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Meiotic Dna Re-Replication And The Recombination Checkpoint

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MEIOTIC DNA RE-REPLICATION AND THE RECOMBINATION CHECKPOINT

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DEDICATION

I dedicate this dissertation to my parents, Hani and Selma, and sisters, Roxanne and Sarah. To all of you, I am eternally indebted for your unconditional love and support. To my mother and father, who have taught me that hard work prevails, I am ten thousand times grateful for your push to challenge myself and aspire for great things. Thank you to my sisters, who have taught me that despite hardships humor can be the best motivational medicine. I am blessed to have a family that consists of my best friends. Your unremitting encouragement, support, and love eased the process of completing the work herein.

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CHAPTER 1

INTRODUCTION

The processes of DNA replication and programmed recombination during meiosis must be carried out with careful precision to ensure the generation of healthy gametes. Situations of aberrant replication or recombination can lead to the loss of genetic information, improper segregation of chromosomes, or aneuploidy, which is an abnormal number of chromosomes. Within this dissertation, our aim was to better understand the mechanisms that control meiotic DNA replication and the links to meiotic recombination. We have developed a system in the budding yeast *Saccharomyces cerevisiae* to induce meiotic DNA re-replication, which occurs when a cell participates in more then one round of DNA replication. Using our meiotic DNA re-replication phenotype, we wanted to uncover the meiotic machinery that would normally prevent this event from occurring. We found that inducing a meiotic recombination checkpoint (pachytene checkpoint) was able to prevent meiotic DNA re-replication. This connection has allowed us to use the pachytene checkpoint as a means to better understand meiotic DNA re-replication, and in addition, to use DNA re-replication as a means to better understand the pachytene checkpoint. We have uncovered new insight into meiotic DNA re-replication and the pachytene checkpoint. Understanding these meiotic mechanisms will allow us to better understand how aberrations that lead to genetic disorders could occur in the developing gametes.

I. The cell cycle and meiosis

S. cerevisiae contains one major cyclin dependent kinase (CDK) known as Cdk1 or Cdc28. This kinase is known to complex with many types of cyclins to regulate progression of the cell cycle. Its ability to be activated and inactivated by associating with cyclins or specialized inhibitors promotes the progression from one stage of the cell cycle to the next, which makes this kinase extremely important. During the G1-S transition of the cell cycle the CDK inhibitor Sic1 protein controls Cdk1 when complexed with the B-type cyclins, Clb5 and Clb6 (Schwob et al., 1994; Schneider et al., 1996; Tyers, 1996). Once the Sic1 is removed, Cdk1-Clb5, -6 is active and transitions the cell into S phase where DNA synthesis will occur. When the cell is in transition from S to G2, Cdk1 is complexed with Clb3 and Clb4, which is thought to aid in proper spindle assembly, and when the cell progresses from G2 to M, Cdk1 is complexed with Clb1 and Clb2 (Figure 1).

The association of Sic1 with Cdk1-Clb5, -6, inactivates these kinase complexes (Schwob et al., 1994). Sic1 phosphorylation leads to its degradation through ubiquitin-dependent proteolysis, which activates the Cdk1-Clb5, -6 complexes and allows the cell to enter S phase (Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997). The Cdk1-G1 cyclin complexes Cdk1-Cln1 and Cdk1-Cln2 are responsible for the phosphorylation and subsequent degradation of Sic1 (Deshaies, 1997). Once the Cdk1-Clb5, -6 complexes are active they additionally contribute to Sic1 destruction and catalyze phosphorylation of proteins such as Sld2 and Sld3 that promote DNA replication.

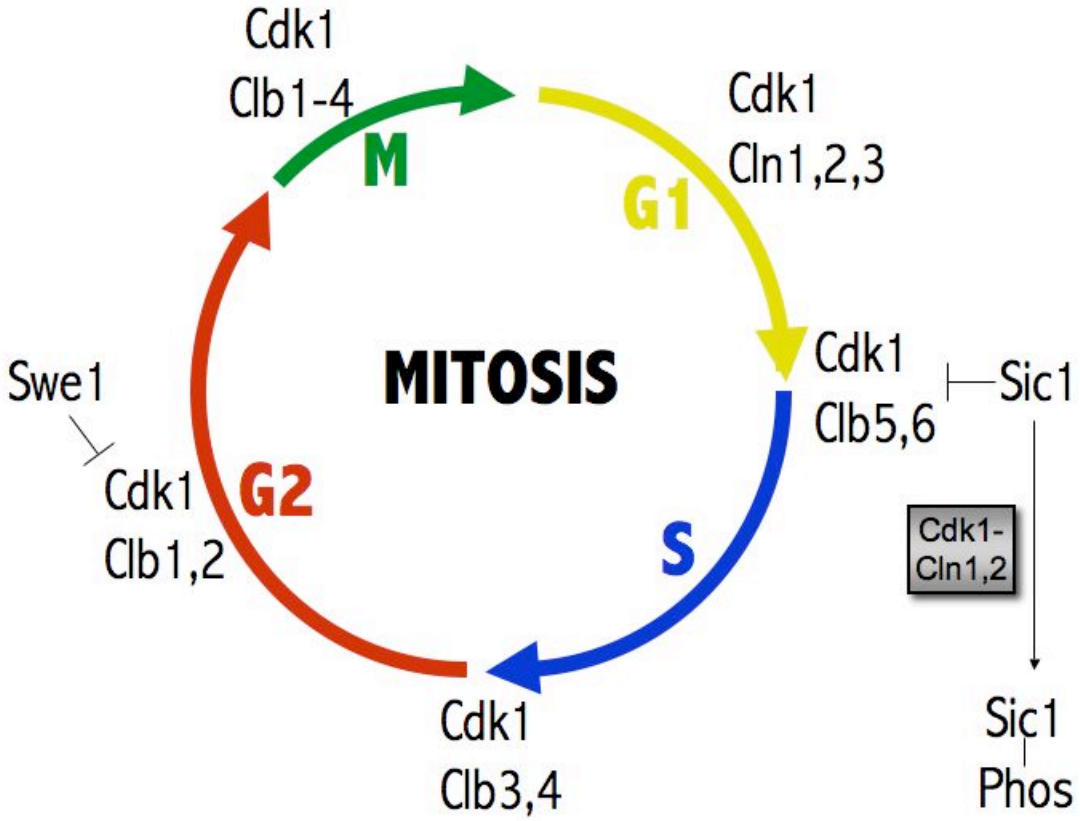


Figure 1. Cyclin dependent kinase 1 (Cdk1) and the cyclins it associates with during the cell cycle.
The schematic shows the Cdk1-cyclin complexes required for the cell cycle. The inhibitor Sic1 is depicted, which controls the G1 to S transition. Sic1 phosphorylation at many sites signals its degradation through the ubiquitin proteasome pathway.

In addition, they act to prevent DNA re-replication (see review (Blow and Dutta, 2005)).

DNA replication is not limited to the mitotic cell cycle but must also occur in meiosis. Meiosis consists of one round of DNA replication that is the precursor to two rounds of chromosome segregation, which will ultimately lead to the production of four haploid cells in yeast. In the first round of division (MI), the homologous chromosomes pair and separate, which is often termed reductional division. During the next division (MII), sister chromatids separate, which is termed equational division. Cdk1-Clb5, -6 are required for initiation of meiotic DNA replication (Dirick et al., 1998; Stuart and Wittenberg, 1998; Benjamin et al., 2003), and recombination (Smith et al., 2001; Henderson et al., 2006), as opposed to Cdk1-Clb1, -3, -4, complexes which are required for meiotic divisions (Grandin and Reed, 1993; Dahmann and Futcher, 1995). Similar to mitosis, Sic1 binds to and inactivates Cdk1-Clb5 and Cdk1-Clb6 complexes (Dirick et al., 1998). However, Cdk1-G1 cyclin complexes do not operate in meiosis and, therefore, cannot be responsible for meiotic Sic1 degradation (Colomina et al., 1999). The destruction of Sic1 liberates Cdk1-Clb5 and Cdk1-Clb6 complexes (Stuart and Wittenberg, 1998). Cdk1 is not required for Sic1 removal in meiosis (Benjamin et al., 2003) suggesting that an undefined protein kinase must be catalyzing Sic1 phosphorylation in meiosis.

Ime2 is a meiosis specific serine-threonine protein kinase required for efficient initiation of meiosis (Smith and Mitchell, 1989) that shares some structural similarity with Cdk1 (Hunter and Plowman, 1997) but does not require

cyclins for activity (Hui et al., 2002). The transcription factor Ime1 stimulates the transcription of the early meiotic genes, including *IME2* (Mitchell et al., 1990; Vershon and Pierce, 2000). It has been shown that Ime2 (Foiani et al., 1996) and Cdk1-Clb5, -6 complexes (Dirick et al., 1998) are required for meiotic S phase. An *ime2*Δ mutant has a meiotic entry defect and displays an absence of Sic1 disappearance (Dirick et al., 1998). Our lab has shown Ime2 catalyzes phosphorylation at a non-Cdk1 consensus site on the protein RPA (Clifford et al., 2005). In addition, we have shown that Ime2 does not share the same phosphorylation profile as Cln2 *in vitro* using Sic1 as the substrate (Sawarynski et al., 2007). These data suggest that during meiosis Ime2 does not phosphorylate Sic1 at the same sites as Cdk1-Cln1, -2. This disfavors the hypothesis that Ime2 functionally replaces Cdk1-Cln1, -2. Recently, it was shown that Ime2 and Cdk1 can phosphorylate similar substrates, but their kinase activities are at distinct sites (Holt et al., 2007). The phosphorylation of Ime2 and Cdk1 at distinct sites of substrates might have similar functional consequences and might help explain the functional overlap between Cdk1 and Ime2. It has also been shown that Ime2 can phosphorylate Sic1 at some Cdk1 sites, but this is inefficient to promote the destruction of Sic1 (Sedgwick et al., 2006). Therefore, the mechanism of Sic1 disappearance, which allows for entry into meiotic S phase, is still in question (Figure 2).

