk1 substrates and de-phosphorylates them. Protein levels were analyzed by immunoblotting of whole cell extracts **(Figure 15A)**. We added 1NA-PP1 seven hours later, when the majority of cells in both cultures were in metaphase I. We observed that cells loose spindles and Ndt80, suggesting that Cdc28 and Ime2 were inhibited. 2 hours after inhibition, we washed-away the inhibitor by filtration and resuspended cells in fresh medium, with or without inhibitor. As judged by immunofluorescence staining of tubulin and Cdc14, and immunoblotting of Ndt80, cells that were resuspended in the presence of 1NA-PP1 remained without spindles, Cdc14 released and Ndt80 degraded. By contrast, cells that were washed and resuspended in media without inhibitor re-assembled spindles and re-synthesized Ndt80, suggesting that Cdk1 and Ime2 activity was restored, respectively. In both cultures, Dbf4, Clb5, Clb6, and Cdc5 persisted until the end of the experiment. Next, we used flow cytometry to measure cellular DNA content (**Figure 15C**). However, we did not observe re-replication. This might be due to the active Cdc14 phosphatase. As judged by immunofluorescence staining, cells released Cdc14 upon inhibition. However, Cdc14 recapture was not efficient after washout.

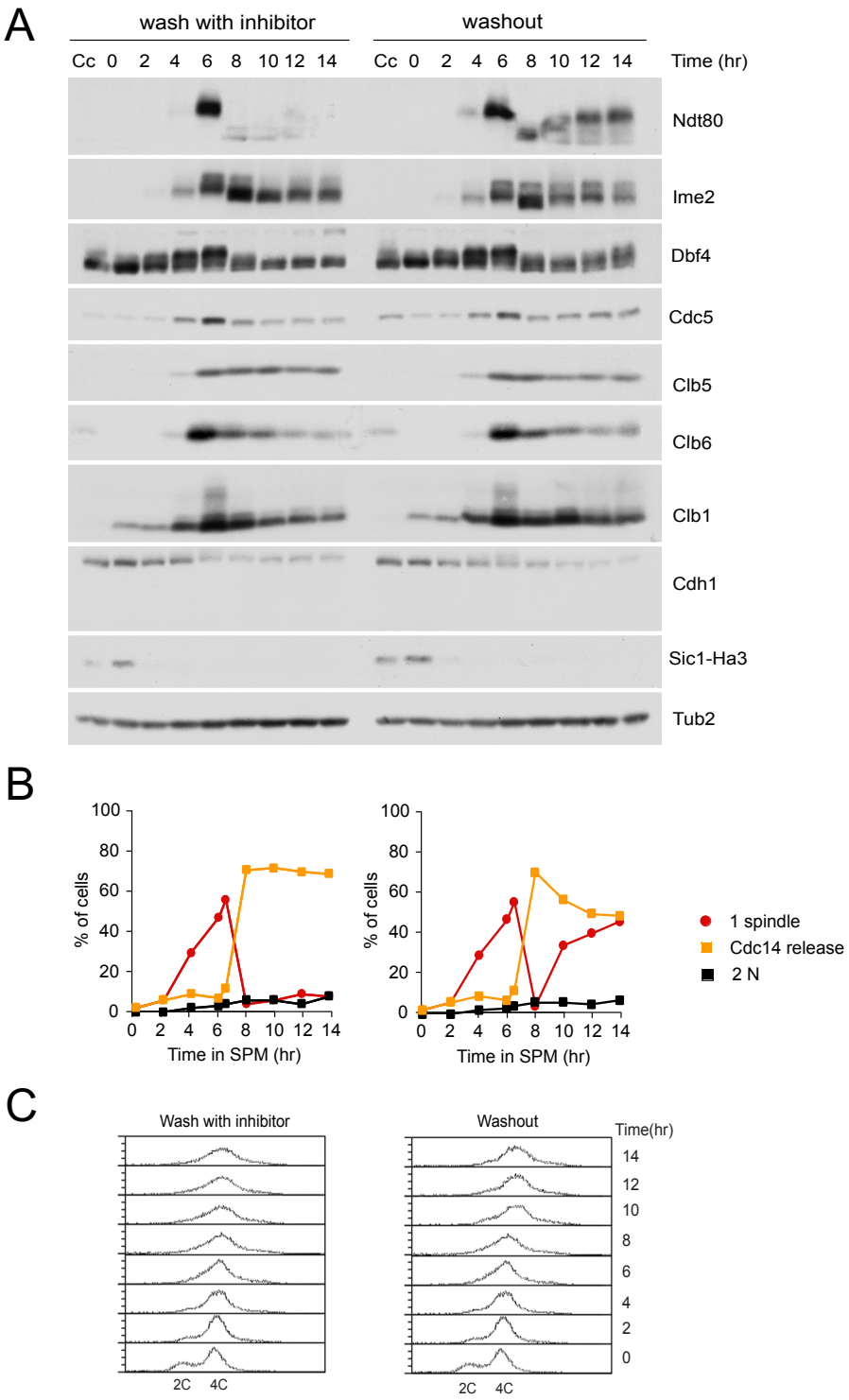


Figure 15. Inhibition and re-activation of both Ime2-as and Cdc28-as2. *APCoff* P_{CLN1}-*SIC1 dbf4-3mDB ime2-as cdc28-as2 cells* (Z29602) were treated with 1NA-PP1 (20 μM) at 7 hr in SPM. At 9 hr in SPM, cells were washed by filtration with conditioned SPM containing (20 μM, control) or lacking (washout) 1NA-PP1. **(A)** Immunoblot detection of protein levels along the time course. **(B)** Immuno-fluorescence detection of Cdc14, bipolar spindles (α-tubulin) and the number of nuclei (DAPI). (**C**) The DNA content of cells stained with propidium iodide.

2.4.3. Deletion of *SWE1* restores Cdk1 activity

In order to trigger a new round of DNA replication, Cdk1 and Ime2 activity must first be reduced to a very low value so that origins of replication can be licensed. Then, kinase activity must increase sufficiently to phosphorylate licensed origins, causing them to begin the replication process. Regulation of Cdk1 activity involves several separate components. Cdk1 is also inhibited by phosphorylation on its tyrosine 19 residue by Swe1 (homolog of Wee1) (Lim et al., 1996; Sia et al., 1996). The Swe1-mediated inhibitory phosphorylation of Tyr19 of Cdc28 is reversed by the tyrosine phosphatase Mih1 (homolog of Cdc25) in order to promote entry into mitosis (Russell et al., 1989). The conserved network of Wee1, Cdc25, and Cdk1 is a simple example of bistability (Domingo-Sananes et al., 2011). On the one hand, there is mutual inhibition between Cdk1 and Wee1, creating a double negative feedback loop. On the other hand, the mutual activation between Cdk1 and the phosphatase Cdc25 creates a positive feedback loop. In order to move our system from the low kinase state to the high kinase state, we deleted *SWE1*. We induced *APCoff swe1Δ ime2-as cdc28-as2* cells to enter meiosis and collected samples every two hours. Immunofluorescence staining of α-tubulin and Cdc14 was used to visualize the spindles and release of the Cdc14 phosphatase from the nucleolus in fixed cells, respectively **(Figure 16B)**. Protein levels were analyzed by immunoblotting of whole cell extracts **(Figure 16A)**. Flow cytometry analysis was used to measure cellular DNA contents (**Figure 16C**). We added 1NA-PP1 seven hours later, when the majority of cells were at metaphase I. Two hours after inhibition, we washed-away the inhibitor by filtration and resuspended cells in fresh medium, with or without inhibitor. Upon addition of 1NA-PP1, Ndt80 lost its phosphorylation, spindles disassembled, and Cdc14 was released indicating the low kinase state. As judged by immunofluorescence staining of α-tubulin and Cdc14, and immunoblotting of Ndt80, cells that were washed and resuspended in media without inhibitor assembled spindles and re-synthesized Ndt80, suggesting that Cdk1 and Ime2 activity was restored. In both cultures, Dbf4, Clb5, and Cdc5 persisted until the end of the experiment. However, Cdc14 recapture was not efficient after washout. We did not observe re-replication. This might be due to the active Cdc14 which make low kinase state more stable even though *SWE1* is deleted.

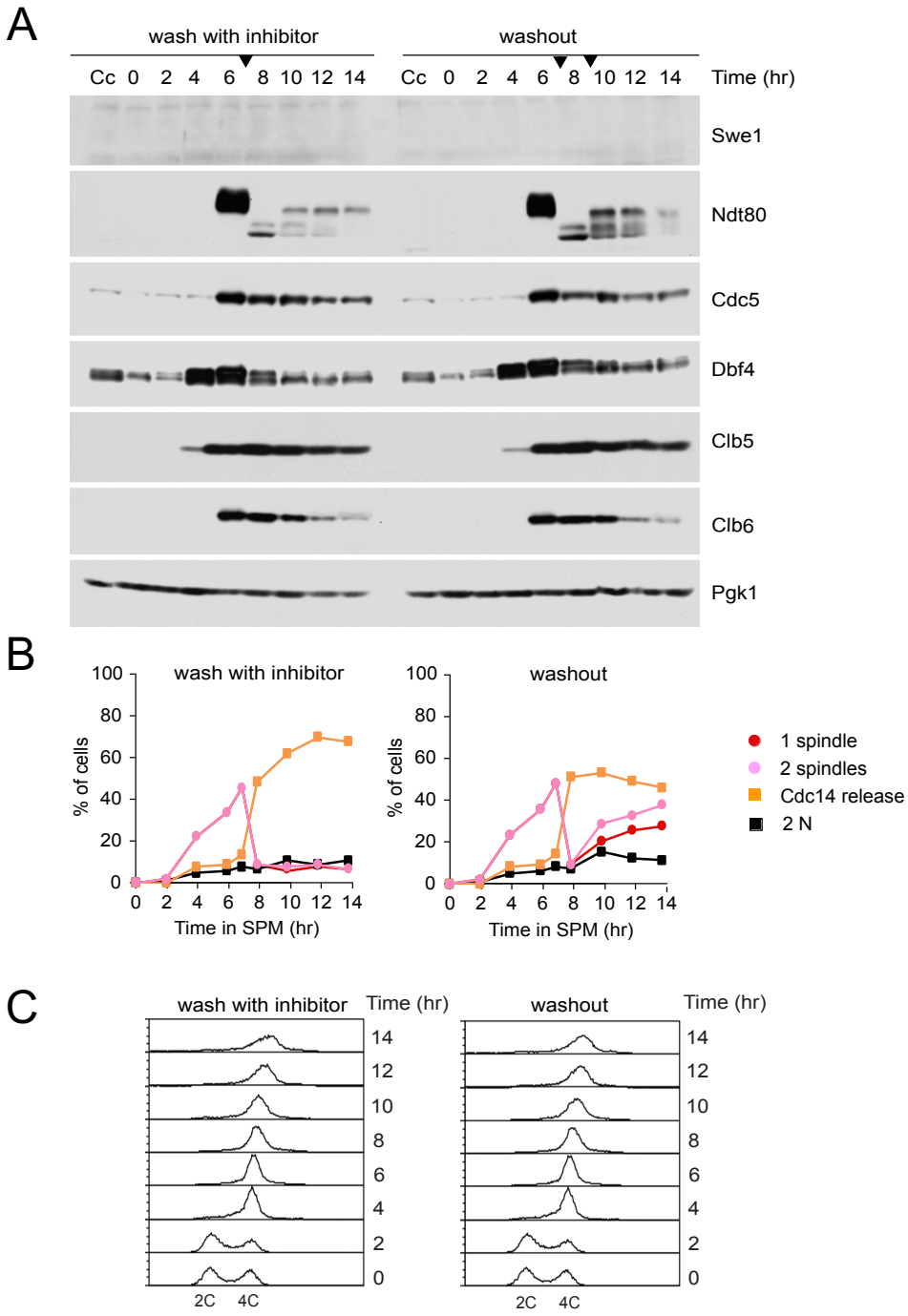


Figure 16. Inhibition and re-activation of both Ime2-as and Cdc28-as2 in *swe1Δ* cells. *APCoff swe1Δ ime2-as cdc28-as2 cells* (Z34212) were treated with 1NA-PP1 (20 μM) at 7 hr in SPM. At 9 hr in SPM, cells were washed by filtration with conditioned SPM containing (20 μM, control) or lacking (washout) 1NA-PP1. **(A)** Immunoblot detection of protein levels along the time course. **(B)** Immunofluorescence detection of Cdc14, bipolar spindles (α-tubulin) and the number of nuclei (DAPI). (**C**) The cellular DNA content of cells stained with propidium iodide.