II. Origin-dependent replication

The activity of Cdk1-Clb5, -6 initiates DNA synthesis, but prior to S phase

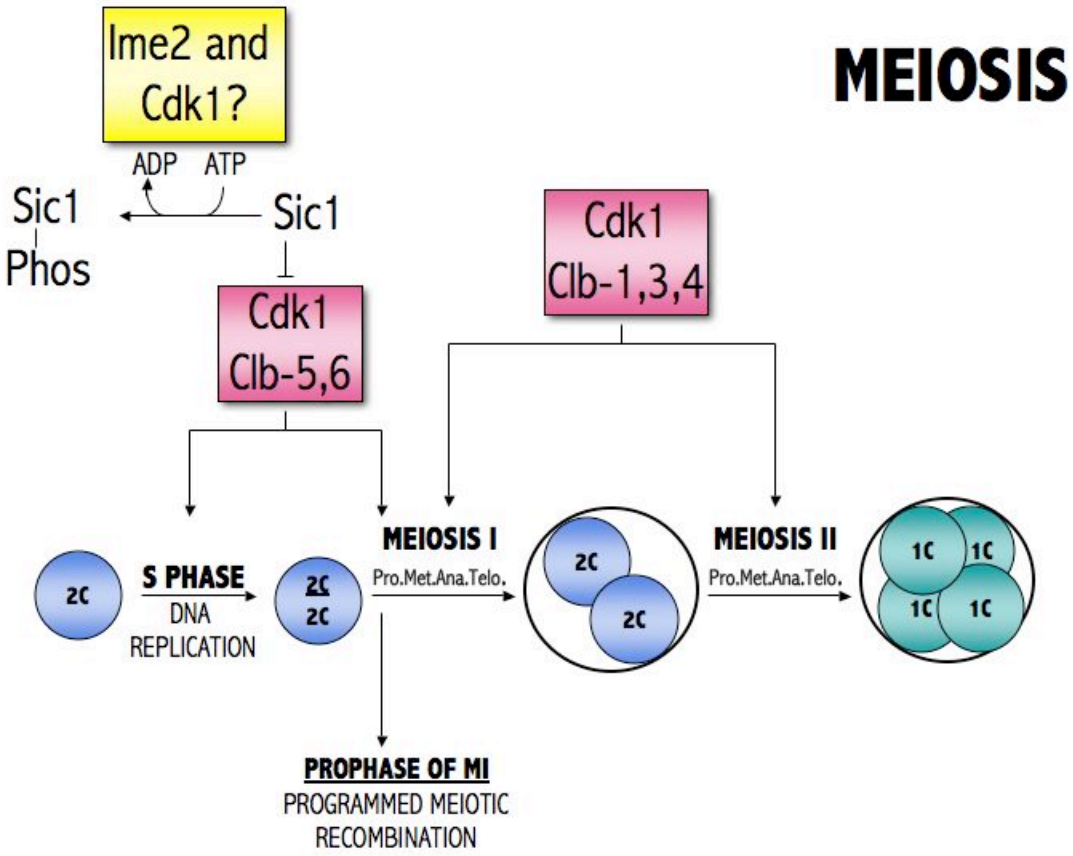


Figure 2. Cdk1 partners with certain cyclins to promote proper progression through the meiotic program.
The schematic shows the Cdk1-cyclin complexes that aid in the different phases of meiosis. Note that the cyclins Cln1, Cln2, and Clb2, which function in mitosis, do not function in meiosis.

a number of orchestrated events must occur in G1. These events are centered on the sites where replication is initiated. Origins define the sites and have proven to be important components for understanding the earliest events in DNA replication. In yeast, origins are often referred to as autonomously replicated sequences (ARS), and are defined by a rich AT consensus sequence, which is not seen in any other organism (Theis and Newlon, 1997). Approximately 200-400 origins replicate the DNA of the sixteen *S. cerevisiae* chromosomes (Raghuraman et al., 2001; Wyrick et al., 2001). In most species, recruiting the replication machinery to the origins on the chromosome regulates DNA replication. The DNA replicated from a single origin is termed a replicon (Jacob and Brenner, 1963). In eukaryotes, there are many origins along the chromosome, and since replication occurs bi-directionally, eventually the replicons will join to form a complete genomic duplication.

The consensus sequence of the ARS is required for the binding of the six-subunit ATPase complex called the ORC (origin recognition complex) (Bell and Stillman, 1992; Diffley and Cocker, 1992; Bell and Dutta, 2002). In G1, ORC recognizes and binds to the origin. It then recruits proteins Cdc6 and Cdt1, and in cooperation they together load the MCM2-7 (minichromosome maintenance) helicase onto the origin to form the pre-RC (pre-replicative complex). Next in S phase, Cd1k and Ddk (Dbf4-dependent kinase) function with Sld2,-3 (Tanaka et al., 2007; Zegerman and Diffley, 2007), Dpb11, Cdc45, and the GINS complex to activate the MCM2-7 helicase. Once activated, the MCM2-7 helicase is thought to unwind the DNA to allow for loading of DNA polymerases and initiation of DNA

synthesis. The complete mechanism of MCM2-7 activation is still unknown, but it is essential that the two proteins Cdc45 and GINS be recruited (Figure 3) (see reviews (Fu and Walter, ; Sclafani and Holzen, 2007)).

III. DNA re-replication

It is important for a cell to undergo only one round of DNA replication per cell cycle or meiotic event, as aneuploidy can lead to cell death or alternatively in humans, contribute to other diseases. Mechanisms that prevent DNA re-replication during the cell cycle have been largely conserved in eukaryotes, with some variation through evolution. After DNA replication has initiated, origin re-licensing must be inhibited to prevent any subsequent rounds of DNA replication. This involves inhibition of proteins responsible for origin licensing (pre-RC assembly). Experiments have shown that pre-RC assembly is inhibited by Cdk1 activity (Dahmann and Futcher, 1995). The events known to occur are Cdk1 inhibitory phosphorylation of ORC (Nguyen et al., 2001; Vas et al., 2001), Cdk1-mediated phosphorylation of Cdc6 and its subsequent degradation (Jallepalli and Kelly, 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., 2000). All these events must occur to prevent DNA re-replication during the cell cycle, and they further emphasize that the prevention of DNA re-replication is due to a direct inhibition of the proteins required for licensing.

Prevention of DNA re-replication is equally important in meiosis, but few studies have been conducted that define whether the same mechanisms that

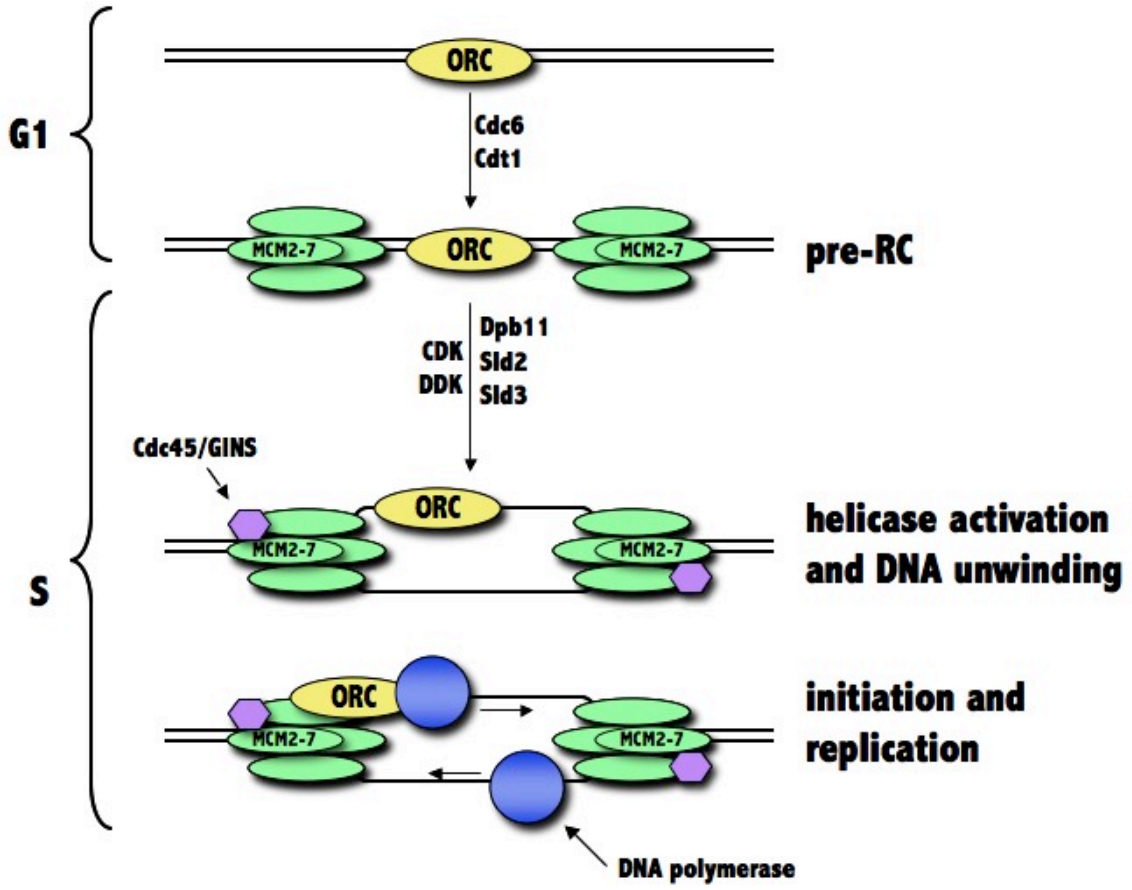


Figure 3. Proteins recruited to origins for eukaryotic DNA replication
This schematic shows proteins responsible for origin-dependent replication. Not shown is a protein RPA, which is known to bind to single strand DNA and aid in the replication process. The pre-RC is formed in G1, and then activated in S phase by the combined action of CDK and DDK.

prevent mitotic DNA re-replication also prevent meiotic DNA re-replication. There are two stages during which re-replication must be blocked in meiotic cells. One is between MI and MII, and the other is during the S phase window. Understanding how meiotic S phase cells block re-replication is a major focus of this dissertation.

A few groups, including ours, have shown the induction of meiotic DNA re-replication. Strich et al., 2004 showed that the B-type cyclins Clb1 and Clb5 can induce re-replication in meiotic cells when overexpressed by specific promoters, and that the spores are viable, haploid, and display normal Mendalian traits. In addition, they showed that re-replication was enhanced when additional mutation was introduced in proteins involved in the formation of the synaptonemal complex (SC). The cells with over replicated DNA produce asci containing up to twenty spores, which were termed “multads”. These data suggest that re-replication control is different in mitotic and meiotic cells because overexpression of Clb1 does not cause re-replication during the cell cycle.

Rice et al., 2005 were also able to show evidence of meiotic DNA re-replication. In their system, an activated allele of *CDC28*, the gene that encodes Cdk1, or the deletion of *SWE1,* the gene that encodes a protein kinase that catalyzed inhibitory phosphorylation of Cdk1, was able to complete several rounds of meiotic DNA replication. These cells exhibited a phenotype termed “multispore”. They also found that multispore asci required Spo11, a transesterase essential for meiotic homologous recombination. This suggests

that cells must initiate homologous recombination for the multispore formation to occur.

IV. Homologous recombination

Homologous recombination occurs in both mitosis and meiosis, although the purposes are different. In the cell cycle of *S. cerevisiae*, recombination serves to repair DNA damage (Game et al., 1980; Kunz and Haynes, 1981) where sister chromatids are the preferred homologs as substrates (Kadyk and Hartwell, 1992). This is quite different than meiotic recombination, due to the fact that homologous recombination in meiosis is a programmed event and it prefers the use of homologous chromosomes as substrates. In fact, during meiosis mechanisms are in place that prevents the use of sister chromatids as substrates (Wan et al., 2004). Programmed meiotic recombination occurs during prophase of MI. In meiosis, a primary function of recombination is to ensure proper chromosome segregations by establishing a physical connection between homologous chromosomes. Meiotic recombination is comprised of tightly arranged events in which homologous chromosomes must undergo pairing, recombination, and synapsis before they segregate to opposite poles at the first meiotic division.

Repair of un-programmed DNA double strand breaks

During meiosis, DNA double strand breaks (DSBs) are programmed and required for homologous recombination to occur. In mitotic cells, DNA damaging

agents such as radiation and different types of chemicals, can elicit DSBs, which can lead to cell death or chromosomal abnormalities. Homologous recombination, where the sister chromatid is the preferred template, is the predominant accurate form of DSB repair mechanism in mitotic cells. However, an alternative mechanism can occur, named break-induced replication (BIR), in which strand invasion leads to the formation of a unidirectional DNA replication fork and subsequent duplication of an entire chromosome arm (see review (McEachern and Haber, 2006)).

BIR events begins as one ended recombination events for two possible reasons: 1) only one end of the DSB is free, or 2) only one of the two strands of the DSB succeeded in strand invasion of a homologous chromosome. BIR is also known to play a key role in repair of stalled or broken replication forks (Kuzminov, 1995; Seigneur et al., 1998; Michel, 2000), as well as in the maintenance of eroding telomeres. Laundblad and Blackburn first demonstrated that cells incapable of maintaining chromosome ends by telomerase somehow managed to maintain telomere sequence (Lundblad and Blackburn, 1993). This eventually led to the understanding that BIR is involved in telomere elongation when telomerase is absent.

For homologous recombination to occur properly as a repair mechanism in mitotic cells, a few critical proteins must be present. Mitotic cells have one major protein that catalyzes strand exchange; Rad51. Therefore, if this protein is nonfunctional the cell must compensate and choose a different pathway to repair DSBs. The cell can turn to BIR to repair the DSB because it can occur in the

absence of Rad51. This is actually a common pathway in *rad51Δ* cells. BIR can prime DNA synthesis of the invading strand, and requires Rad52 and Rad59 to promote invasion of the 3' ssDNA end (Cortes-Ledesma et al., 2007), followed by the requirement of DNA Polα primase complex and Polδ to initiate new DNA synthesis (Lydeard et al., 2007). The subunit Pol32 of DNA Polδ was found to be essential for BIR, which is interesting because it was previously understood to be the non-essential subunit of Polδ when functioning in replication and gene conversion events (Lydeard et al., 2007).

The events following programmed DNA double strand breaks

Homologous recombination that occurs in prophase of the first meiotic division is initiated form programmed DNA double strand breaks (Szostak et al., 1983; Sun et al., 1989). Cdk1-Clb5, -6 complexes are required for initiation of meiotic recombination (Figure 2) (Smith et al., 2001; Henderson et al., 2006). DSBs are processed in a proteinaceous structure, the synaptonemal complex (SC), which forms along the meiotic chromosome. The formation of the SC depends on recombination, and in turn some aspects of recombination depend on the SC (Paques and Haber, 1999). To understand the complexity of how DSBs are formed, note that eleven genes are required for DSB formation. These include *RAD50, SPO11, MRE11, XRS2, MEI14, MER1, MER2, MRE2, REC102, REC104,* and *REC114* (Paques and Haber, 1999). In addition to the eleven genes, *RED1, HOP1,* and *MEK1* genes are implicated in the formation of the SC; specifically, they are involved in the formation of axial elements between sister

chromatids and are required for full levels of meiotic DSBs (Mao-Draayer et al., 1996; Xu et al., 1997).

Although there are many genes that have roles in DSBs formation, the Spo11 transesterase has been proposed to be the direct protein involved in generating DSBs early in meiotic prophase (Keeney et al., 1997). It is important to note that no breaks are observed when any of the eleven genes are deleted (Malone et al., 2004). DSBs occur along several points of the chromatids, after which the 5' ends are resected by a 5' to 3' exonuclease, which creates 3' single stranded overhangs on either side of the break (Aylon and Kupiec, 2004; Bannister and Schimenti, 2004).

Rad51 and Dmc1 play a critical role in strand invasion and catalyze invasion of the 3' tails into intact homologous nonsister chromatids (Collins and Newlon, 1994; Hunter and Kleckner, 2001). The 3' overhang also acts as a primer for the initiation of DNA synthesis. This process leads to the formation of a double Holliday junction (DHJ) in mid-prophase (Holliday, 1964; Schwacha and Kleckner, 1995). Dmc1 was originally identified in a screen for meiotically induced genes (Bishop et al., 1992), and when mutated it accumulates DSB recombination intermediates and arrests late in meiotic prophase (Bishop et al., 1992). Rad51 is necessary for both mitotic and meiotic recombination, whereas Dmc1 is specific to meiosis. The two proteins are detectable on meiotic chromosomes by immunostaining (Bishop et al., 1992; Dresser et al., 1997; Shinohara et al., 2000).

V. Checkpoint control

Proper completion of either mitosis or meiosis requires an orderly sequence of events. Many regulatory mechanisms are in place to ensure that orderly progression is maintained so that late events follow the early events. In 1989, Leland Hartwell and Ted Weinert studied these controls in mitosis and termed them as 'checkpoints'. Twenty-one years later, scientists have uncovered a number of checkpoints that can regulate distinct stages of the cell cycle and the meiotic program (Hartwell and Weinert, 1989).

The pachytene checkpoint

In meiosis, more than 200 DSBs are introduced into the genome (Hochwagen and Amon, 2006). If cells initiate chromosome segregation before programmed meiotic recombination, entire chromosomes or chromosome fragments could be lost. The meiotic recombination checkpoint (also known as the pachytene checkpoint) delays progression into meiosis until DSBs are repaired. This checkpoint operates to prevent chromosome segregation when recombination intermediates are present. Many proteins are involved in the pachytene checkpoint, which is described below (Figure 4).

a. Dmc1

When mutants of proteins required for strand invasion, such as *DMC1*, are produced, the recombination/pachytene checkpoint is activated. These mutants

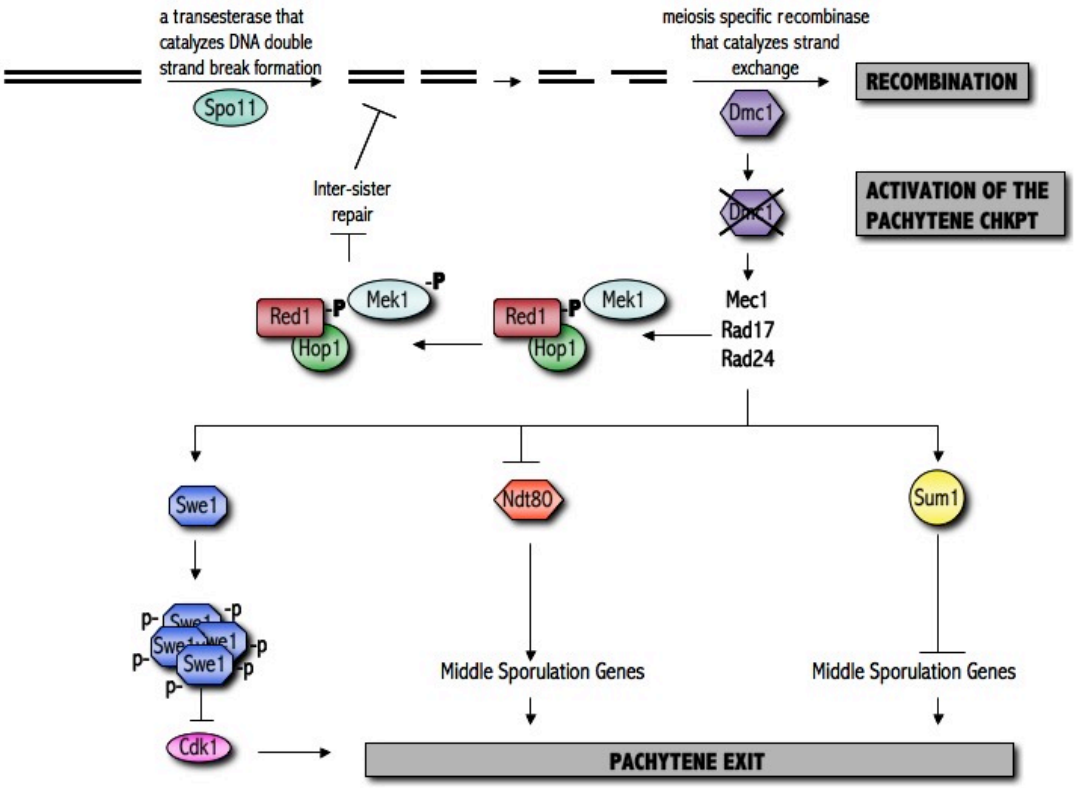


Figure 4. Activation of the pachytene checkpoint.
Unprocessed recombination intermediates by a gene deletion of *DMC1* can activate the pachytene checkpoint. The three main downstream targets prevent exit from pachytene (sub stage of prophase) by manipulating Cdk1 and inhibiting middle sporulation genes. Once recombination intermediates are processed, the signal is released and cells resume through meiosis.

cause the DNA to fail invasion into the homologous chromosome and result in an accumulation of large amounts of hypersected DSBs. This will cause a delay in prophase I (Bishop et al., 1992; Leu et al., 1998; Gerton and DeRisi, 2002). The large amounts of hypersected DNA are coated with Rad51, and it is suggested that this nucleoprotein may constitute a signal (Lydall et al., 1996; Shinohara et al., 1997). Mutants in *DMC1* cause an arrest in late G2/prophase that requires checkpoint genes *RAD17, RAD24,* and *MEC1* (Lydall et al., 1996).

b. Mitotic checkpoint genes that are also active in meiosis

The Rad17, Rad24, and Mec1 proteins were first characterized in the context of the mitotic DNA damage checkpoint. In 1996, Lydall et al. showed that these same proteins are also involved in the pachytene checkpoint (Lydall et al., 1996). By creating double mutants, it was revealed that *RAD17, RAD24,* and *MEC1* genes allow *dmc1* mutant cells to progress through meiosis. The *RAD17* gene is a conserved recognition protein that shares structural similarities with the proliferating cell nuclear antigen (PCNA). PCNA forms a homotrimeric ring structure around the DNA, and is commonly referred to as the “sliding clamp” that recruits polymerases onto the DNA (Thelen et al., 1999). Two proteins that Rad17 commonly associated with are Ddc1 and Mec3 (Paciotti et al., 1998; Kondo et al., 1999). The human homolog of this complex is commonly referred to as the “9-1-1 complex” (Carballo and Cha, 2007). One function of the complex involves a 3’ to 5’ exonuclease activity, which may be required to convert DSBs

into a recognizable triggering substrate for a checkpoint response (Zhang et al., 2001).