2.5. Degradation of the Cdc14 phosphatase does not cause re-replication between meiotic divisions

Cdc14 is a highly conserved, dual-specificity phosphatase responsible for the dephosphorylation of many substrates in mitosis and meiosis. Cdc14 dephosphorylates Cdh1, the late-mitosis APC/C activator, thereby activating it (Visintin et al., 1998; Zachariae et al., 1998; Jaspersen et al., 1999). This results in the proteolysis of B type cyclins, antagonizing Clb-Cdk1 activity. Cdc14 also dephosphorylates Sic1, the mitotic Cdk1 inhibitor, and Swi5, the Sic1 transcription factor. These events result in the stabilisation and accumulation of Sic1 in cells, leading to complete Clb-Cdk1 inhibition (Knapp et al., 1996; Toyn et al., 1997; Visintin et al., 1998). Furthermore, Cdc14 opposes Clb-Cdk1 activity by reversing the phosphorylation of many Cdk1 targets (Bloom et al., 2011). Replication factors, such as Sld2, Dbp2, Pol12, Cdc6, Orc2 and Orc6, were identified as possible Cdc14 substrates (Bloom and Cross, 2007; Jin et al., 2008, Zhai et al., 2010). In our experiments, we found that Cdc14 was released from nucleolus upon inhibition of Cdc28 and Ime2 (**Figure 15B, 16B**). Cdc14 stayed released even after re-activation of the kinases. Cdc14, the Cdk-counteracting phosphatase, could set a threshold that Cdk activity must overcome to achieve net substrate phosphorylation (Bouchoux and Uhlmann, 2011). We wanted to elucidiate the effect of Cdc14 inactivation on re-replication. To this end, we used an auxin-inducible degron (AID) system to degrade Cdc14. The AID system allows rapid and efficient depletion of target proteins in response to auxin and enables the generation of conditional mutants of essential proteins in yeast (Nishimura et al. 2009). Auxin represents a family of plant hormones that control gene expression during growth and development. Auxin family hormones, such as indole-3-acetic acid (IAA; a natural auxin), bind to the F-box transport inhibitor response 1 (TIR1) protein and promote the interaction of the ubiquitin ligase SCF-TIR1 (a form of SCF complex containing TIR1) and the auxin or IAA (AUX/IAA) transcription repressors. SCF-TIR1 recruits a ubiquitin-conjugating enzyme that then polyubiquitylates AUX/IAAs resulting in rapid degradation by the proteasome (Dharmasiri et al., 2005; Kepinski et al., 2005; Tan et al., 2007; Nishimura et al., 2009). We tagged Cdc14 with AID on C-terminus. In the absence of auxin, the growth rate of *CDC14-AID* cells was indistinguishable from that of wild-type cells. Consistent with the essential Cdc14 function, *CDC14-AID* cells

failed to grow on medium containing IAA. Next, we tested *CDC14-AID* in our kinase inhibition and re-activation system. We induced *APCoff* P_{CUP1}*-OsTIR1 cdc14-AID ime2-as cdc28-as2 cells* to enter meiosis. We collected samples every two hours for immunoblotting of whole cell extracts and FACS analysis **(Figure 17 A, B)**. We added 1NA-PP1 seven hours later, when the majority of cells was in metaphase I. One hour after inhibition, we washed-away the inhibitor by filtration and resuspended the cells in fresh medium, with or without inhibitor. To one of the wash-out cultures, we added CuSO4 and IAA to deplete Cdc14 after wash-out since Cdc14 is needed for the dephosphorylation of replication proteins as well. We observed that Cdc14 was efficiently depleted one hour after wash-out and Dbf4, Cdc5, Clb5, and Clb6 stayed until the end of the experiment. However, cells did not re-replicate even after depletion of Cdc14 as judged by the flow cytometry.

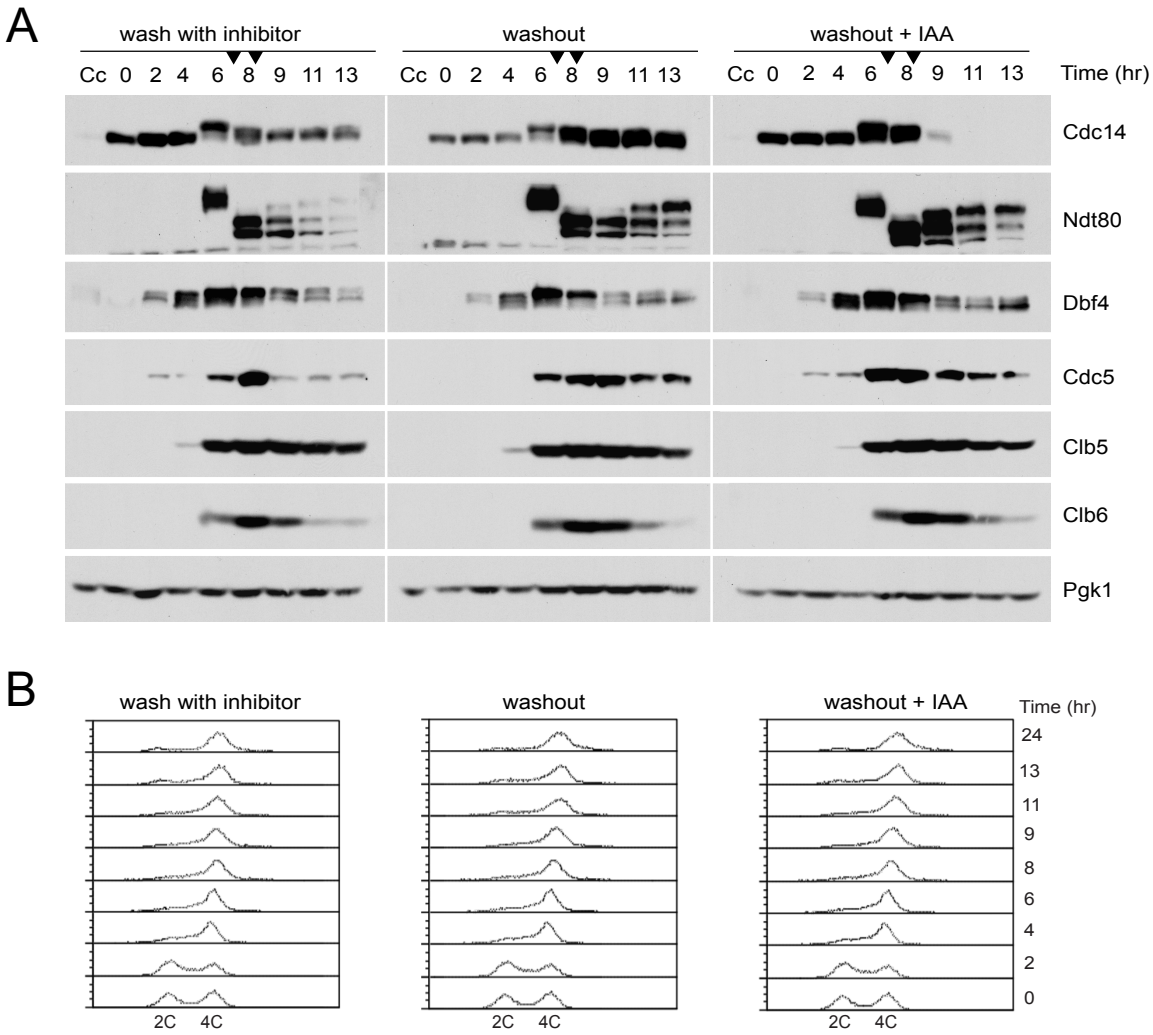


Figure 17. Degradation of Cdc14 after inhibition and reactivation of Ime2-as and Cdc28-as2 does not promote re-replication. *APCoff cdc14-AID ime2-as cdc28-as2* cells (Z33412) were treated with 1NA-PP1 (20 μM) at 7 hr in SPM. At 8 hr in SPM, cells were washed by filtration with conditioned SPM containing (20 μM, control) or lacking (washout) 1NA-PP1. Immediately after washout, 10 μM CuSO4 and 2 mM IAA was added. **(A)** Immunoblot detection of protein levels along the time course. (**B**) The cellular DNA content of cells stained with propidium iodide.

2.6. Restoring Ime2 activity after kinase inhibition is not enough to induce re-replication

Ime2 is a conserved protein kinase involved in the regulation of various key events in meiosis, such as the initiation of DNA replication, the expression of meiosis-specific genes and sporulation (Irniger et al., 2011). In order to understand how Ime2 can promote re-replication, we first tested the sole effect of Ime2 between MI and MII. To manipulate cells between meiotic divisions, it was necessary to synchronize cultures undergoing meiosis. To this end, we used the

cdc20-3-meiotic-arrest/release (*cdc20-3-mAR*) system. It is a modified version of the *CDC20-mAR* system (Arguello-Miranda et al., 2017). In the *cdc20-3-mAR* system, the endogenous *CDC20* gene is placed under control of the mitosis-specific *CLB2* promoter, and an additional copy of temperature-sensitive *CDC20* is expressed by the copper-inducible *CUP1* promoter. After induction of meiosis, *CDC20-mAR* cells arrest at metaphase I due to the lack of Cdc20. Addition of $CuSO_4$ induces Cdc20 and results in cells progressing synchronously through anaphase I, metaphase II, and anaphase II within 120 minutes. With the manipulation of *cdc20-3*, we can inactivate or reactivate Cdc20 by shifting the temperature (Katarzyna Jonak, unpublished). Because of the degradation of cyclins by APC/C-Cdc20, Cdk1 is inactivated. We combined the *cdc20-3-mAR* system with an Ime2-as allele. We induced the cultures to enter meiosis, and released cells from the metaphase I-arrest by adding $CuSO_4$. We added 1NA-PP1 50 min later, when the majority of cells in both cultures were in anaphase I. We expected that cells would enter the low kinase state. Then, 60 min later, we washed away the ATP analog to reactivate Ime2-as and shifted the temperature to inactivate Cdc20, meaning that we re-arrested the cells. We collected samples for immunoblot detection of whole cell extracts and FACS analysis of DNA content (**Figure 18**). We found that inhibition and reactivation of Ime2 resulted in the dephosphorylation and re-phosphorylation of Ndt80. Strikingly, Clb5 was degraded upon Cdc20 release and reaccumulated upon deactivation of *cdc20-3* by temperature shift, indicating oscillations in Cdc28 activity (**Figure 18A**). Next, we analyzed cellular DNA content by flow cytometry and showed that cells mostly completed premeiotic DNA replication within 3 hours. Inactivation and reactivation of Ime2-as did not lead to an increase in the DNA content of the washed-out strain (**Figure 18B**). Here, Cdk1 was inactivated by APC/C-Cdc20 activation, which degrades the cyclins. However, it is not certain that APC/C-Cdc20 can inhibit Cdc28 fully between meiosis I and II. This experiment supports the idea of partial inactivation of Cdk1 during the MI-MII transition in budding yeast. Additionally, Ime2 is a highly unstable protein (Sari et al., 2008) and it might not be fully active even after wash-out of cultures. We aimed to elucidate the relevance of Ime2 instability and the partial inhibition of Cdc28. We decided to use *cdc28-as2* and *ime2-as* alleles combined with a highly active version of *IME2* under an inducible promoter. It was previously shown that the C-terminal

domain of Ime2 is important for mediating protein instability and deletion of a 241 amino acid C-terminal region resulted in a highly stabilized protein. The stabilized, truncated Ime2 protein is highly active *in vivo* (Sari et al. 2008). To control *IME2ΔC* expression, we used the *GAL* promoter and a chimeric transcription factor consisting of the DNA-binding domain of Gal4 and the hormone-binding domain from the human estrogen receptor (Gal4-ER) (Benjamin et al., 2003). We induced *APC-off cdc28-as2* and *ime2-as* cells expressing the truncated *IME2ΔC* from the inducible *GAL1* promoter (P_{GAL1}-*IME2ΔC)* to synchronously enter meiosis. We collected samples every two hours for indirect immunofluorescence staining of tubulin, Cdc14 and DAPI, for immunoblot detection of whole cell extracts, and for FACS analysis of DNA content until the end of experiment (t=12 hr) **(Figure 19)**. As soon as cultures synchronously entered metaphase I (t=7 hr), we added 1NA-PP1 to inhibit Ime2-as and Cdc28-as. Then, we washed-away the inhibitor by filtration and resuspended the culture in fresh medium, with or without inhibitor two hours after inhibition (t=9 hr). For the wash-out cultures, we added Estradiol (5 μM) just after to induce the active, stabilized form of Ime2. The *APCoff* P_{GAL}*- ime2ΔC cdc28-as2 ime2-as* cells entered meiosis with normal kinetics. Upon addition of 1NA-PP1, Ndt80 lost its phosphorylation, spindles disassembled, and Cdc14 was released from nucleolus indicating the low kinase state. Washing-away the inhibitors triggered the re-accumulation of spindles and addition of β-estradiol triggered the accumulation of Ime2 and efficient re-appearance of Ndt80 (**Figure 19A, B**). Immunofluorescence microscopy of fixed cells demonstrated that Cdc14 was released upon inhibition but not fully recaptured after wash out (**Figure 19B**). Western blot analysis of whole cell extracts showed that in both cultures, cyclins and Cdc5 protein levels remained constant. Nevertheless, we did not observe DNA re-replication after inhibition and reactivation of the kinases. It indicates that inducing active and stable Ime2 is not sufficient to promote re-replication. Thus, there must be other mechanisms inhibiting re-replication.

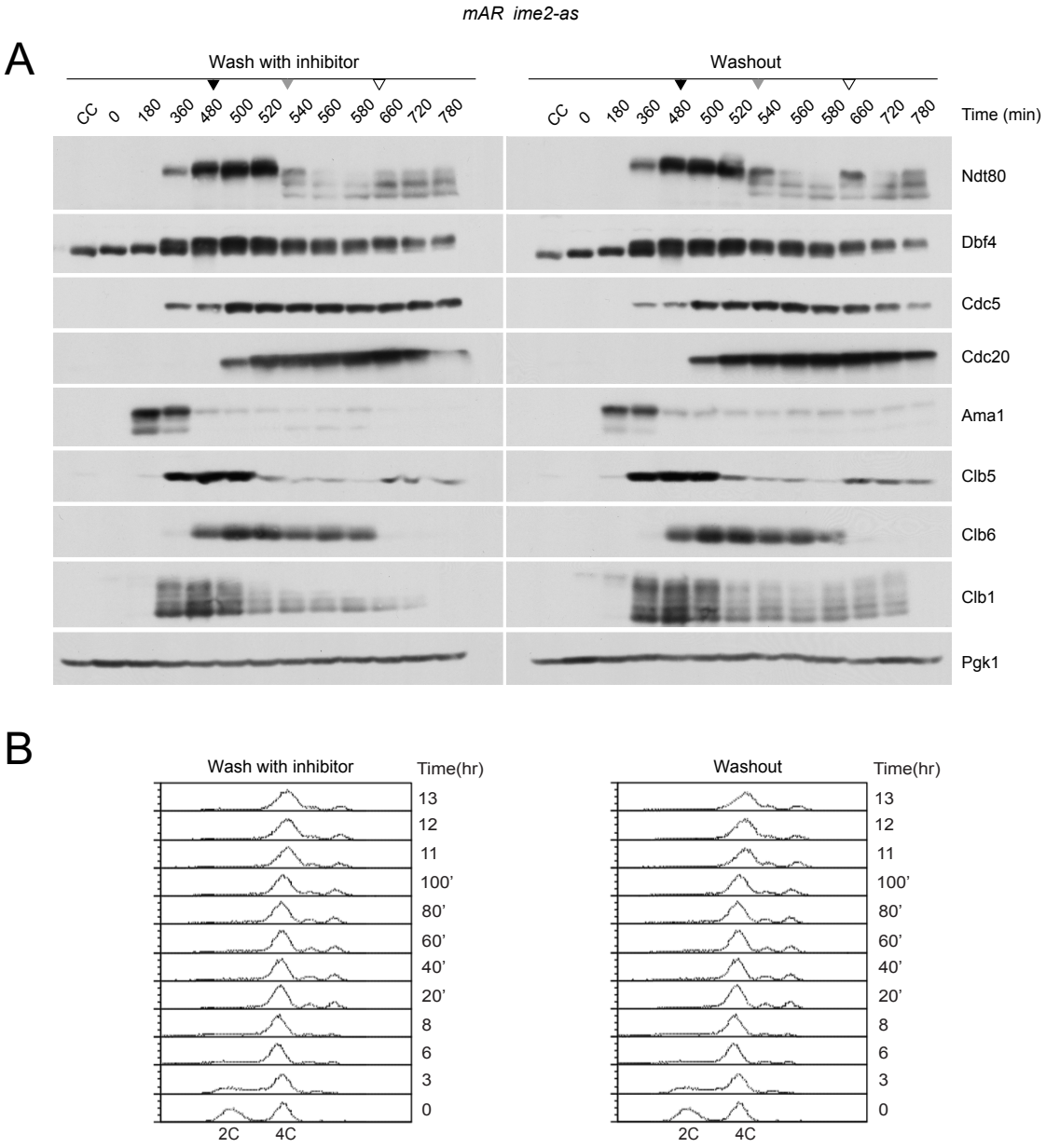


Figure 18. Ime2 alone is not sufficient to promote re-replication. Meiotic time course of *ime2-as cdc20-3-mAR dbf4-3mDB APCoff* (Z33678) strains. Cells were released into anaphase I with CuSO4 (10 μM, 480 min), treated with 1NA-PP1 (20 μM, 530 min) and washed away with 10 volumes of conditioned SPM with (20 μM) or without 1NA-PP1 and the temperature was switched to 36.5 °C (590 min). **(A)** Immunoblot detection of protein levels along the time course. (**B**) The cellular DNA content of cells stained with propidium iodide.

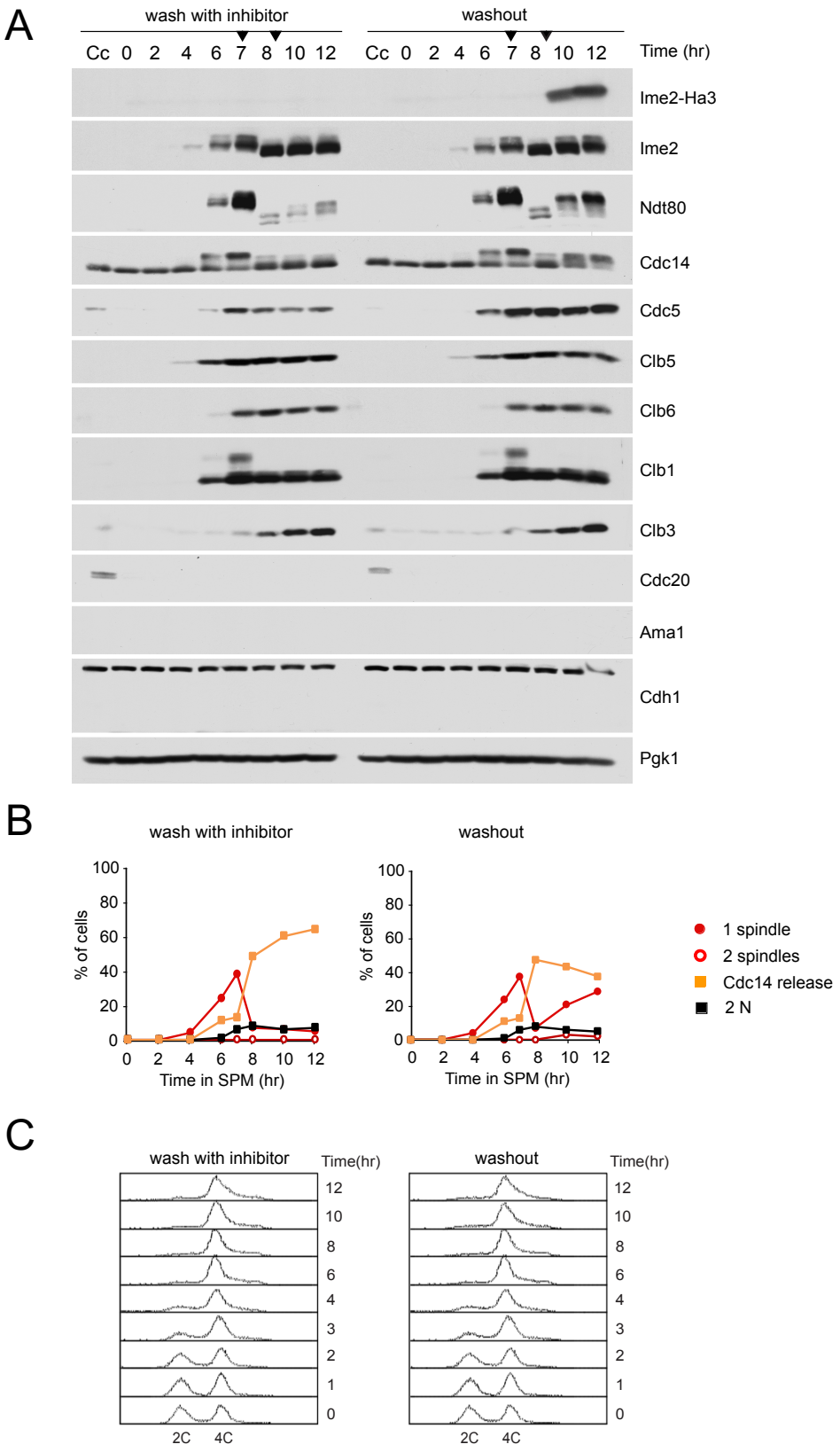


Figure 19. Expression of Ime2-ΔC after washout produced active and stable Ime2.
(Legend on the next page)

2.7. The polo-like kinase Cdc5 does not affect re-replication

The data collected so far showed that Cdc5 stayed at constant levels in our wash-out cells. Therefore, we asked whether Cdc5 can inhibit re-replication. We considered the possibility that downstream steps of DNA replication could also be inhibited during the meiotic divisions. Despite pre-RC components accumulated upon inhibition of Cdk1 and Ime2, we could not trigger the activation of the helicase. Most helicase-activation proteins are present throughout the meiotic divisions, including Cdc45, Psf2 (a member of the GINS complex), Sld3, and Dpb11. However, Sld2 is degraded upon entry into MI and did not reaccumulate until the completion of MII (Phizicky et al., 2018). Sld2 is essential for replication initiation (Kamimura et al., 1998; Yeeles et al., 2015) and its degradation represents a robust mechanism to inhibit helicase activation. Recent studies showed that both Cdk1 and the polo-like kinase Cdc5 were candidates for regulating meiotic Sld2 protein levels (Reusswig et al., 2016; Phizicky et al., 2018). In order to investigate whether the kinase activity of Cdc5 has an effect on inhibition of re-replication, we used an allele of *CDC5*, which has a catalytic site mutated in a way that makes it accessible for the small-molecule inhibitor CMK (pyrrolopyrimidine chloromethylketone). CMK resembles the ATP molecule, which enables it to enter the catalytic site of the analog-sensitive mutant of Cdc5 (called *cdc5-as*) and to bind a reactive cysteine residue covalently. CMK does not affect the wild-type protein because it cannot enter the ATP-binding cleft (Snead et al., 2007). To observe the effect of the inhibition of Cdc5's activity, we induced *APCoff ime2-as cdc28-as2 cdc5-as* cells to undergo synchronous meiosis. When the cultures entered metaphase I (t=8 hr), we added 1NA-PP1 and washed-away the inhibitor two hours later (t=10 hr). Next, we inactivated Cdc5-as by adding CMK (t=12 hr).

Figure 19. Expression of Ime2-ΔC after washout produced active and stable Ime2. *APCoff* P_{GAL}*- ime2ΔC cdc28-as2 ime2-as* cells (Z34142) were treated with 1NA-PP1 (20 μM) at 7 hr in SPM. At 9 hr in SPM, cells were washed by filtration with conditioned SPM containing (20 μM, control) or lacking (washout) 1NA-PP1. Immediately after washout, 5 μM estradiol was added for washed-out culture. **(A)** Immunoblot detection of protein levels along the time course. **(B)** Immunofluorescence detections of tubulin (spindles), Cdc14 and DAPI **(C)** The DNA content of cells stained with propidium iodide.

Protein levels were analyzed by immunoblotting of whole cell extracts **(Figure 20A)**. Immunofluorescence microscopy was used to visualize spindles and Cdc14 **(Figure 20B)**. Flow cytometry analysis was used to measure DNA contents (**Figure 20C**). This strain entered meiosis with wild-type kinetics as judged from accumulation of proteins and spindles. After we added 1NA-PP1, cells lost spindles and the phosphorylation of Ndt80 and released Cdc14, indicating the inactivation of Cdc28 and Ime2. After we washed away the inhibitor, we observed the re-appearance of Ndt80 phosphorylation. Since the inhibitors to inactive Cdc28-as2, Ime2as and Cdc5-as are different, we could inhibit these kinases at different times. As expected, inactivation of Cdc5-as resulted in the loss of phosphorylation of Ndt80 and Ime2. However, Sic1 also accumulated upon the inactivation of Ime2 and Cdc28, and degradation of Sic1 was not efficient. As already mentioned in the previous chapters, Sic1 is a strong inhibitor of Cdk1 and this could negatively regulate the activation of re-replication.

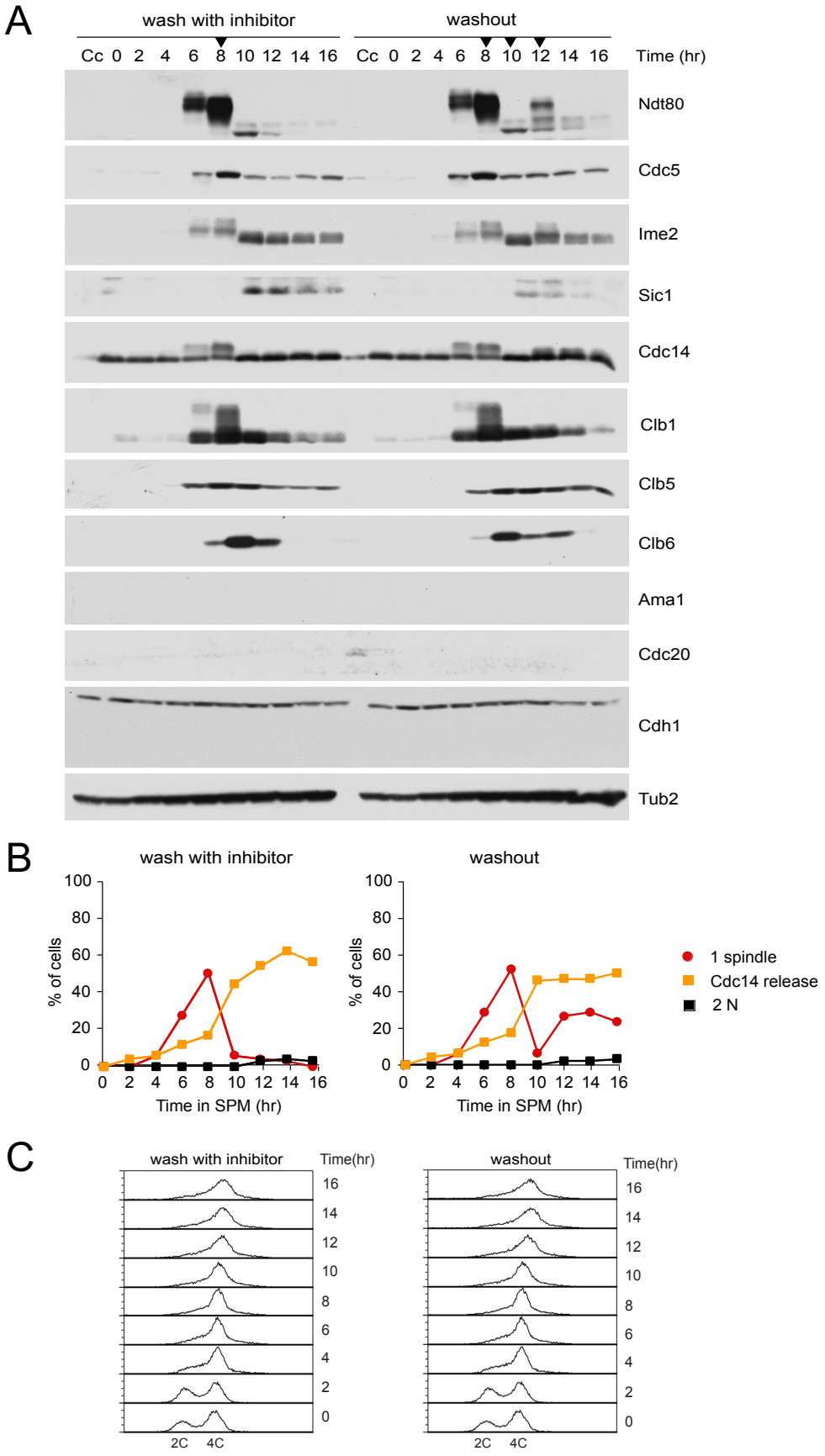


Figure 20. Inhibition of Cdc5-as after wash-out does not promote re-replication. (Legend on the next page).