The Rad17-Ddc1-Mec3 complex interacts with another mitotic DNA damage checkpoint protein, Rad24, which contains a clamp loading function that may act to load the Rad17-Ddc1-Mec3 PCNA-like complex (Lowndes and Murguia, 2000; Venclovas and Thelen, 2000). In 2001, Zhang et al. proposed that Rad24 might function as the initial sensor by binding to the DSB, after which the Rad17p sliding complex would be recruited and therefore create a platform which allows for downstream signaling (Zhang et al., 2001).

Rad24 and the Rad17-Ddc1-Mec3 complex are considered to act upstream of Mec1, which was initially described as a mitosis entry checkpoint gene I (Weinert, 1992). Recent understanding of its role in meiosis has suggested that it is essential for and meiotic chromosomal processes. In fact, many meiotic processes that Mec1 is known to be involved in, which include S-phase progression, recombination, inter-homolog bias, and chromosome synapses (Carballo and Cha, 2007). In addition, Mec1 plays a central role in the localization and phosphorylation of many proteins including Mek1 (Roeder and Bailis, 2000) and Hop1 (Carballo and Cha, 2007). In the pachytene checkpoint, Rad24 and Rad17-Ddc1-Mec3 complex recognize incomplete recombination and activate the Mec1 kinase, as in mitosis (Bailis and Roeder, 2000). The active kinase then phosphorylates the meiosis specific kinase, Mek1.

c. Mek1, Red1, Hop1

In *S. cerevisiae,* the meiotic chromosomal proteins Mek1, Red1, and Hop1 are involved in the pachytene checkpoint. The mutants of these proteins are known to have defects in recombination products, but no apparent prophase arrest in the mutant cells (Rockmill and Roeder, 1990; Leem and Ogawa, 1992). Mek1 is a protein kinase whose phosphorylation requires the initiation of meiotic recombination (Bailis and Roeder, 1998), as well as certain proteins known to be involved in sensing DNA damage (Rad17, Rad24, and Mec1). Mek1 exists in a complex with two other meiosis specific chromosomal core components, Hop1 and Red1. *RED1* and *HOP1* genes encode proteins that are components of axial elements (AEs) essential for SC development and production of viable spores (Rockmill and Roeder, 1990; Hollingsworth and Ponte, 1997; Smith and Roeder, 1997). The condensation of the sister chromatids along a protein core generate AEs, which are the precursors to the SC (Hollingsworth and Ponte, 1997). Yeast two-hybrid analysis and co-immunoprecipitation assays have revealed that Red1 and Hop1 proteins interact with each other as well as co-localizing with AEs (Hollingsworth and Ponte, 1997; Smith and Roeder, 1997; de los Santos and Hollingsworth, 1999). The same assays have revealed that Red1 and Mek1 also directly interact with each other (Bailis and Roeder, 1998).

Within the pachytene checkpoint, it is thought that Hop1 binds to DSB sites recruiting Red1, after which phosphorylation of Red1 may provide a recognition sequence for Mek1 binding (Wan et al., 2004). Once bound, Red1 is required for the phosphorylation of Mek1 (Woltering et al., 2000) by the kinase

Mec1 (Bailis and Roeder, 2000). It is in this way that Red1 acts as an adapter protein between Mec1 and Mek1 (Hochwagen and Amon, 2006). Other models that have suggested Red1 being a substrate for Mek1 (Roeder and Bailis, 2000), but evidence within the field favors the model defined by Wan et al. Recent evidence suggests that these proteins operate in the pachytene checkpoint to prevent inappropriate repair between sister chromatids (Figure 5) (Niu et al., 2007).

d. Pch2

The protein Pch2 functions in wild type cells and pachytene arrested cells. Until this point, we have discussed induction of the pachytene checkpoint through the deletion of *DMC1*. Research has shown that in addition to *DMC1*, deletion of *HOP2* or *ZIP1* can also activate the pachytene checkpoint (Rockmill and Roeder, 1991; Bishop et al., 1992; Sym et al., 1993; Leu et al., 1998). Pch2 was originally identified in a screen to search for novel genes able to bypass the *zip1*Δ induced checkpoint arrest (San-Segundo and Roeder, 1999). Zip1 is a component of the synaptonemal complex. Previously, little was known about Pch2, but recent reports have shown that its functions are beyond being a member of the AAA (ATPases associated with diverse cellular activities) family, which is highly conserved and involved in a large variety of processes (Beyer, 1997). Pch2 is required for the progression of recombination during normal pachytene, because the absence of Pch2 reduces and delays progression of crossover and non-crossover events (Borner et al., 2008). In *zip1*Δ pachytene arrested cells, the

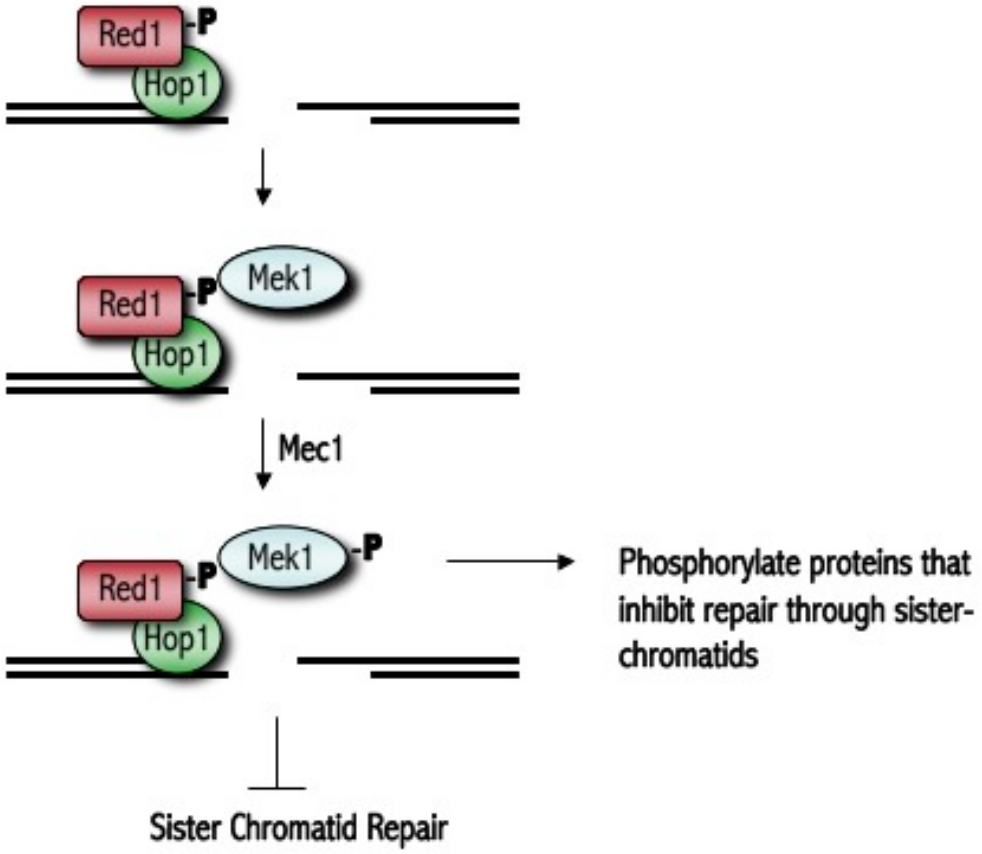


Figure 5. A biological pathway that prevents the use of sister chromatids as repair during meiosis.
This model shows how Mek1, Red1, and Hop1 prevent the use of sister chromatids for repair during meiosis. These proteins are also targeted during the pachytene checkpoint to prevent repair of the unprocessed recombination intermediates, which initially activated the checkpoint.

crossover pathway is blocked, and it is thought that Pch2 keeps the stalled recombination complexes intact to persist arrest. But if Pch2 is absent in arrested cells, then the recombination complexes can deteriorate, which allows for DNA events to progress. Other researches have shown that *pch2*Δ can bypass *dmc1*Δ arrest, but only about 50% of the cells are able to progress through to the meiotic divisions (San-Segundo and Roeder, 1999). Therefore, meiotic arrest by *dmc1*Δ is partially dependent on Pch2.

e. Downstream targets Swe1, Ndt80, and Sum1

Swe1 is a kinase that can inhibit Cdk1 by phosphorylation on Tyr19 (Booher et al., 1993)) and is thought to function in the pachytene checkpoint. Mitotically, Swe1 most efficiently inhibits Cdk1 when complexed with Clb2, modestly inhibits Cdk1-Clb3, -4, and does not inhibit Cdk1-Clb5 -,6 or Cln complexes (Hu and Aparicio, 2005). Swe1 is not required for the DNA damage checkpoint (Amon et al., 1992), but it was found to be essential for the morphogenesis checkpoint (Lew, 2000) and the pachytene checkpoint (Leu and Roeder, 1999). Bud formation requires the polarization of the actin cytoskeleton, but when stress perturbs the actin cytoskeleton the morphogenesis checkpoint is triggered (Lew and Reed, 1995; McMillan et al., 1998). The checkpoint turns on a cell cycle arrest in G2 by blocking degradation of Swe1 (Sia et al., 1998), and inhibiting Mih1, the Cdc25-family phosphatase that de-phosphorylates Cdk1 at Tyr19 (Harrison et al., 2001). Many factors go into play in the degradation of

Swe1, but, mainly, Swe1 hyper-phosphorylation makes the protein susceptible to ubiquitin degradation (Kaiser et al., 1998).