In budding yeast, Sld2 and Sld3 are the minimal set of Cdk1 targets required for DNA replication. S-Cdk1 phosphorylation of one residue of Sld2, T84, is critical for the interaction between Sld2 and Dpb11. A phosphomimetic mutant Sld2-T84D can productively bind to Dpb11 *in vivo* (Tanaka et al., 2007; Zegerman and Diffley, 2007). We observed that Sld2 was degraded upon entry into Metaphase I (data not shown). Thus, we tested the effect of overexpression of Sld2 in our system. We introduced phospho-mimicking *SLD2-T84D* as a second copy expressed from the inducible *GAL* promoter in our strains. We induced *APCoff ime2-as cdc28-as2* P_{GAL}*-Sld2-T84D* cells to undergo synchronous meiosis. When yeast cultures synchronously entered metaphase I (t=8 hr), we added 1NA-PP1 and washed-away the inhibitor two hours after inhibition (t=10 hr). Next, we expressed Sld2 by adding estradiol. Protein levels were analyzed by immunobloting of whole cell extracts **(Figure 21A).** Flow cytometry analysis was used to measure cellular DNA content (**Figure 21B**). Immunoblot analysis showed that Sld2 was highly expressed in the inhibited and washout cultures. Phosphorylation of Ndt80 was recovered for the washout culture indicating the activities of Ime2 and S-phase Cdk1 were present until the end of the experiment. However, overexpression of *Sld2-T84D* did not result in re-replication after inactivation and reactivation of the kinases. We conclude that there should be an upstream mechanism besides Cdc5 and Sld2 for preventing re-replication.

Figure 20. Inhibition of Cdc5-as after wash-out does not promote re-replication. *APCoff cdc5-as ime2-as cdc28-as2* cells (Z31937) were treated with 1NA-PP1 (20 μM) at 8 hr in SPM. At 10 hr in SPM, cells were washed by filtration with conditioned SPM containing (20 μM, control) or lacking (washout) 1NA-PP1. At 12 hr in SPM, cells were treated with CMK (10 μM). **(A)** Immunoblot detection of protein levels along the time course. **(B)** Immunofluorescence detections of tubulin (spindles), Cdc14 and DAPI. **(C)** Flow cytometry analysis of cellular DNA content of cells stained with propidium iodide.

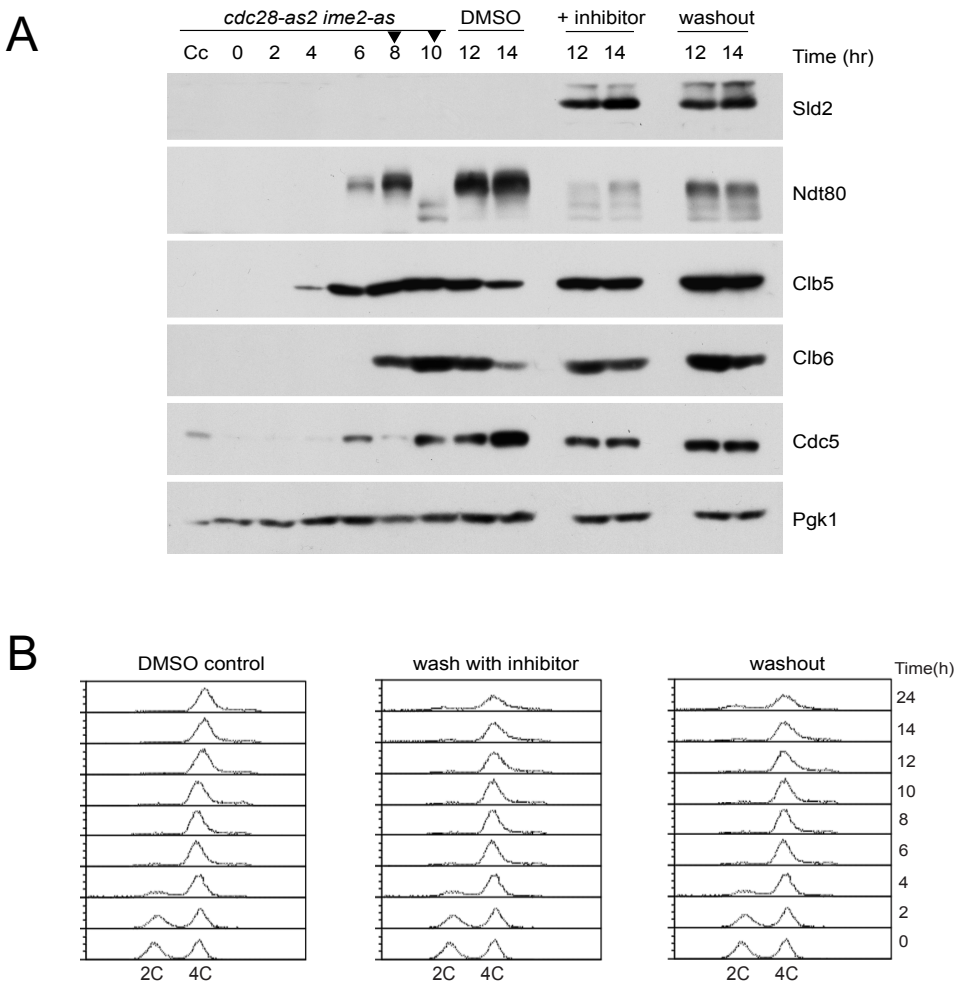


Figure 21. Overexpression of phosphomimic Sld2 after wash-out does not promote re-replication. *APCoff ime2-as cdc28-as2 dbf4-3mDB pGAL-Sld2-T84D* (Z33977) strains were treated with 1NA-PP1 (20 μM) and DMSO at 8 hr in SPM. At 10 hr in SPM, cells were washed by filtration with conditioned SPM containing (20 μM, control), (DMSO, control) or lacking (washout) 1NA-PP1. Immediately after washout, 5 μM estradiol was added for washed-out and inhibited cultures. **(A)** Immunoblot detection of protein levels along the time course **(B)** The cellular DNA content of cells stained with propidium iodide.

2.8. Cells do not form CMG complexes even after kinase activation

So far, we investigated possible control mechanisms of DNA re-replication including Cdk1, Ime2, Swe1, and Cdc5 kinases and the Cdc14 phosphatase between meiosis I and II. Next, we asked whether we might fail to activate pre-RC. Clb5,6-Cdk1s as well as the Dbf4-Cdc7 kinase (DDK) complex trigger pre-RC activation (Labib, 2010). Clb5-Cdk1 phosphorylates the replication proteins Sld2 and Sld3, which interact with Cdc45 and lead to the recruitment of Dpb11 to origins. Sld3 and the helicase-activating protein Cdc45 are recruited to Mcm2-7 via DDK-dependent phosphorylation (Heller et al., 2011). Another complex, known as GINS, is then recruited. The formation of the Cdc45-Mcm2-7-GINS (CMG) complex activates the helicase and triggers unwinding of the double-stranded DNA (Muramatsu et al., 2010). This prompted us to investigate the helicase-activating protein, Cdc45, and its interaction with the helicase. We induced *APCoff cdc28-as2 ime2-as* with wild-type *CDC45* and expressing Cdc45 with a C-terminal ha3 tag to synchronously undergo meiosis. We added 1NA-PP1 when majority of cells in metaphase I (t=7 hr) and washed-away the inhibitor two hours after inhibition (t=9 hr). We withdrew samples for anti-HA immunoprecipitation, followed by immunoblot detection of Mcm2, Mcm4 and Mcm7 **(Figure 22)**. We found that Mcm2, Mcm4, Mcm7 and Cdc45 were present at constant levels throughout the experiment and Cdc45-Ha3 co-purifies with Mcm components in premeiotic S-phase at the time point 0 and 3. However, Cdc45 did not interact with Mcm components when we inactivated Cdc28-as2 and Ime2-as or after wash-out of the inhibitor. We concluded that CMG complex was not formed even though Pre-RC was formed. Previous studies showed that MCM phosphorylation by Dbf4-dependent kinase (DDK) promotes the recruitment of Cdc45 to the MCM ring in S-phase (Sheu and Stillman, 2006; Tanaka et al., 2011). After these results, we sought to confirm the activity of Cdc7 in our system. First, we examined the interaction between Mcm2-Ha3 with Dbf4 and Cdc7 through co-immunoprecipitation analyses. We showed that Mcm2 interacts with both Dbf4 and Cdc7 in metaphase I-arrested cells (**Figure 23**). Moreover, this interaction stayed when we inactivated and re-activated the Cdc28 and Ime2 kinases.

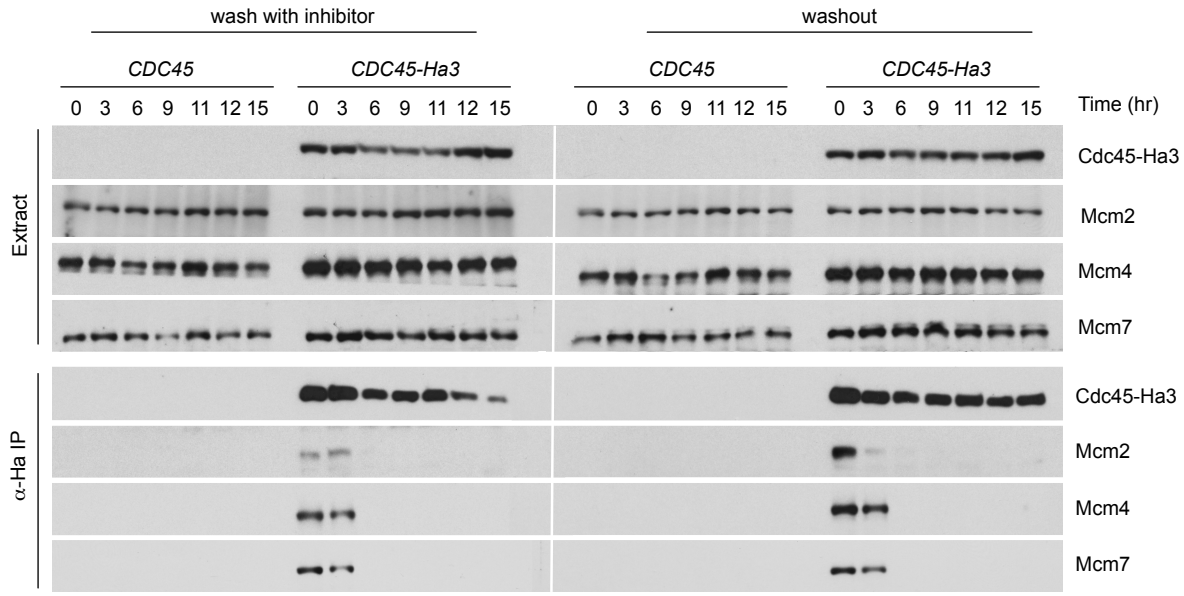


Figure 22. Mcm2, Mcm4 and Mcm7 co-purify with Cdc45-Ha3 from extracts of meiotic cells in premeiotic S phase but not after inhibition of kinases and wash-out. Co-immunoprecipitation of Cdc45 and Mcm2-7 components. Immunoblot analysis of whole cell extract and anti-Ha immunoprecipitates from *cdc28-as2 ime2as CDC45* (Z30540) and *CDC45-Ha3* (Z31274) strains.

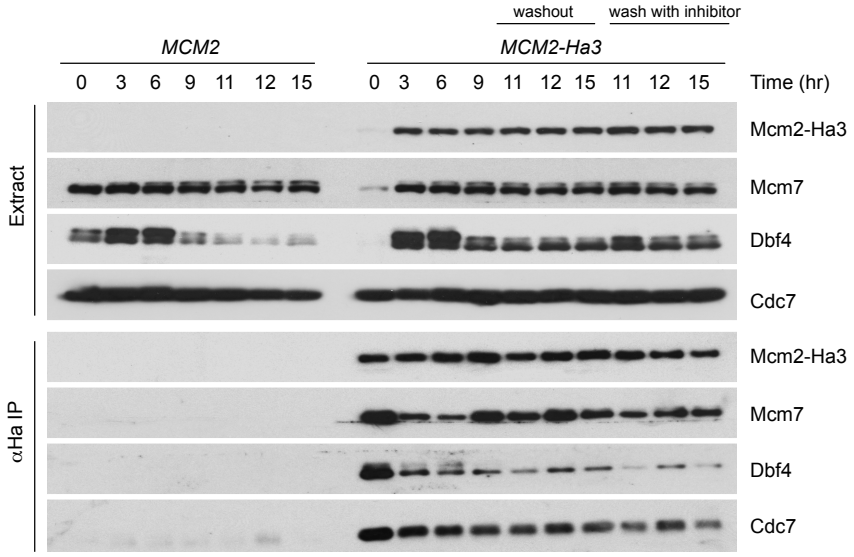


Figure 23. Cdc7 and Dbf4 co-purify with Mcm2-Ha3 from extracts of meiotic cells. Immunoblot analysis of whole cell extract and anti-Ha immunoprecipitates from *cdc28-as2 ime2as MCM2* (Z30540) and *MCM2-Ha3* (Z30539) cells.

To show DDK activity after wash-out, we imaged *cdc28-as2 ime2-as* strains, expressing mCherry-tagged Cdc7 and GFP-tagged tubulin (**Figure 24**). We induced the cultures to synchronously enter meiosis and added 1NA-PP1 when the majority of cells were in metaphase I. One hour after inhibition, we washed the cultures with or without the inhibitor and started filming of the cells. As judged by the tubulin signal, cells that inactivated the kinases did not have spindles. By contrast, cells that were washed and resuspended in medium without inhibitor accumulated spindles, suggesting that Cdk1 kinase activity was restored. The diffused nuclear signal of Cdc7 was present in 45 % of inhibited cells and 70 % of reactivated cells, indicating that Cdc7 was active in majority of cells.

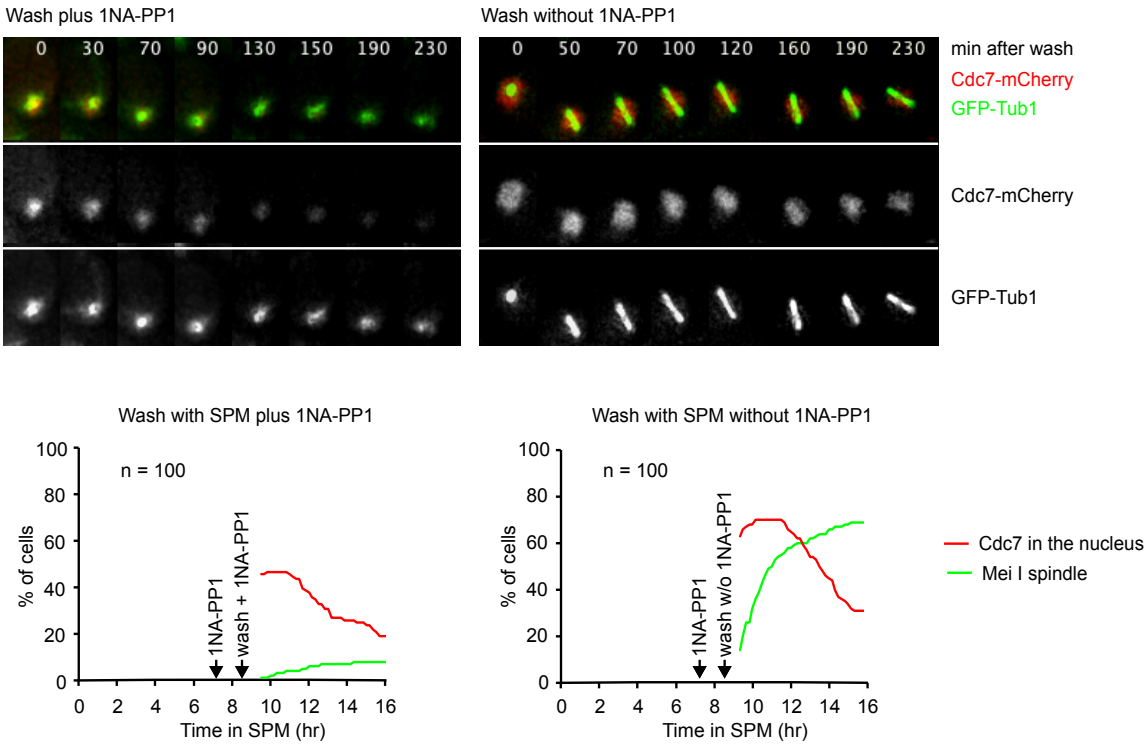


Figure 24. Cdc7 stays in the nucleus after wash-out. *APCoff cdc28-as2 ime2as* cells (Z34834) were treated with 1NA-PP1 (10 μM) at 7 hr 40 min in SPM. At 8 hr 40 min in SPM, cells were washed by filtration with conditioned SPM containing (20 μM, control) or lacking (washout) 1NA-PP1. GFP-Tub1 and Cdc7-Mcherry were imaged every 10 min starting from 9 hr 30 min in SPM. Top, representative time-lapse images of the cells are shown. Below, graphs display the percentage of cells with Meiosis I spindles and Cdc7 nuclear signal.

2.9. Cdc6 overexpression does not induce re-replication

To examine whether the over-expression of Cdc6 after inhibition and reactivation of kinases can induce re-replication, we induced *APC-off cdc28-as2* and *ime2-as* cells expressing *CDC6* from the inducible *GAL1* promoter (P_{GAL1}-*CDC6)* to synchronously enter meiosis. When the cells were induced with estradiol after wash-out, high levels of Cdc6 accumulated like in the inhibited control, consistent with previous experiments. Strikingly, in our wash-out strain, we found that Cdc6 accumulated upon inhibition of kinases and was degraded again after washing away the inhibitor (**Figure 25A**). We concluded that Clb-Cdk1 kinase activity was sufficient to phosphorylate Cdc6 leading to its degradation. According to immunoblot analysis of proteins, phosphorylation of Ndt80 was recovered, and Clb5 and Dbf4 were present until the end of the experiment. However, re-replication was not detectable by flow cytometry in wash-out or wash-out plus Cdc6 overexpressing strains (**Figure 25B**). Here, we showed the clear inhibition and re-activation of Cdc28 and Ime2 kinases. These observations suggest that there must be additional mechanisms to prevent re-replication between MI and MII.

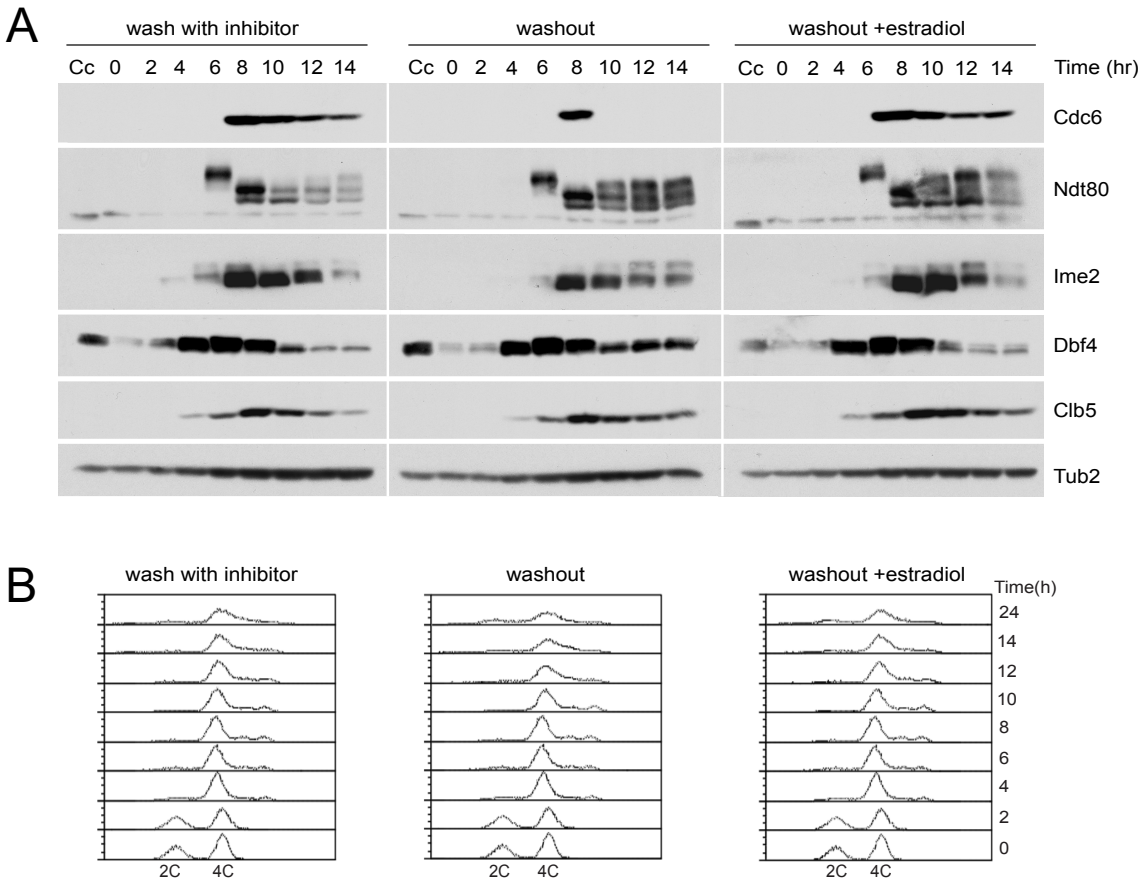


Figure 25. Overexpression of *CDC6* after inhibition and reactivation of Cdc28 and Ime2. *APCoff* P_{GAL}*-CDC6 ime2-as cdc28-as2* cells (Z32643) were treated with 1NA-PP1 (20 μM) at 7 hr in SPM. At 9 hr in SPM, cells were washed by filtration with conditioned SPM containing (20 μM, control) or lacking (washout) 1NA-PP1 and estradiol (10 μM) was added immediately after washout. **(A)** Immunoblot detection of protein levels along the time course. **(B)** Flow cytometric analysis of cellular DNA content of cells stained with propidium iodide.

3. Discussion

In this study, we used budding yeast to address a fundamental question regarding the regulation of meiosis: how do meiotic cells undergo two sequential rounds of chromosome segregation without an intervening S-phase? The complete inhibition of DNA replication outside S phase of the cell cycle and meiosis is very important since reinitiating replication can cause genome instability and tumorigenesis. The hallmark of meiosis is the formation of gametes with a haploid set of chromosomes from diploid germ cells. Another round of replication between MI and MII would prevent haploidization. Without genome haploidization, fertilization would result in zygotes whose chromosome number doubles in each generation. In this case, accurate chromosome segregation would be impossible. In proliferating cells, DNA replication and chromosome segregation are driven by cyclin-dependent kinase (CDK) activity. Cdk1 activity is low in G1 phase and high in S, G2, and M phases. At the end of mitosis, Cdk1 activity decreases and a new cycle starts with the next G1 phase. In meiotic cells, Cdk1 activity is low in G1 phase and high in S, G2 and MI phases. Subsequently, the activity of Cdk1 drops between MI and MII, and is high again in MII. When we apply the mitotic model of replication control to meiotic cells, we expect to see re-replication between meiosis I and -II. Thus, there must be meiosis-specific mechanisms to prevent replication during the MI-MII transition. One potential candidate is the conserved meiosis-specific kinase Ime2, which is active during the MI-MII transition (Benjamin et al., 2003). Ime2 promotes the G1-S transition (Dirick et al., 1998) and shares many substrates with Cdk1 (Holt et al., 2007). Our strategy to address the question of how re-replication is prevented between MI and MII was to create the oscillation of the kinases Ime2 and Cdk1 and the counteracting phosphatases in order to induce re-replication.

3.1. Mechanisms known to prevent re-replication in mitotic cells

DNA replication is a highly regulated process involving a number of licensing and replication factors that function in a carefully orchestrated order to faithfully replicate DNA. To ensure genome stability, DNA must be replicated once and only once during each cell cycle. DNA re-replication was extensively studied in

mitotic cells of budding yeast and prevention of DNA re-replication is due to the direct inhibition of the proteins required for licensing. The events known to occur are Cdk1-inhibitory phosphorylation of ORC (Nguyen et al., 2001), Cdk1-mediated phosphorylation of Cdc6 and its subsequent degradation (Jallepalli et al., 1997; Elsasser et al., 1999; Drury et al., 2000), and Cdk1-promoted nuclear export of Cdt1 and Mcm2-7 (Labib et al., 1999; Nguyen et al., 2001). All these events must occur to prevent DNA re-replication during the cell cycle. Strikingly, it has been shown that re-replication can be induced in arrested cells by artificially oscillating Sic1 levels since this will cause oscillations in Cdk1 activity. By increasing the expression of Sic1 using the *GAL1* promoter, pre-RC components are established at origins, and then decreasing expression of Sic1 allows for an increase in Cdk1 activity and subsequent origin firing (Dahmann et al., 1995).

The complex control of DNA replication initiation in eukaryotic organisms is highly conserved. Cdk1 levels are also important for regulating the replication in metazoans (reviewed in Nishitani and Lygerou, 2004). For example, fluctuations of cyclin E levels are required to drive endoreplication cycles in *Drosophila* (Follette et al., 1998). Additionally, in higher eukaryotes, geminin serves as a major DNA replication safeguard. Geminin is a protein present in metazoans, but not in yeast, that suppresses origin licensing through its ability to bind and inactivate Cdt1 (reviewed in Porter, 2008). Table 1 shows the main mechanisms to prevent re-replication in different species.