When activated within the pachytene checkpoint, it is thought that Swe1 can prevent pachytene exit by its accumulation and phosphorylation, which in turn has an inhibitory phosphorylation function on Cdk1 (Leu and Roeder, 1999). Although it is unclear which Cdk1-Clb complex Swe1 inhibits to aid in pachytene arrest, it is known that exit from pachytene requires Cdk1 (Shuster and Byers, 1989; Xu et al., 1995) upregulation of Clb1, and to a lesser extent upregulation of Clb3, -4 (Grandin and Reed, 1993; Dahmann and Futcher, 1995). When originally defined in the pachytene checkpoint, *swe1*Δ mutants were found to enter meiotic divisions in *dmc1*Δ-induced checkpoint arrested cells as efficiently as wild type cells in the YAB36 *S.cerevisiae* strain background (Leu and Roeder, 1999). However, others have found that in a different strain background (SK1), only 10-30% of *swe1*Δ mutants bypass the *dmc1*Δ-induced pachytene checkpoint and enter meiotic divisions (Pak and Segall, 2002).

Another target of the pachytene checkpoint is Ndt80, which is a transcriptional activator of approximately 150 middle sporulation genes (MSGs). As part of its function, Ndt80 binds to the middle sporulation elements (MSEs) located in the promoter region of the MSGs (Chu et al., 1998; Chu and Herskowitz, 1998; Hepworth et al., 1998). Ndt80 induces transcription of genes required for nuclear divisions (*CLB1)* and spore formation (*SMK1*), in addition to stimulating transcription of itself (*NDT80).* In *dmc1*Δ-induced checkpoint cells, Ndt80 is inactive and MSGs are not expressed. Activation of the pachytene

checkpoint prevents the accumulation and phosphorylation of Ndt80, which depends on Ime2 (Tung et al., 2000; Benjamin et al., 2003).

The third downstream target of the pachytene checkpoint is Sum1, a transcriptional repressor of Ndt80 targets. Deletion of *SUM1* can bypass *dmc1*Δ-induced arrest (Lindgren et al., 2000; Pak and Segall, 2002). When cells are mitotically dividing, Sum1 represses the expression of many sporulation genes (Xie et al., 1999; Pak and Segall, 2002). Sum1 binds MSE sites in the promoter regions of MSGs, but its important to note that it does not bind to the MSEs of all the MSGs (Xie et al., 1999). Ndt80 and Sum1 can compete for binding at MSEs, but small changes in the sequence of the MSE can affect which protein binds (Pierce et al., 2003). Some MSE sites are Ndt80-dependent activator sites, and some MSE sites are Sum1-dependent repressor sites. Therefore expression level of the MSGs is dictated, in part, by the amount of Ndt80 and Sum1 present and the affinity of the proteins for the MSE site (Xie et al., 1999). In terms of the pachytene checkpoint, it is possible that Sum1 functions to repress the MSGs to aid in pachytene arrest. When Sum1 binds to DNA, it recruits other proteins that participate in targeted histone deacetylation that represses corresponding genes by establishing an inactive chromatin state (Pijnappel et al., 2001; McCord et al., 2003).

G1-S DNA damage checkpoint

Just as an accumulation of recombination intermediates activates the pachytene checkpoint during meiosis, another checkpoint is activated during the

G1 to S transition in response to DNA damage in mitosis. Damage could occur as a result of a replication fork collapse, ionizing radiation (IR), chemical compounds such as benzo(a)pyrene, or endogenous compounds such as free radicals. These inducers can yield DNA double stranded breaks (DSBs) or single stranded DNA (ssDNA), which have been shown to initiate the damage signal (Garvik et al., 1995).

The key components of the G1-S DNA damage checkpoint in *S.cerevisiae* are the phosphoinositol-3-related kinases (PIKK) Mec1 and Tel1. The human homolog of Mec1 is ATR (ataxia-telangiectasia and Rad3-related), and null mutations, which cause a complete inactivation of the gene, have serious health consequences. Namely, mutations in ATR are known to contribute to the autosomal recessive disorder Seckel syndrome. Patients diagnosed with Seckel syndrome, or microcephalic primordial dwarfism, have dramatic microcephaly and developmental delay (Goodship et al., 2000). A protein kinase that is functionally redundant to Mec1 is Tel1, whose human homolog is ATM (ataxia-telangiectasia mutated). Mutations in ATM can contribute to ataxia-telangiectasia (AT), which is a neurodegenerative disorder with a predisposition to cancer (Abraham, 2001).

Both Mec1 and Tel1 function as signal transducers and do not appear to directly recognize DNA damage. Rather, Mec1 and Tel1 recognize specific complexes, which have already recognized the initial damage. When DNA damage elicits single strand DNA (ssDNA), the protein RPA (replication protein A) binds to ssDNA and triggers the recruitment of the protein kinase Mec1, which

causes a series of cascading events (Zou and Elledge, 2003). Tel1 is recruited to the DNA by the end-binding Mre11-Rad50-Xrs2 complex (Nakada et al., 2003), and is more important for maintaining normal telomere length (Lustig and Petes, 1986; Ritchie et al., 1999). Another complex that recognizes DNA damage sites is the Rad24-Rfc2-5 complex, which in turn helps load the PCNA-like Ddc1-Mec3-Rad17 complex (*S.cerevisiae* homologs of the 9-1-1 complex) (Kondo et al., 2001; Melo et al., 2001). These complexes load independently of Mec1, but are required for functioning of Mec1 (de la Torre-Ruiz et al., 1998). Once all the proteins and kinases have been recruited to the DNA damage site, several proteins become rapidly phosphorylated in a Mec1/Tel1 dependent manner (Lowndes and Murguia, 2000; Abraham, 2001). Some downstream effectors of Mec1/Tel1 signaling are the kinases Rad53 and Rad9.

Once DNA damage has been detected, Mec1/Tel1 are recruited to site where they catalyze phosphorylation of Rad9. First, this hyper-phosphorylated form of Rad9 promotes Mec1-Rad53 interaction specifically by Mec1-mediated phosphorylation of Rad53 and its subsequent activation (Sweeney et al., 2005). Then Rad9 acts a scaffold to facilitate Rad53 auto-phosphorylation (Emili, 1998; Sun et al., 1998; Gilbert et al., 2001). Now active, the protein kinase Rad53 can phosphorylate the transcriptional regulator Swi6 during G1 of the cell cycle. This will in turn inhibit a transcriptional activator Swi4, required for transcription of G1 cyclins, causing a G1 arrest until damage can be repaired.

VI. Health consequences

There are many health consequences associated with aberrant DNA metabolism and some are associated with cancer. For example, the DNA damage checkpoint described above is abolished in patients with AT (Painter et al, 1982). Also, the tumor suppressor gene p53, which can detect DNA damage, is mutated in over 50% of solid tumors (Hollstein et al., 1991). As in the cell cycle, aberrant DNA metabolism during meiosis can have serious health consequences, as illustrated by diseases such as Down's syndrome resulting from misegregation. Errors in DNA replication and recombination during meiosis can have equally profound health effects resulting from point mutations, chromosomal translocations, and changes in ploidy.

Specifically, chromosomal translocations have been linked to many types of cancer. These events are rearrangements of DNA between non-homologous chromosomes. One common type of translocation seen in chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL) is the translocation of a region of chromosome 22 to the *ABL1* gene of chromosome 9 (Kurzrock et al., 2003).

Alternatively recombination between non-allelic DNA segments that share high sequence similarity can cause serious health consequences. Usually within the chromosome there are regions of low copy repeats (LCRs), which share $\geq$ 90% sequence identity over one thousand base pairs. Within these regions, homologous recombination can occur and lead to detrimental effects of the developing gametes. Research within this field has yet to answer if NAHR (non-

allelic homologous recombination) events occur in meiosis or in mitotically dividing germ line cells. Regardless, these events lead to many inherited genetic disorders. To name a few, Hunter's Syndrome is a lysosomal storage disease caused by the inversion of a portion of *IDS* (iduronate sulfatase gene), and β-Thalassaemia is an anaemia caused by the deletion of the β-globin gene (see review (Sasaki et al.)). It is clear from these examples that meiotic recombination, as well as the DNA replication process that precedes it, must be meticulously controlled to avoid the generation of systemic and potentially harmful genetic alterations.

CHAPTER 2

MATERIALS AND METHODS

I. Strains and Plasmids

Yeast strains used in this study were in the W303 background (Table 1 and Table 2). Plasmids encoding galactose-inducible Sic1 derivatives with HA and 6xHis tags at the C–terminus were kindly provided by Raymond Deshaies. One version, referred to here as Sic1WT^{HA}, is degraded properly during vegetative growth, while the other, referred to here as Sic1ΔP^{HA}, is resistant to degradation during vegetative growth due to mutations of multiple Cdk1-targeted phosphorylation sites (Verma et al., 1997). Both proteins contain a T2A mutation, while Sic1ΔP^{HA} contains additional T5GP, S33A, and S76A mutations. Approximately 1kb DNA regions were PCR amplified with BamHI-tailed primers using the following templates: pNH59-2 (Hollingsworth et al., 1990), kindly provided by Dr. Jacqueline Segall (University of Toronto), for *HOP1pr*. This product was inserted upstream of *SIC1WT^{HA}* and *SIC1ΔP^{HA}* at the BamHI site in the two plasmids, and the sequences of cloned promoters were verified. The resulting plasmids were then digested with Sse8387I (Amersham) or its isoschizomer SbfI (New England Biolabs) for integration at the *URA3* locus of various strains. All insertions were verified by PCR. A spontaneous revertant of the diploid strain containing *HOP1pr-SIC1ΔP^{HA}* (YGB495) to uracil auxotrophy was isolated by counter-selection with fluoro-orotic acid (Toronto Research

Table 1. Haploid yeast strains.

Name	Relevant Genotype
W303	MATa ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100
YGB502	MATa ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 $SIC1^{13myc}$::kanMX6

Table 2. Diploid yeast strains.
All yeast strains listed are congenic with YGB138.