Species	Pathways to prevent rereplication
Budding yeast *(Saccharomyces cerevisiae)*	Cdt1, Mcm2-7 nuclear export Cdc6 destruction Inhibition of Cdc6 transcription Orc2/6 Phosphorylation ORC6 inactivation by Clb5 binding
Fission Yeast (*Schizosaccharoymces pombe*)	Cdt1 and Cdc6 destruction Cdc2-Cdc13 binding to Orc2 and phosphorylation
Worms *(Caenorhabditis elegans)*	Cdt1 destruction (Cul4-Ddb1^{Cdc2}) GMN-1 (geminin)
Flies *(Drosophila melanogaster* and *Sciara coprophila)*	Cdt1 destruction (Cul4-Ddb1^{Cdc2}) Geminin CDK inhibition of pre-RC assembly
Frog Embryo (*Xenopus laevis)*	Cdt1 destruction (Cul4-Ddb1^{Cdc2}) Geminin CDK inhibition of ORC binding
Mammals	Cdt1 destruction (Cul4-Ddb1^{Cdc2}, SCF^{Skp2}) Geminin CDK inhibition of ORC binding

Table 1. Species-specific pathways that prevent re-replication in somatic cells (Arias and Walter, 2007).

3.2. Ime2 and Cdk1 cooperate to inhibit pre-RC formation in budding yeast

In meiotic cells, there is another mechanism involved in the control of DNA replication, namely Ime2. Previous studies have shown that Ime2 can robustly inhibit helicase loading by multiple mechanisms, similar to G1-Cdk1 (Holt et al., 2007). These observations strongly suggest that Ime2 separates in time helicase loading from helicase activation during the meiotic G1-S transition. Even though Cdk1 levels decrease between meiosis I and II, Ime2 levels stay constant throughout meiosis. Thus, we speculated that Ime2 serves to prevent re-replication together with Cdk1 between meiotic divisions. Previous studies have mostly focused on the idea of Cdk1 and Ime2 acting separately (Holt et al., 2007; Iwabuchi et al., 2000), but have not systematically addressed how specific proteins and steps of replication are inhibited by these and other kinases. First, we tested these models in *Saccharomyces cerevisiae* and found that Ime2 inhibition alone and Cdk1 inhibition alone do not result in Mcm2-7, Cdt1, and Cdc6 accumulation. Strikingly, simultaneous inhibition of Ime2 and Cdk1 caused

strong Mcm2-7, Cdt1, and Cdc6 accumulation. Another question is whether these pre-RC components are loaded onto replication origins and whether ORC is still active. It has been shown recently that ORC was bound to the replication origins throughout both meiotic divisions. Furthermore, substantial dephosphorylation of ORC after the inhibition of Cdc28 and Ime2 has been observed (Phizicky et al., 2018). We concluded that one of the ways to prevent DNA replication between the meiotic divisions is inhibiting helicase loading by Cdk1 and Ime2. Each kinase is sufficient for preventing origin licensing. Inhibiting early steps of replication makes sense, as there is less danger of aberrant DNA unwinding and polymerase recruitment. Simultaneous inhibition of Ime2 and Cdk1 caused the formation of pre-RC. Next, we sought to fire origins.

3.3. Experimental oscillations of Cdk1 and Ime2 for promoting re-replication

Even though simultaneous Ime2 and Cdk1 inhibition resulted in the accumulation of pre-RC components, they do not activate the helicase. Activation of the replicative helicase is the commitment step of replication initiation (reviewed in Bell and Labib, 2016) and preventing this step would be sufficient for suppressing replication before initial DNA unwinding and synthesis. Cells achieve once-per-cell-cycle replication initiation by dividing the replication initiation process into two non-overlapping phases: licensing and firing (reviewed in Blow and Dutta, 2005; Siddiqui et al., 2013). In biochemical terms, licensing corresponds to the low kinase state, while firing corresponds to the high kinase state, which results in the activation of the replicative helicase. In the mitotic cell cycle, oscillations in Clb-Cdk1 activity induce a complete round of DNA replication as mentioned in previous chapters (Dahmann et al., 1995). Here, we tried to create artificial oscillations of both Ime2 and Cdk1 in metaphase I-arrested cells to promote re-replication. First, we inactivated Ime2-as and Cdc28-as by adding an ATP analog. Then, we washed-away the inhibitor by filtration to re-activate the kinases. However, by inhibiting Cdk1 and Ime2, we induce a state in which it is difficult to re-activate the kinases and re-inactivate the opposing phosphatases. This might result from a bistable switch and irreversible transition into the low kinase state.

Bistability is characterized by two stable steady states which are self-maintaining and resistant to small perturbations (Novak et al., 2010). In the bistability, two stable steady states are separated by unstable region that can be described as a

mountain ridge separating two valleys (Tyson et al., 2001). Proportionally small increases in input are buffered until a threshold is reached, thereafter a major change in the output occurs (Fisher et al., 2012). Biological bistable systems usually contain a combination of positive and double-negative feedback loops. A simple example of bistability is the conserved network composed of Wee1, Cdc25, and Cdk1, which control entry into M phase (Domingo-Sananes et al., 2011; Nurse, 1990). Cdk1 and Wee1 inhibit one another by direct phosphorylation, a double negative feedback loop. On the other hand, Cdc25 contributes to Cdk1 activation by removing inhibitory phosphorylations. This allows Cdk1 to activate more Cdc25, which engages the positive feedback loop. In order to move the system from the low kinase state to the high kinase state, we deleted *SWE1*. Swe1 (homolog of Wee1) is a kinase that inhibits Cdk1 by phosphorylation on Tyr19 (Booher et al., 1993). There is mutual inhibition between Cdk1 and Swe1, a double negative feedback loop. Both kinases inhibit one another by direct phosphorylation. In our experimental setup, *SWE1*-deleted cells entered meiosis with normal kinetics and artificial oscillation of Cdk1 and Ime2 kinases at metaphase I did not cause re-replication. Decreasing levels of Cdk1 and Ime2 lead to the activation of Cdh1 and Sic1 via the Cdc14 phosphatase. A simplified network of factors regulating Cdk1 is depicted in Figure 26. The level of Sic1 was shown to play a major role in triggering irreversible transitions in the cell cycle by inhibiting the activity of Cdk1 (reviewed in Reed, 2003). In order to start DNA replication, Sic1 is degraded and Cdh1 is inactivated, allowing Cdk1 activity to rise. Therefore, we depleted also the potential enemies, namely Cdh1 and Sic1, of Cdk1 for switching the system to the high kinase state. However, we did not observe re-replication.

We conclude that either there is a missing replication factor or we could not create robust oscillations of the kinases due to the bistable switches. First, we explored two more mechanisms of kinases: Cdc5 and DDK.

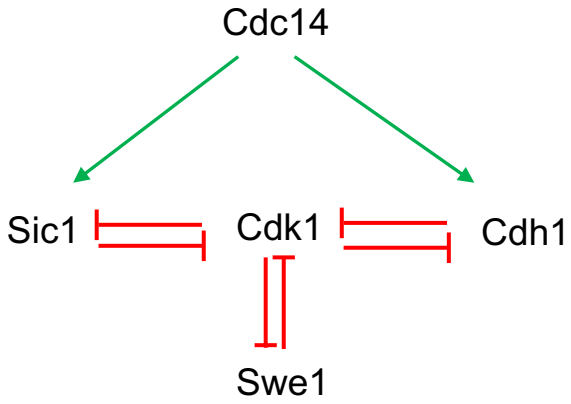


Figure 26. Simplified network involving Cdk1-Clb, APC-Cdh1, Sic1, Cdc14, and Swe1. Double negative feedback loops, mediated by APC-Cdh1, Sic1, and Swe1 suppresses Cdk1 and Cdk1 also inhibits Sic1, Cdh1, and Swe1. Green arrows represent activating interactions, and red blunt-ended lines represents inhibition of activity.

3.4. What is missing to induce re-replication?

We found that additional inhibitory mechanisms might be represented by the degradation of Dbf4 and Sld2. Dbf4 binds to Cdc7 to form the kinase DDK which is required for helicase activation. Dbf4 is degraded at meiotic anaphase I (Matos et al., 2008) suggesting a mechanism for preventing DNA re-replication. Recently developed in vitro systems, which recapitulate the molecular events culminating in origin firing, have further demonstrated that DDK is absolutely essential for the initiation of DNA replication (Heller et al., 2011; Yeeles et al. 2015). Here, we bypassed the degradation of Dbf4 by expressing a stabilized protein and/or using the *APCoff* (*ama1Δ, PHSL1-Cdc20 and PHSL1-Cdh1)* system. However, we did not observe activation of the helicase or re-replication.

Previous studies propose that Cdk1 and Cdc5 inhibit Mcm2–7 activation during the meiotic divisions by promoting Sld2 degradation (Phizicky et al., 2018). In mitotic cells, degradation of the firing factor Sld2 is mediated by Cdk1, DDK, Mck1, and Cdc5 kinases (Reusswig et al., 2016). The firing reaction depends on the specific firing factors Sld2, Sld3, and Dpb11. CDK facilitates the firing by phosphorylating Sld2 and Sld3 and thereby enabling these proteins to interact with Dpb11 (Kamimura et al., 1998; Masumoto et al., 2002; Tanaka et al., 2007; Zegerman and Diffley, 2007). Then, this complex activates the helicase (Gambus et al., 2006; Heller et al., 2011; Muramatsu et al., 2010; Yeeles et al., 2015). In our experiments, constant levels of Cdc5 were present even when we inhibited Cdk1

and Ime2. Thus, we speculated that the polo-like kinase Cdc5 might inhibit re-replication between meiosis I and -II. However, depletion of Cdc5 (data not shown) or inhibition of Cdc5-as did not result in re-replication. In particular, we tested the overexpression of phosphomimic Sld2 in our system, which did not result in re-replication. So far, we investigated the kinases Cdk1, Ime2, DDK, Cdc5, and Swe1 to induce re-replication in metaphase I-arrested cells. Meiotic cells use many mechanisms to inhibit DNA replication between the meiotic divisions. We considered the possibility that re-activation of the kinases is not efficient due to persistent phosphatase activity. We mostly focused on the kinases in this study. However, the counteracting phosphatases are equally important to solve this problem. The phosphatases have their own bistable states, which make the low and high-kinase state more stable and switching between them more difficult.

3.5. Phosphatases for preventing re-replication in budding yeast

The cell cycle and meiosis comprise a series of sequential events. The major mechanisms that govern these complicated processes are reversible protein phosphorylations catalyzed by many different types of kinases and phosphatases. Although the central role of protein kinases has been intensely studied, the importance of protein phosphatases is often overlooked. Different types of phosphatases have been implicated in cell cycle regulation, such as protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), Cdc14, and Mih1 (homolog of Cdc25). Mih1 is a protein tyrosine phosphatase involved in mitotic entry and regulates the phosphorylation state of Cdk1 by reversing the Swe1-mediated inhibitory phosphorylation (Russel et al., 1989).

Cdc14 is thought to be the major Cdk-counteracting phosphatase in mitotic cells of budding yeast. It is sequestered in the nucleolus for most of the cell cycle, and only released and activated upon anaphase onset through the FEAR (Cdc fourteen early anaphase release) and MEN (mitotic exit network) pathways to counteract several Cdk1-dependent phosphorylations of substrates (reviewed in Stegmeier and Amon, 2004; Sullivan and Morgan, 2007). In budding yeast meiosis, activation of the FEAR network is required for exit from meiosis I but does not lead to the activation of origins of replication. The important substrates of MEN-activated Cdc14 include Cdh1 and Swi5 (Visintin et al., 1998).

Dephosphorylation of Cdh1 by Cdc14 activates APC/C-Cdh1 activity, which in turn targets cyclins for destruction. Parallel to this pathway, dephosphorylation of the transcription factor Swi5 triggers its nuclear import to activate *CDC6* and *SIC1* transcription (Piatti et al., 1995; Visintin et al., 1998). Recently, Cdc14 was shown to have additional substrates, such as replication initiation proteins Orc2, Orc6, Cdc6 and Mcm3 and it resets the competency of replication licensing during mitotic exit in budding yeast (Zhai et al., 2010). Based on our results, Cdc14 was released upon inhibition of Ime2-as and Cdc28-as, leading to formation of pre-RCs. However, it was not fully recaptured after reactivation of the kinases. Thus, we used Cdc14-AID to degrade Cdc14 at specific times. However, degradation of Cdc14 did not induce re-replication in our experimental setup. We speculate that there might be other phosphatases that inhibit re-replication since the sites phosphorylated by Ime2 are resistant to dephosphorylation by Cdc14 (Holt et al.,2007). This also helps to explain how Cdc14 allows MI exit without promoting nuclear accumulation of Mcm2-7 necessary for licensing.

PP2A is a good candidate for preventing re-replication. It is a major counteracting phosphatase of Cdk1 and can also dephosphorylate Ime2 sites (Holt et al., 2007). PP2A is a highly conserved serine-threonine phosphatases involved in diverse cellular processes. PP2A phosphatase consists of 3 subunits: a catalytic subunit (Pph21/Pph22), a scaffold subunit (Tpd3), and one of two regulatory subunits (Rts1/Cdc55) (Sneddon et al., 1990; Healy et al., 1991; Shu et al., 1997). In budding yeast, meiotic cells lacking PP2A-Cdc55 activity undergo premature exit from meiosis I, which results in a failure to form bipolar spindles and to divide nuclei. This defect is mainly due to its role in negatively regulating the FEAR pathway. PP2A-Cdc55 prevents nucleolar release of Cdc14 by counteracting phosphorylation of the nucleolar protein Net1 by Cdk1 (Kerr et al., 2016). In addition to its role in Cdc14 release, PP2A was investigated in regulating DNA replication initiation. PP2A antagonizes the DNA damage checkpoint protein Chk1 (Petersen et al., 2006), and its function is required for Cdc45 loading onto chromatin (Chou et al., 2002). A potential role for PP2A phosphatase in DNA replication initiation could be to oppose the action of Cdk1 and Ime2 kinases. However, such a role remains to be investigated.

A role for dephosphorylation in controlling DNA replication initiation was established recently for the protein phosphatase 1. The Rap1-interacting factor Rif1 is able to recruit PP1 phosphatase to MCM subunits, resulting in their dephosphorylation (Davé et al., 2014; Hiraga et al., 2014; Mattarocci et al., 2014). Therefore, higher levels of DDK are required for the induction of origin firing, since the MCM phosphorylation rate must exceed its dephosphorylation rate. Conversely, DDK can bind directly to Rif1 and inhibit its interaction with PP1 (Hiraga et al., 2014). Therefore, as DDK levels increase during S phase, MCM phosphorylation is promoted and dephosphorylation is inhibited. The resulting feedback loop allows for a rapid switch from low MCM phosphorylation in G1 to high MCM phosphorylation in S phase. There is further evidence that Rif1-PP1 controls additional aspects of DNA replication initiation. For example, in yeast, Rif1-PP1 may antagonise Cdk1 phosphorylation. In *RIF1*-deleted yeast strains, phosphorylation of Sld3 is increased and this strain can partially rescue the phenotype of temperature-sensitive origin firing factors including Dpb11, Cdc45, and Sld3 (Mattarocci et al., 2014). Rif1-PP1 involvement in DNA replication control appears to be conserved throughout eukaryotes, both *Xenopus* egg extract and HeLa cell studies support the findings in yeast (Poh et al., 2014; Yamazaki et al., 2012). In this study, we mostly focused on kinases and the Cdc14 phosphatase but interesting questions remain, such as PP2A and PP1 involvement in DNA re-replication.

Although this work has significantly expanded our knowledge of how DNA replication is prevented between MI and MII, many questions remain unresolved with regard to the induction of re-replication. In summary, the kinase/ phosphatase ratio is important for the commitment point and the onset of replication. We believe that if robust oscillations of kinases Cdk1 and Ime2 can be created, the activities of replication proteins should also oscillate. Then, re-replication might occur at the MI-MII transition. Future work should address whether re-inactivation of PP2A allows re-replication.

4. Materials 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*). Mating of the corresponding haploid strains produced diploid strains. Mutations in diploid strains are homozygous unless stated otherwise. Genotypes of strains used in this work are listed in **Table 2**. The following alleles have been described previously: *CDC20* under the control of the mitotic *SCC1* promoter (Okaz et al., 2012), the analog-sensitive alleles *cdc28-as1* (Bishop et al., 2000), *ime2-as* (Benjamin et al., 2003) and *cdc5-as* (Snead et al., 2007), *ama1Δ::NatMX4* (Oelschlaegel et al., 2005) and a Gal4-estrogen receptor fusion under the control of the *GPD1* promoter (P_{GPD1}-*GAL4-ER*) used for estradiol inducible expression (Benjamin et al., 2003). mCherry or tdTomato were used for tagging with red fluorescent protein (RFP) (Shaner et al., 2004). eGFP, sfGFP and neonGreen were used for tagging with green fluorescent protein (GFP) (Sheff and Thorn, 2004; Pedelacq et al., 2006; Shaner et al., 2013).

4.2 Construction of plasmids and yeast strains

One-step PCR C-terminal epitope tagging was used to generate the strains containing Myc, Ha or Pk tagged proteins (Knop et al., 1999; Wach et al., 1994). Mcm7 and Cdc7 were tagged at the C-terminus with mCherry by one-step gene tagging (Sheff and Thorn, 2004). To visualize Cdt1 and Sic1 in live-cell imaging experiments, PCR-generated cassettes were used for C-terminal tagging of Cdt1 and Sic1 with NeonGreen (Shaner et al., 2013). Cdc14 was tagged at the C-terminus with AID by one-step gene tagging and transformed into a yeast strain containing *OsTIR1* gene under the control of the cupper *CUP1* promoter. The AID containing plasmid was a present from Valerie Borde and the P_{CUP1}-*OsTIR1-9Myc* strain was kindly provided by Neil Hunter. The P_{GAL}-*SLD2-D84* strain was kindly provided by Philip Zegerman. Tagged proteins are fully functional as judged from normal proliferation and sporulation of homozygous diploids. PCR-generated fragments were used for gene replacement with cassettes conferring resistance to the antibiotics G418, nourseothricin, or hygromycin B (Goldstein and

McCusker, 1999). To suppress *CDC5*, *CDC20* and *CDH1* gene expression in meiotic cells, endogenous promoters were replaced with cassettes containing the mitosis-specific *SCC1* and *HSL1* promoters (Okaz et al., 2012). For inducible expression of stabilized *IME2*, 241 amino acids from C-terminal of its ORF was deleted and the truncated *IME2* was cloned behind the *GAL1* promoter in the yeast integrative plasmid YIplac128 (*LEU2*) (Katarzyna Jonak, Zachariae Lab). The temperature-sensitive *cdc20-3* was generated by PCR-mediated site-directed mutagenesis and cloned behind the copper-inducible *CUP1* promoter in the yeast integrative plasmid YIplac204 (*TRP1*) (Katarzyna Jonak, Zachariae Lab).
To restrict *SIC1* expression in meiosis, the endogenous promoter was replaced with a cassette containing the mitosis-specific promoter of *CLN1*. *CLN1* promoter was amplified from SK1 genomic DNA and cloned into pAG32 cassette (Goldstein and McCusker, 1999) for PCR-mediated one step promoter replacement in yeast. For inducible expression of *CDC6*, its ORF was cloned behind the *GAL1* promoter in the yeast integrative plasmid YIplac211 (*URA3*). PCR-mediated site-directed mutagenesis was used to generate another analog-sensitive version of Cdc28. Phenylalanine in the ATP-binding cleft was changed to alanine (F88A). This mutant *cdc28-as2* allele was transformed into *cdc28-4* temperature-sensitive cells by PCR and positive clones were selected by selection for growth at elevated temperatures.

4.3. Meiotic time course experiments

Entry into meiosis was induced at 30 °C as described before (Oelschlaegel et al., 2005). Strains grown on YP-glycerol plates were spread on YPD plates and grown for 24 hr to produce a lawn. Cells were inoculated into YEPA (YP plus 2% K-acetate) medium to OD600 ~0.3 and grown for 11-12 hr. At the end of this period, the OD600 reached ~1.8 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 sporulation medium (SPM, 2% K-acetate), centrifuged one more time, and finally resuspended in 100 ml of SPM, resulting in an final OD600~3.4 in 2.8L Fernbach flasks on an orbital shaker at 200 rpm. OD measurements were made at 600 nm with an Ultrospec 3100pro UV/Visible Spectrophotometer (Amersham Biocsience). Before measuring the OD, samples were briefly sonicated. In experiments in which thermo-sensitive mutant strains

were used, all the procedures prior to induction of meiosis were done at 25 °C. YEPA synchronization at 25 °C was done for 14 hr (OD600 ~1.7). After meiotic induction, the temperature was shifted to the respective restrictive temperature at times indicated for each experiment. A 5 mM stock solution of ß-estradiol (Sigma) was made in DMSO and stored at -20 °C until use. In time course experiments, including *cdc28-as1* strains, the inhibitor 1NM-PP1 (Cayman Chemicals) was added to a final concentration of 5 μM from a stock solution of 5 mM in DMSO, stored at -20°C until use. In time course experiments, including *ime2-as* and *cdc28-as2* strains, the inhibitor 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. In time course experiments, including *cdc5-as,* the inhibitor CMK (AccendaTech, Tianjin, P.R. China) was added to a final concentration of 20 μM from a stock solution of 20 mM in DMSO, stored at -20°C until use. In time course experiments, including Cdc14-AID, the inhibitor IAA (Sigma-Aldrich) was added to a final concentration of 2 mM from a stock solution of 2 M in DMSO, stored at -20°C until use. At the indicated time points, samples were collected for trichloroacetic acid (TCA) protein extracts, immunofluorescence, and flow cytometric analysis of cellular DNA content.

4.4. Indirect immunofluorescence

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. Indirect immunofluorescence was performed with cells fixed overnight at 4 °C in 3.7% formaldehyde as described (Salah and Nasmyth, 2000). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Cells containing two distinct masses of DNA were scored as undergoing the meiosis I division. Cells with 3-4 DNA masses perform meiosis II. The following primary antibodies were used: monoclonal mouse anti-Myc (1:5, 9E10), monoclonal rat anti-tubulin (1:250, Serotec YOL 1/34), monoclonal mouse anti-Tub4 (1:30, Zachariae lab), Goat anti-Cdc14 (1:20, sc-12045 Santa Cruz). Affinity purified secondary antibodies conjugated to CY3 (1:200, Jackson ImmunoResearch), CY5 (1:50, Jackson ImmunoResearch), Alexa488 (1:200, Chemicon) were used for detection. For quantifications, 100 cells per sample were

counted.

4.5. Chromosome spreads

Chromosome spreading was performed essentially as described (Loidl et al., 1998). 1 ml of culture was withdrawn from a meiotic culture (OD600 ~3.5). Cells collected by centrifugation were resuspended in 200 µl of spheroplasting solution (2% potassium acetate, 1.2 M sorbitol) and treated for 15 min at 30 °C with 10 mM of DTT. Cell walls were removed by incubation with zymolyase 100T (50 µg/ml, Amsbio) until at least 90% of cells burst upon dilution into water plus 2% SDS. The reaction was stopped by the addition of 1 ml of cold stop solution (0.1 M MES, 1 mM EDTA, 0.5 mM $MgCl_2$, pH 6.4). Spheroplasts were centrifuged (1 min, 3000 rpm) and resuspended in 100 µl of Stop solution. The following reagents were applied consecutively to a glass slide containing 20 µl of spheroplast suspension: 40 µl of fixative solution (4% paraformaldehyde, 3.4% sucrose), 80 µl of Lipsol (1%), and 80 µl of fixative solution. After mixing briefly, spheroplasts were gently spread over the slide with a glass rod. After overnight fixation and drying at room temperature, slides were washed in PBS (50 mM potassium phosphate buffer pH 7.4, 0.15 M NaCl) for 15 min. Before immunostaining, spreads were blocked for 30 min with PBS-BSA (PBS, 1% BSA). Primary antibodies were diluted in PBS-BSA, added to the slides, and incubated overnight at 4 ºC or for 4 hr at room temperature. After incubation with primary antibodies, slides were washed in PBS for 20 min and then secondary antibodies were added. Chromosome spreads were incubated with secondary antibodies for 2 hr. After 20 min of washing in PBS, slides were mounted with 40 µl pd-DAPI (10% PBS, 1mg/ml phenylenediamine, 0.05 µg/ml DAPI, 90% glycerol, pH 8.0). The following primary antibodies were used: monoclonal Mouse anti-Ha (1:50, 12CA5) and rabbit anti-Tub4 (1:500, Zachariae lab). Secondary antibodies from donkey conjugated to Alexa488 (Chemicon), and CY3 (Jackson ImmunoResearch) were used for detection.

4.6. Live-cell imaging

4.6.1. Experimental setup

Imaging of living cells undergoing meiosis was performed essentially as described (Okaz et al., 2012). Cultures were induced to enter meiosis as described above and diluted to OD600 = 0.2 with pre-warmed SPM. 0.3 ml aliquots were

applied to 8-well Ibidi chamber slides (Ibidi, Ibitreat 80826) coated with Concanavalin A (Sigma C5275, 0.5 mg/ml in PBS) to give 20-30 cells per field of view. Imaging was performed on a DeltaVision Elite system controlled by the SoftWoRx5.0 software (Applied Precision). Optical components 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 HQ2 CCD camera. Images were acquired in the green and the red channels every 10 min for at least 12 hr using a 12% neutral density filter (TrueLight Additional ND filter, ND090-37 12%T from Lumencor) and exposure times of 50-300 ms. T% was set to 5-10 for the green channel and to 32-50 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).

4.6.2. 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 6-8 fields of view were individually followed through meiosis. Percentages of meiotic events were then calculated for each time point using Microsoft Excel. The resulting graphs show percentages of meiotic events over time. Alternatively, a cellular event was chosen as a reference and set to t = 0 in each cell. The meiosis of 100 cells was aligned and the percentages of other events (e.g., duplication of SPBs) were calculated at 10 min intervals before and after the reference event. Representative cells were cut out from the original image files. Stacks of the merge and the single channels were combined using the "Stack Combiner" plugin. The acquisition times were printed on the images with the "Time stamper" tool. Selected frames of the movie were fused into a single image using the "Montage" tool.

4.7. Analysis of proteins

4.7.1. Preparation of protein extracts

Protein levels were analyzed by breaking cells with glass beads in trichloroacetic acid (TCA) followed by SDS polyacrylamide gel electrophoresis and immunoblotting. For each sample, cells from 8-10 ml of sporulating culture were

spun down, washed in 1 ml of 10% TCA and frozen. Samples were thawed on ice, and resuspended in 200 µl 10% TCA. Cells were broken by vigorous shaking with zirconium beads (diameter 0.5 mm) (Roth, 11079105z) for 25 min. After a low speed centrifugation (3000 rpm), the protein pellet was boiled in SDS-sample buffer (62.5 mM Tris-HCl pH 6.8, 10 % Glycerol, 2% SDS, 0.01% bromophenol blue) for 10 min. Samples were centrifuged at 14000 rpm for 10 min and the protein concentration in the soluble extract was determined using the BioRad protein assay. Absorbance was measured at 595 nm with an Ultrospec 3100pro UV/Visible Spectrophotometer (Amersham Biocsience).