Name	Relevant Genotype
YGB138	*MATa/α ade2-1/" ura3-1/" leu2-3,112/" his3-11,15/" trp1-1/" can1-100/"*
YGB495	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3*
YGB513	*SIC1/SIC1^{13myc}::kanMX6*
YGB514	*ura3-1/ura3-1::HOP1pr::SICWT^{HA}::URA3 SIC1/SIC1^{13myc}::kanMX6*
YGB515	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 SIC1/SIC1^{13myc}::kanMX6*
YGB583	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 ndt80Δ::kanMX4/"*
YGB604	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/"*
YGB613	*ura3-1/ura3-1::HOP1pr::SICWT^{HA}::URA3*
YGB617	*ura3-1::HOP1pr::SICΔP^{HA}::URA3/"*
YGB672	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 pol32Δ::kanMX4/"*
YGB673	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 mek1Δ::kanMX4/"*
YGB678	*ura3-1/ura3-1::HOP1pr::SICWT^{HA}::URA3 pol32Δ::kanMX4/"*
YGB679	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/" mek1Δ::kanMX4/"*
YGB687	*swe1Δ::kanMX4/"*
YGB688	*ura3-1/ura3-1::HOP1pr::SICWT^{HA}::URA3 swe1Δ::kanMX4/"*
YGB689	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 swe1Δ::kanMX4/"*
YGB697	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/" swe1Δ::kanMX4/"*
YGB700	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/" pch2Δ::kanMX4/"*
YGB703	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 pch2Δ::kanMX4*
YGB712	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 hop1Δ::kanMX4/"*
YGB713	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/" hop1Δ::kanMX4/"*
YGB721	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 red1Δ::kanMX4/"*
YGB722	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/" red1Δ::kanMX4/"*
YGB757	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 SIC1/SIC1^{13myc}::kanMX6 dmc1Δ::natR/"*
YGB758	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 rad9Δ::kanMX4/"*
YGB759	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/" rad9Δ::kanMX4/"*
YGB764	*dmc1Δ::natR/"*
YGB765	*dmc1Δ::natR/" swe1Δ::kanMX4/"*
YGB785	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 sum1Δ::kanMX4/"*
YGB786	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/" sum1Δ::kanMX4/"*
YGB788	*ura3-1/ura3-1::HOP1pr::SICΔP^{HA}::URA3 dmc1Δ::natR/" mec1Δ::LEU2/" sml1Δ::kanMX4/"*

Name	Relevant Genotype
YGB789	*ura3-1/ura3-1::HOP1pr::SIC∆P^{HA}::URA3 mec1∆::LEU2/" sml1∆::kanMX4/"*
YGB792	*dmc1∆::natR/" sum1∆::kanMX4/"*
YGB807	*SIC1^{13myc}::kanMX6/"*
YGB808	*ura3-1/ura3-1::HOP1pr::SIC∆P^{HA}::URA3 SIC1^{13myc}::kanMX6/"*
YGB809	*ura3-1/ura3-1::HOP1pr::SIC∆P^{HA}::URA3 SIC1^{13myc}::kanMX6/" dmc1∆::natR/"*

Chemicals), which indicated loss of the *HOP1pr-SIC1*ΔP^{HA} element (Boeke et al., 1984). For construction of 2X *SIC1*ΔP^{HA}, we inserted the digested *SIC1*ΔP^{HA} plasmid at the *URA3* locus of 2 separate haploids. We checked each haploid by PCR to ensure integration of one plasmid using primers RS1 (5' tgaaaacctctgacacatgcag 3') and RS2 (5' cttgattagggtgatggttcacg 3'). We then mated the haploids and performed tests to select for a diploid.

Deletion mutations were generated in haploids of mating types *MATa* and *MAT*α by site-specific integration of markers PCR-amplified from the genomic DNA of previously characterized deletion mutants (Baudin et al., 1993). Deletion mutants produced with a *kanMX4* marker were resistant to the drug G418 (Mediatech Inc.). Where necessary, deletion mutants were switched to *natR* markers using the p4339 plasmid kindly provided by Charles Boone (University of Toronto), which allowed for nourseothricin resistance (Werner BioAgents). All haploid mutants were verified by PCR. Forward and reverse primers were designed that recognized the marker and/or the ORF of the corresponding gene. Based on different combinations of forward and reverse primers recognizing the marker and/or the ORF of the corresponding gene, we were able to verify complete deletion of all genes in this report. Deletion mutants were constructed to produce *MATa gene of interest*Δ and *MAT*α *gene of interest*Δ *SIC1*ΔP^{HA} that were then mated. The resulting diploid strain contained homozygote deletions of one of the following genes (*MEC1, MEK1, RED1, HOP1, PCH2, SWE1, RAD9, SUM1*), and was used to test whether the deletion affected our DNA re-replication phenotype. To test whether the homozygote deletion alleviated our

checkpoint block to DNA re-replication, we constructed strains by mating haploid deletion mutants *MATa gene of interest*Δ *dmc1*Δ with *MATα gene of interest*Δ *dmc1*Δ *SIC1*ΔP^{HA}. We performed subsequent mating tests to ensure proper selection of diploids. We then performed PCR checks, using the same PCR primer combinations used to check the haploids. Once the homozygote deletion mutant was selected, we were able to analyze its behavior meiotically by synchronous sporulation. The *ndt80*Δ mutant was constructed similarly (Sawarynski et al., 2009).

Heterozygote and homozygote *SIC1*^{13myc} strains were first constructed in haploids by the method described in (Longtine et al., 1998). Briefly, the 13myc tag was PCR amplified to have regions of homology to the C-terminal end of the *SIC1* gene using the plasmid pFA6-13myc::kanMX6 (Longtine et al., 1998). Once constructed in a haploid (YGB502), we mated with a subset of strains that contained other mutations and deletions of interest. We then sporulated the diploids and performed a series of tetrad dissections to isolate mating types that contained different combinations of mutants, which we could use to generate heterozygote and homozygote *SIC1*^{13myc} diploid strains (YGB 513, 514, 515, 757, 787, 807, 808, 809)

II. Alpha Factor and Synchronous Sporulation

All yeast incubations were conducted at 30°C. Mitotic growth was conducted with rich media (YPD). To conduct a synchronized mitotic time course we diluted a saturated cell population to an OD_{600} of 0.2 and incubated for 2 hrs

at 30°C. Next, the yeast pheromone α factor was added to a final concentration of 2.5μM. Cells were incubated for an additional 2 hrs at 30°C, α factor was removed by washing with sterile water, and cells were resuspended in fresh YPD to conduct a mitotic time course. Aliquots were taken every 15 minutes, up to 75 minutes, and analyzyed by flow cytometry and SDS-PAGE.

Meiosis was induced by starvation based on an established procedure for synchronous sporulation (Padmore et al., 1991). In this method, yeast cells were taken from an overnight YPD culture and diluted to an OD_{600} of 0.2 in YPA media (1% yeast extract / 2% peptone / 2% potassium acetate). They were grown for 15.5 -16 hrs and then switched to a sporulation medium, SPM, consisting of 0.3% potassium acetate and 0.02% raffinose supplemented with leucine, arginine, and histidine each at 250 μM, tryptophan at 100 μM, and uracil at 50 μM. Yeast strains were normalized in the SPM medium to the yeast strain with the lowest OD_{600}. Aliquots were taken at 0hr and subsequent time points for 2, 4, 6, 8, 10, 12, 24 hours depending on the experiment.

III. Whole Cell Lysate Preparation and Western Blot Analysis

Denatured whole-cell extracts were prepared as previously described (Kushnirov, 2000). Within individual experiments cells harvested were normalized to the same optical density. The following description is based on 2ml aliquot of yeast cells. Cells were harvested by centrifugation then stored at -80°C until processing. Aliquots were treated with 100-200μl of 0.1N NaOH. Cells were then placed on ice for 10 minutes, centrifuged to remove NaOH, and vortexed in 50μl

of 1X SDS/PAGE buffer to dissolve the pellets. 1X SDS/PAGE buffer was adapted from Laemmli formula (Laemmli, 1970), which contains 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.002% bromophenol blue. Samples were then heated at 95°C for 5 minutes and subjected to SDS/PAGE, and the separated proteins were transferred to nitrocellulose membranes (GE Healthcare) in 25 mM Tris/192 mM glycine/20% methanol. For most applications, samples were loaded onto a 10% SDS-PAGE gel. Primary antibodies included mouse anti-hemagglutinin monoclonal (HA-11, Covance), rat anti α-tubulin polyclonal (Serotec), and mouse anti-myc (Santa Cruz). Signals were generated with IRDye 800-conjugated goat anti-rat (Rockland), or Alexa Fluor 680 goat anti-mouse (Invitrogen) secondary antibodies. Protein bands were visualized with a Li-Cor Odyssey infrared imaging system.

IV. RNA Analysis

For Northern blotting, total RNA was isolated using a kit from Epicentre, and then subjected to electrophoresis through a 1.2% agarose gel in 20 mM MOPS, pH 7.0 / 5 mM sodium acetate /1 mM EDTA / 0.74% formaldehyde. The separated RNA was transferred to Hibond-N+ nylon membrane (Amersham) in 10X SSC buffer by capillary elution. Probes specific to *SIC1* (ORF nucleotides 305-783), *HOP1* (ORF nucleotides 269-778) and *ACT1* (277-870) were generated by PCR amplification using genomic DNA as a template and then labeled with [α−^{32}P] dCTP (PerkinElmer) using a random primer DNA labelling kit (Roche). Hybridization was conducted with individual probes overnight at 65°C.

Radioactivity was detected through Phosphoimager (GE Healthcare) analysis.

V. DNA Content/Flow Cytometry

Cells were harvested by centrifugation, resuspended in 70% ethanol and stored at 4°C. Aliquots of the fixed cells were washed once with 50 mM Tris–HCl, pH 7.5, resuspended in 1 ml of the same buffer, and then treated with 250 µg RNase A for 1 hour at 37°C followed by 250 µg proteinase K for 1 h at 37°C. The digested samples were incubated with 10X SYBR Green I (Molecular Probes) at 4°C overnight, sonicated briefly and analyzed by with a FacsCalibur and FACSCantoII flow cytometer (BD Biosciences). DNA content histograms were generated and analyzed using WinMDI freeware.