4.7.2. Western blotting and immunodetection of proteins

Samples of 50-100 µg of protein were separated in 8% polyacrylamide SDS gels. Semidry western blotting (0.45 mA/cm2 for 1 hr) was used to transfer proteins to a PVDF membrane (Immobilon P, Millipore). Membranes were then blocked for 1 hour in PBS buffer containing 0.1 % Tween 20 and 4% non-fat milk powder (PBS-T), and incubated with primary antibody for 1 hour at room temperature. After four washes of 5 minutes in PBS-T, the membrane was incubated with secondary antibody (1:5000) conjugated to horseradish peroxidase for two hours at room temperature. After four washes of 5 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 exposed to a Kodak X-omat machine.

For immunodetection, mouse monoclonal antibodies were used to detect Ha (12CA5, 1:500), myc (9E10, 1:200, Evan et al., 1985), pK9 (1:5000, Serotec) and Pgk1 (1:40000, Invitrogen). Rabbit polyclonal antibodies were used for the detection of Ndt80 (1:10000, a gift from Kirsten Benjamin), Ama1 (1:2000, Oelschlaegel et al., 2005), Cdh1 (1:5000, Zachariae lab), Cdc5 (1:5000, Matos et al., 2008), Cdc20 (1:5000, Camasses et al., 2003), Dbf4 (1:5000, Matos et al., 2008), Tub2 (1:20000, a gift from Wolfgang Seufert), Sic1 (1:400, Santa Cruz sc-50441). Goat polyclonal antibodies were used for the detection of Clb1 (1:300, Santa Cruz sc-7647), Clb4 (1:400, Santa Cruz sc-6702), Clb5 (1:200, Santa Cruz sc-6704), Clb6 (1:500, Santa Cruz sc-7166), Sum1 (1:200, Santa Cruz sc-26441), Ime2 (1:300, Santa Cruz sc-26444), Cdc14 (1:1000, sc-12045 Santa Cruz), Swe1 (1:500, Santa Cruz sc-7171).

4.7.3. Co-immunoprecipitation assay

To analyze protein-protein interactions, extracts for immunoprecipitations were prepared as described previously (Oelschlaegel et al., 2005). Briefly, 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 I mmunoprecipitation. Cells were resuspended in B150 buffer (50 mM HEPES KOH pH 7.4, 150 mM KOAc, 20 mM β-glycerophosphate, 5 mM magnesium acetate, 0.1% Triton X-100, 10% glycerol) containing protease inhibitors (Complete Roche, 5 mM Pefabloc, 2 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml pepstatin, and 2 mg/ml leupeptin). Cell breakage was performed using zirconium 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 14000 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.

4.8. Measuring of the cellular DNA content

In order to determine the cellular DNA content, cells were permeabilized overnight using 70% ethanol. On the next day, cells were incubated with 20 µl RNaseA (10 mg/ml) at 37 °C for 4 hours, treated with 0.5 ml pepsin (5 mg/ml in 55 mM HCl) at 37 °C for 30 min, washed once with FACS buffer (200 mM Tris/Cl pH 7.5, 211 mM NaCl, 78 mM $MgCl_2$) and resuspended in 0.5 ml FACS buffer containing 55 µl of propidium iodide. The stained cells (40 µl) were diluted in 1 ml 50 mM Tris/Cl, pH 7.8, and the DNA content was measured with a Becton Dickinson Accuri C6 flow cytometer using C6 software. Overlay graphs are prepared by FCS express de Novo software.

4.9. Abbreviations

AID	auxin-inducible degron
APC/C	anaphase-promoting complex/cyclosome
as	analog sensitive
ATP	adenosine triphosphate
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CMG	Cdc45-Mcm2-7-GINS
CMK	pyrrolopyrimidine chloromethylketone
DAPI	4′, 6′-diamino-2-phenylindole
DDK	Dbf4 dependent Cdc7 kinase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
FEAR	Cdc Fourteen Early Anaphase Release
GFP	green fluorescent protein
IAA	indole-3-acetic acid
NA	numerical aperture
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pre-RC	pre-replication complex
RFP	red fluorescent protein
SCF	Skp1-cullin-F-box protein family of ubiquitin ligases
SDS	sodium dodecylsulfate
SPB	spindle pole body
SPM	sporulation medium
TCA	trichloroacetic acid
YEPA	yeast peptone medium plus 2% potassium acetate
YPD	yeast peptone dextrose medium
YPG	yeast extract peptone glycerol

Table 2. List of *Saccharomyces cerevisiae* SK1 strains used in this work

Figure	Strain	Genotype
7	**Z22116**	*MCM7-link-mCherry-SpHIS5 SPC42-GFP::His3MX6 cdc20::P_{SCC1}-CDC20::KanMX6*
7	**Z22288**	*MCM7-link-mCherry-SpHIS5 SPC42-GFP::His3MX6 cdc28-as1 cdc20::P_{SCC1}-CDC20::KanMX6*
7	**Z22289**	*MCM7-link-mCherry-SpHIS5 SPC42-GFP::His3MX6 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc20::P_{SCC1}-CDC20::KanMX6*
7	**Z22451**	*MCM7-link-mCherry-SpHIS5 SPC42-GFP::His3MX6 cdc28-as1 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc20::P_{SCC1}-CDC20::KanMX6*
11A,B	**Z22786**	*cdc28-as1 SIC1-Ha3::His3MX6 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KlTRP1*
11 A,B	**Z22787**	*cdc28-as1 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 SIC1-Ha3::His3MX6 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KlTRP1*
11 A,B	**Z22788**	*ime2::KanMX4::P_{IME2}-ime2-as-LEU2 SIC1-Ha3::His3MX6 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KlTRP1*
11 A,B	**Z22789**	*SIC1-Ha3::His3MX6 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KlTRP1*
10	**Z23749**	*CDC6-pk9::KlTRP1 SIC1-Ha3::His3MX6 PDS1myc18::KlTRP1 ime2::KanMX4::P_{IME2}-IME2-LEU2 cdc20::P_{SCC1}-CDC20::KanMX6*
10	**Z23750**	*CDC6-pk9::KlTRP1 SIC1-Ha3::His3MX6 cdc28-as1 ime2::KanMX4::P_{IME2}-IME2-LEU2 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KlTRP1*
10	**Z23751**	*CDC6-pk9::KlTRP1 SIC1-Ha3::His3MX6 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KlTRP1*

10	**Z23752**	*CDC6-pk9::KlTRP1 SIC1-Ha3::His3MX6 cdc28-as1 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc20::P_{SCC1}-CDC20::KanMX6 PDS1myc18::KlTRP1*
11C	**Z23971**	*SIC1-neonGreen::TRP1 CNM67-tdTomato::NatMX4 cdc28-as1 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc20::P_{SCC1}-CDC20::KanMX6*
11C	**Z23972**	*SIC1-neonGreen::TRP1 CNM67-tdTomato::NatMX4 ime2::KanMX4::P_{IME2}-IME2-LEU2 cdc20::P_{SCC1}-CDC20::KanMX6*
14	**Z29132**	*Sic1::HphMX4::P_{CLN1}-SIC1-Ha3::TRP1*
14	**Z29179**	*SIC1-Ha3::TRP1*
15	**Z29602**	*cdc28-as2 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc20::P_{SCC1}-CDC20::HphMX4 hct1::PHSL1-HCT1::BleMX4 ama1Δ::NatMX4 dbf4::KanMX4::P_{DBF4}-dbf4-3mDB-Ha3::TRP1 Sic1::HphMX4::P_{CLN1}-SIC1-Ha3::TRP1*
9	**Z29665**	*CDT1-neonGreen::TRP1 CNM67-tdTomato::NatMX4 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc20::P_{CLB2}-CDC20::HphMX4*
9	**Z29666**	*CDT1-neonGreen::TRP1 CNM67-tdTomato::NatMX4 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc28-as1 cdc20::P_{CLB2}-CDC20::HphMX4*
9	**Z29667**	*CDT1-neonGreen::TRP1 CNM67-tdTomato::NatMX4 ime2::KanMX4::P_{IME2}-IME2-LEU2 cdc20::P_{CLB2}-CDC20::HphMX4*
9	**Z29668**	*CDT1-neonGreen::TRP1 CNM67-tdTomato::NatMX4 ime2::KanMX4::P_{IME2}-IME2-LEU2 cdc28-as1 cdc20::P_{CLB2}-CDC20::HphMX4*
8	**Z30489**	*MCM2-Ha3::His3MX6 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc28-as2 cdc20::P_{SCC1}-CDC20::URA3*

23	**Z30539**	*MCM2-Ha3::His3MX6 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc28-as2 cdc20::P_{SCC1}-CDC20::URA3 hct1::P_{HSL1}-HCT1::BleMX4 ama1Δ::NatMX4 Sic1::HphMX4::P_{CLN1}-SIC1-Ha3::TRP1*
22,23	**Z30540**	*ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc28-as2 cdc20::P_{SCC1}-CDC20::URA3 hct1::PHSL1-HCT1::BleMX4 ama1Δ::NatMX4 Sic1::HphMX4::P_{CLN1}-SIC1-Ha3::TRP1*
22	**Z31274**	*CDC45-Ha3:: His3MX6 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc28-as2 cdc20::P_{SCC1}-CDC20::URA3 hct1::PHSL1-HCT1::BleMX4 ama1Δ::NatMX4 Sic1::HphMX4::P_{CLN1}-SIC1-Ha3::TRP1*
20 A,B,C	**Z31937**	*cdc5L158G::HphMX4 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc28-as2 ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::URA3 hct1::P_{HSL1}-HCT1::BleMX4*
13B	**Z32606**	*NDT80-sfGFP::KanMX4 ura3::HIS3pscarlet-tub1::URA3 cdc28-as2 ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::HphMX4*
25	**Z32643**	*ura3::P_{GAL}-CDC6-URA3 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc28-as2 ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::HphMX4 hct1::P_{HSL1}-HCT1::BleMX4 his3::PGDP-GAL4(484).ER::HIS*
17	**Z33412**	*CDC14-AID::KanMX4 ura3::P_{CUP1}-OsTIR1-9myc-URA3 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc28-as2 ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::HphMX4 hct1::P_{HSL1}-HCT1::BleMX4*
18A,B	**Z33678**	*trp1::P_{CUP1}-CDC20-3-TRP1 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 cdc20::P_{CLB2}-CDC20::HphMX4 hct1::P_{HSL1}-HCT1::BleMX4 ama1Δ::NatMX4 leu2::P_{DMC1}-AMA1cDNA::LEU2 dbf4::KanMX4::P_{DBF4}-dbf4-3mDB-Ha3::TRP1 PDS1-myc18::His3MX6*
21 A,B	**Z33977**	*cdc28-as2 ime2::KanMX4::P_{IME2}-ime2-as-LEU2 ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::HphMX4 hct1::P_{HSL1}-HCT1::BleMX4 his3::P_{GAL}-SLD2-D84::HIS3*

		ura3::PGDP-GAL4(484).ER::URA3 *leu2:: P_{DBF4}-dbf4-RxxL mutant::LEU2*
13A	**Z34131**	*NDT80-sfGFP::KanMX4 ura3::HIS3pscarlet-tub1::URA3* *cdc28-as1 ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::HphMX4*
19 **A,B,C**	**Z34142**	*cdc28-as2 ime2::KanMX4::P_{IME2}-ime2-as-LEU2* *ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::HphMX4* *hct1::P_{HSL1}-HCT1::BleMX4 leu2::P_{GAL}-IME2-delC::LEU2* *his3::P_{GDP}-GAL4(484).ER::HIS3*
16 **A,B,C**	**Z34212**	*cdc28-as2 ime2::KanMX4::P_{IME2}-ime2-as-LEU2* *ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::HphMX4* *hct1::P_{HSL1}-HCT1::BleMX4 swe1Δ::KanMX4*
12A	**Z34579**	*dbf4::KanMX4::P_{DBF4}-dbf4-3mDB-Ha3::TRP1* *CDC7-link-mCherry::SPHIS5 SPC42-GFP::His3MX6*
12 B	**Z34580**	*dbf4::KanMX4::P_{DBF4}-dbf4-Ha3::TRP1* *CDC7-link-mCherry::SPHIS5 SPC42-GFP::His3MX6*
24	**Z34835**	*cdc28-as2 ime2::KanMX4::P_{IME2}-ime2-as-LEU2* *ama1Δ::NatMX4 cdc20::P_{HSL1}-CDC20::HphMX4* *hct1::P_{HSL1}-HCT1::BleMX4 CDC7-link-mCherry::SPHIS5* *dbf4::KanMX4::P_{DBF4}-dbf4-3mDB-Ha3::TRP1* *ura3::TUB1p-yEGF-tub1::URA3*

Strains are listed for each figure from left to right and/or top to bottom. 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.

5. References

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2. *Arguello-Miranda O, *Zagoriy I, Mengoli V, Rojas J, Jonak K, **Oz T**, Graf P, Zachariae W. 2017. Casein Kinase 1 coordinates cohesin cleavage, gametogenesis, and exit from M phase in meiosis II. Developmental cell 40, 37-52

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