VI. Immunofluorescence

Cells were collected in 10ml aliquots for DAPI staining. Cells were fixed in 1ml 50% EtOH and stored at 4°C. The resulting fixed cells were harvested by centrifugation at 1400 RPM for 2 min at 4°C. Cells were then washed with 1ml 50 mM Tris–HCl, pH 7.5 and resuspended in 250µl of the same buffer. The samples were sonicated at 5 watts for 5 seconds, and slides were immediately prepared by pipeting 4µl of cells and 4µl of VECTAShield Mounting Media containing 4'-6-diamidino-2-phenylindole (DAPI) (VectorLabs). Cells were quickly mixed with the mounting media directly on the slide prior to the addition of the coverslip. The mounting media was allowed to harden at room temperature for at least 30 minutes in the dark. Strains were then analyzed by microscopy (Olympus IX71)

under oil emersion and images were taken (Hamamatsu ORCA-ER). We scored the number of DAPI-staining bodies by cells that had distinct staining bodies (one, two, three, or four bodies). All other cells were not counted. Counts were performed blindly and 200 cells were counted per strain. We analyzed the DAPI staining of *swe1*Δ (YGB687), *dmc1*Δ (YGB764), *swe1*Δ*dmc1* (YGB765), and *sum1*Δ*dmc1*Δ (YGB792). These strains, except *swe1*Δ, were constructed by selecting for spontaneous revertants, which had lost the *HOP1pr::SIC1*ΔP^{HA} element in the diploid strains *dmc1*Δ*SIC1*ΔP^{HA}, *swe1*Δ*dmc1*Δ*SIC1*ΔP^{HA}, and *sum1*Δ*dmc1*Δ*SIC1*ΔP^{HA} by counter-selection with fluoro-orotic acid (Toronto Research Chemicals) (Boeke et al., 1984).

VII. Comparative genome hybridization

Experiments were performed in collaboration with Dr. Grant Brown at the University of Toronto. To perform this method we extracted the genomic DNA and fragmented with DNase I. Biotin-dUTP was incorporated at the 3' ends of the DNA fragments using terminal deoxynucleotidyl transferase (TdT). Once the DNA fragments were biotinylated, they were hybridized to an Affymetrix GeneChip®*S.Cerevisiae* Tiling 1.0R Array. This microarray contains the entire yeast genome and is comprised of 3.2 million match/mismatch probe pairs. In addition, the probes are tiled at an average of five base pair resolution. Once the hybridization process was complete, the chip was washed and probed with streptavidin conjugated to a fluore (streptavidin-phycoerythrin biotinylated anti-streptavidin antibody). Due to the strong affinity streptavidin has for biotin, there

is a high efficiency of binding of biotin to streptavidin. Based on computer analysis we were able to detect signal emission of the fluore. The relative hybridization intensity at a given location on the array is ideally proportional to the relative copy number of those sequences in the genome.

CHAPTER 3

MEIOTIC DNA RE-REPLICATION INDUCIBLE BY MUTATIONS OF THE CDK1 INHIBITOR SIC1

I. Introduction

DNA replication must only occur once in mitosis and meiosis to prevent genomic instability. During the cell cycle, chromosomes are duplicated in S phase then segregated during subsequent M phase. The meiotic program is similar in that the DNA is replicated in pre-meiotic S phase, but there are two successive divisions, MI and MII, without an intervening S phase. Homologous chromosomes segregate during the first division, MI, also termed the reductional division. In the next successive division, MII (equational division), sister chromatids segregate to generate four haploid gametes. Programmed recombination occurs in prophase of MI and is one process that sets mitosis apart from meiosis. This ensures proper chromosome segregation and genetic variability between the haploid gametes. Recombination can occur during mitosis, and often does, but it functions for repair and the sister chromatids serve as the template.

The model organism *S.cerevisiae* has proven to be effective for studying S phase in the cell cycle. There is one major cyclin dependent kinase (Cdk1 or Cdc28) in yeast that combines with G1 cyclins (Cln) or B-type cyclins (Clb) to direct the cell through the cell cycle. As a cell transitions from G1 to S phase,

Cdk-Clb5 and Cdk-Clb6 are responsible for initiating DNA replication (Schwob et al., 1994). To promote the transition from S to G2, which includes proper spindle assembly, Cdk1-Clb3 and Cdk1-Clb4 become active. Finally, Cdk1-Clb1 and Cdk1-Clb2 help govern the transition from G2 to M phase. Different types of inhibitors hold Cdk-Clns and Cdk-Clbs inactive until the cell is ready for their execution at the proper time. Sic1 is an inhibitor of Cdk1-Clb5, -6, which prevents early entry into S phase, and is specific for B-type cyclin-CDK complexes (Schwob and Nasmyth, 1993). When Sic1 is phosphorylated by Cdk-Cln1, -2 it becomes degraded through the ubiquitin pathway, which allows Cdk1-Clb5, -6 to now become active (Deshaies, 1997).

During meiosis, Cdk1 and Ime2, a meiosis-specific kinase, catalyze many phosphorylation events that allow for proper progression through meiosis. They each can phosphorylate the same substrates (Sic1, Cdh1, and components of the pre-RC), but the kinases have different consensus phosphorylation sites (Clifford et al., 2005; Sedgwick et al., 2006; Holt et al., 2007; Moore et al., 2007) (Sawarynski et al., 2007). Cdk1-Cln1,-2 are responsible for the phosphorylation and subsequent degradation of Sic1 in mitosis (Deshaies, 1997), but do not function in meiosis (Dirick et al., 1998; Colomina et al., 1999). It has been proposed that Ime2-dependent degradation of Sic1 can lead to the activation of Cdk1 associated with Clb5 and Clb6 (Dirick et al., 1998; Stuart and Wittenberg, 1998). However, it seems unlikely that Ime2 functionally replaces the Cdk1-Cln1, -2 complexes during meiosis, because Cdk1 and Ime2 have different specificities (Benjamin et al., 2003; Clifford et al., 2005; Sawarynski et al., 2007).

Deletion of the genes encoding *CLB5* and *CLB6* or inactivation of Cdk1, prevents meiotic DNA replication (Dirick et al., 1998; Stuart and Wittenberg, 1998; Benjamin et al., 2003). This provides evidence that Cdk1-Clb5, -6 complexes promote meiotic S phase entry, as they do in mitosis. Also, overexpression of Sic1 mutated at residues required for its degradation prevents mitotic S and meiotic S phase (Stuart and Wittenberg, 1998; Sedgwick et al., 2006). Therefore, it seems likely that Cdk1-Clb5, -6 complexes govern meiotic S phase, but a different mechanism is responsible for Sic1 destruction.

Once DNA replication is initiated, it is crucial that the replication machinery is prevented from re-initiating DNA replication. Mechanisms that prevent DNA re-replication have been largely conserved in eukaryotes, with some variation through evolution. During mitosis, Cdk1-Clb complexes are responsible for preventing DNA re-replication by influencing components of the pre-replication complex (pre-RC) (Dahmann and Futcher, 1995). Specific events that have been shown to prevent DNA re-replication include inactivation of the origin recognition complex (ORC) (Dahmann and Futcher, 1995), export of minichromosome complex (MCM) and Cdt1 from the nucleus (Labib et al., 1999; Tanaka et al., 2007), degradation of Cdc6 (Drury et al., 1997; Elsasser et al., 1999), or a physical interaction between Clb5 and the ORC (Wilmes et al., 2004). It is still unclear whether these same mechanisms are responsible for prevention of meiotic DNA re-replication. Others have shown the induction of meiotic DNA re-replication through over expression of Clb1 or Clb5 (Strich et al., 2004), an activated allele of *CDC28*, or the deletion of *SWE1,* the gene that encodes a

protein kinase involved in the G2 to M transition prevention of CDK (Rice et al., 2005). These data would suggest that different mechanisms prevent meiotic DNA re-replication.

II. Results

Meiosis-specific expression of Sic1WT^{HA} and Sic1ΔP^{HA}

In *S. cerevisiae*, Sic1 is present early in meiosis then becomes degraded as the cell enters S phase (Dirick et al., 1998). Certain phosphorylation sites on Sic1 mark Sic1 for degradation by the ubiquitin pathway in mitosis, and these same phosphorylation sites participate in meiosis (Stuart and Wittenberg, 1998; Sedgwick et al., 2006). Since Cdk1-Cln1, -2 complexes are not active during meiosis and Cdk1 activity is not required for the Sic1 destruction in meiosis, we sought to further investigate the method of Sic1 destruction in meiosis. We designed two versions of Sic1, both of which were placed under the control of the meiosis-specific *HOP1* promoter (*HOP1pr*) as well as encoding hemagglutin (HA) and 6x histidine tags. The first version of Sic1 we constructed is referred to as *HOP1pr-SIC1WT^{HA}* and is considered our wild type strain because this version of Sic1 is properly degraded during mitosis although it contains one mutation at a Cdk1 consensus site (T2A). The second version we constructed, *HOP1pr-SIC1ΔP^{HA}*, contains the T2A mutation as well as T5GP, S33A, and S76A mutations, and it cannot be adequately phosphorylated and subsequently signaled for ubiquitin degradation in the cell cycle (Verma et al., 1997).

To analyze the strains containing these constructs, we subjected them to starvation to induce meiosis. Protein expression revealed that during earlier time-points, protein levels between Sic1WT^{HA} and Sic1ΔP^{HA} were relatively similar, but as the time points progressed levels of Sic1ΔP^{HA} accumulated to a much greater extent then Sic1WT^{HA} (Figure 6A). To ensure that protein level was not due to enhanced transcription, we performed Northern blot analysis (Figure 6B). We noted that transcript levels of *HOP1pr-SIC1WT^{HA}* and *HOP1pr-SIC1ΔP^{HA}* were relatively similar, suggesting to us that the Sic1 protein level is regulated post-transcriptionally. Northern blot analysis revealed that both *SIC1WT^{HA}* and *SIC1ΔP^{HA}* transcripts were induced from the *HOP1pr* elements with patterns nearly identical to those of the *HOP1* transcripts (Figure 6B). Note that recovery of 24hr transcripts was inefficient in all 3 strains.

Next, we wanted to analyze DNA content by flow cytometry (Figure 7). These data revealed similar kinetics between parental cells and *HOP1pr-SIC1WT^{HA}*; however, the *HOP1pr-SIC1ΔP^{HA}* strain, exhibited an impressive degree of DNA re-replication. As indicated by our flow cytometry analysis, we were able to detect cells that had clearly replicated their entire genome two times (8C) (Figure 7B). We also analyzed the 24 hour flow data further by estimating the percentage of cells that have DNA content >4C, and found that more than half of *HOP1pr-SIC1ΔP^{HA}* cells had larger than 4C DNA content (Figure 7B). In summary, we have shown that the same sites required for Sic1 degradation in mitosis are also required for meiotic degradation.

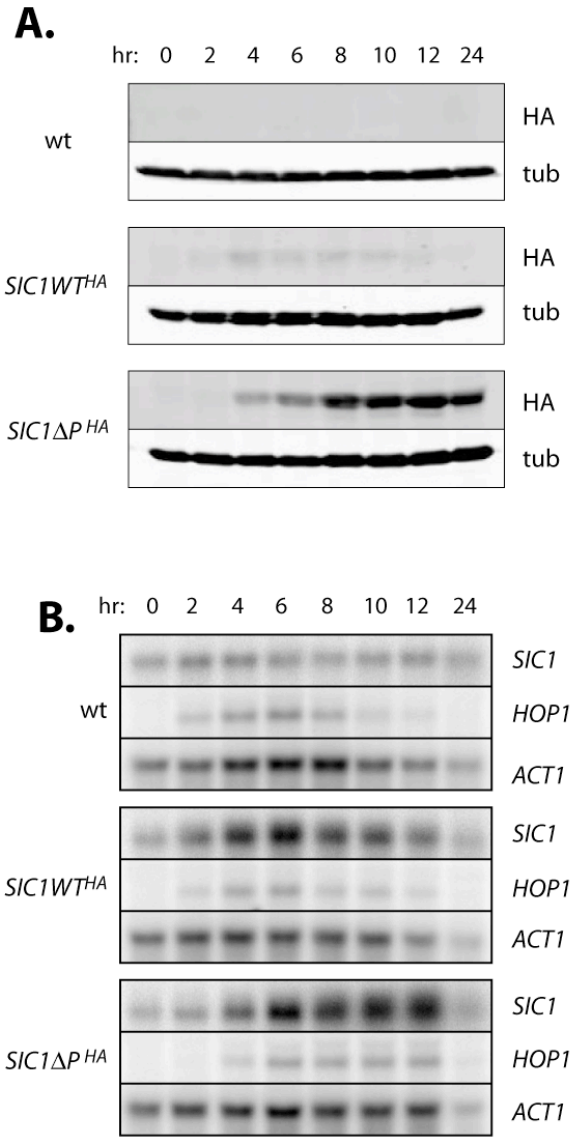
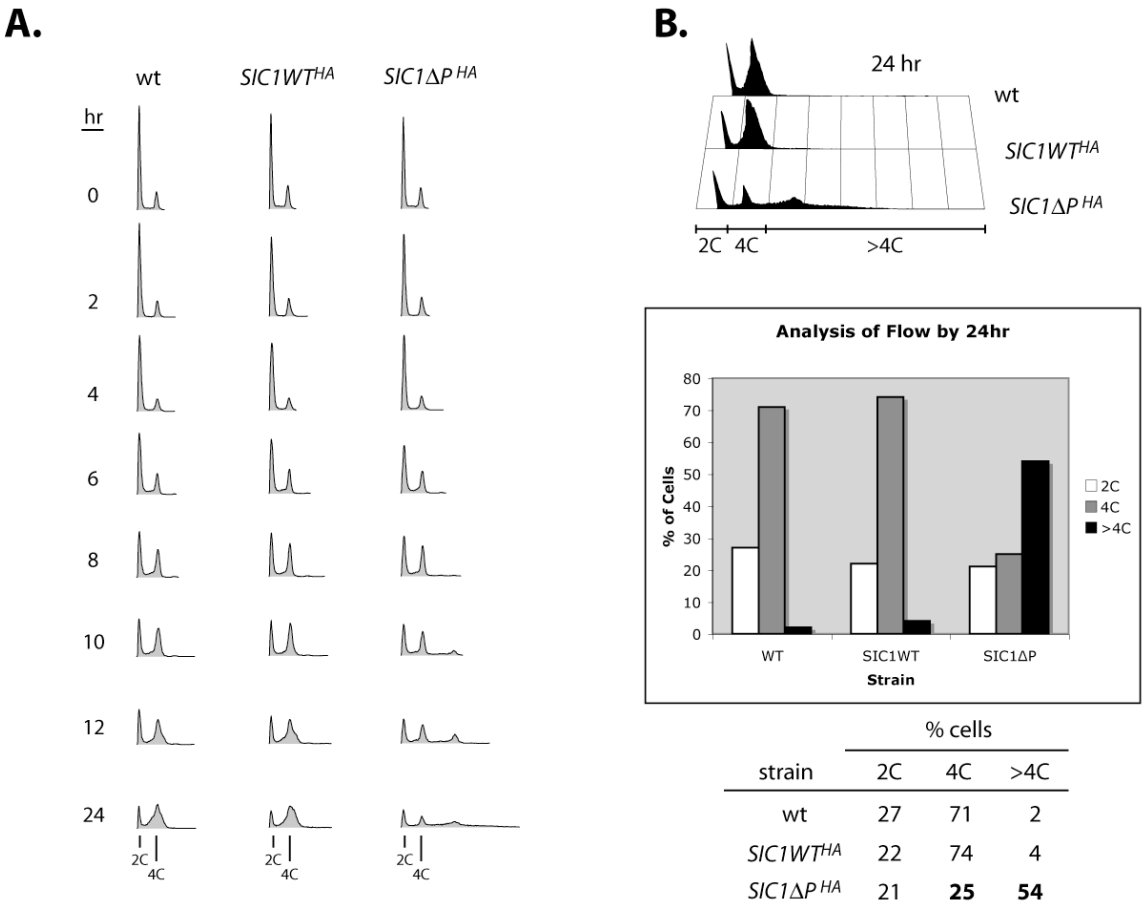


Figure 6. Sic1∆P^{HA} is resistant to degradation.
Cells were induced to enter meiosis and analyzed for various parameters over time. Comparisons were made between the parental strain (WT; YGB138), *HOP1pr-SIC1WT^{HA}* (YGB613), and *HOP1pr-SIC1∆P^{HA}* (YGB495) **A.** Protein analysis of the time course. Sic1 and tubulin protein bands were visualized with antibodies against HA and tubulin. **B.** Northern blot from the same time course. Transcripts *SIC1, HOP1,* and *ACT1* were analyzed by synthesizing ^{32}P radioactive probes labeled.



strain	% cells 2C	4C	>4C
wt	27	71	2
SIC1WT^{HA}	22	74	4
SIC1ΔP ^{HA}	21	**25**	**54**

Figure 7. *SIC1*Δ*P*^{HA} leads to extra rounds of DNA replication.
A. DNA content was analyzed by flow cytometry for the parental strain (WT; YGB138), *HOP1pr-SIC1WT*^{HA} (YGB613), *HOP1pr-SIC1*Δ*P*^{HA} (YGB495), which were induced to undergo meiosis. 2C signifies a population of cells prior to DNA replication, and 4C signifies cells that have completed one round DNA replication. **B.** DNA re-replication can be assessed from the enlarged 24hr histograms. Flow analysis of the 24hr histograms were analyzed to estimate the percentage of cells that re-replicated in *HOP1pr-SIC1*Δ*P*^{HA} when compared to WT and *HOP1pr-SIC1WT*^{HA}.

and that mutations in our *HOP1pr-SIC1ΔP^{HA}* strain produced a DNA re-replication phenotype.

Increasing the expression of Sic1ΔP^{HA} blocks DNA replication and any subsequent rounds of DNA replication

It was interesting that this mutated form of Sic1 caused a re-replication phenotype, because it was previously reported that *SIC1ΔP^{HA}* arrested and did not complete meiotic DNA replication (Stuart and Wittenberg, 1998; Sedgwick et al., 2006). In the previous studies, researchers used a different meiosis-specific promoter, *IME2pr*, so we reasoned this was a possible explanation for our re-replication phenotype. *IME2pr* possibly directs stronger and/or earlier expression. We constructed a strain with *SIC1ΔP^{HA}* under the *IME2pr.* Through comparison with the *HOP1pr* we noted that the level of Sic1ΔP^{HA} was increased with the *IME2pr*, and interestingly we saw that DNA re-replication did not occur (Sawarynski et al., 2009). In fact, the cells were still mainly in G1, which coincided with the previous research that showed cells were unable to complete meiotic DNA replication (Stuart and Wittenberg, 1998; Sedgwick et al., 2006).

A likely explanation for our re-replication phenotype is that the stabilized version of Sic1 inhibits Cdk1 at a certain level that allows for re-initiation of DNA replication; in a wild type cell, active Cdk would prevent re-initiation, as it does in mitosis. We showed that increased expression of *SIC1ΔP^{HA}*, under the *IME2pr*, did not allow for meiotic DNA replication by an accumulation of cells in G1 (Sawarynski et al., 2009). We next determined if increasing expression by adding

an extra copy of *HOP1pr-SIC1∆P^{HA}* would also prevent DNA re-replication. We found that the extra copy increased Sic1∆P^{HA} expression, as expected, and S phase was prevented as shown by the accumulation of cells in the 2C peak (Figure 8) (Brush and Najor, 2009). The *IME2pr* data and the 2X *HOP1pr-SIC1∆P^{HA}* data suggest that increasing protein expression of a non-degradable form of Sic1 lowers Cdk1-Clb5, -6 activity to levels that prevent pre-replication complex (pre-RC) assembly, which initiates DNA replication.

Characteristics of *SIC1∆P^{HA}*-induced meiotic DNA re-replication

We narrowed the mechanism of DNA re-replication down to two possibilities. The first was that DNA re-replication occurred due to a re-initiation of origins prior to meiotic divisions. The second was that re-initiation of origins was occurring between meiotic divisions, which is suppressed normally in meiosis.

Our DAPI data of *HOP1pr-SIC1∆P^{HA}* did not display the proper production of 4 nuclei staining bodies. Rather, many of the cells had one large staining body, which suggested the *HOP1pr- SIC1∆P^{HA}* strain had undergone multiple rounds of DNA replication without nuclear divisions (Sawarynski et al., 2009). To further investigate whether DNA re-replication could occur between meiotic divisions, we constructed a deletion mutant that arrests in the pachytene stage of prophase I. Ndt80 is a transcriptional activator that turns on many middle sporulation genes (Chu and Herskowitz, 1998) required for meiosis. In the absence of Ndt80, cells arrest at pachytene and do not enter the meiotic divisions (Xu et al., 1995). The

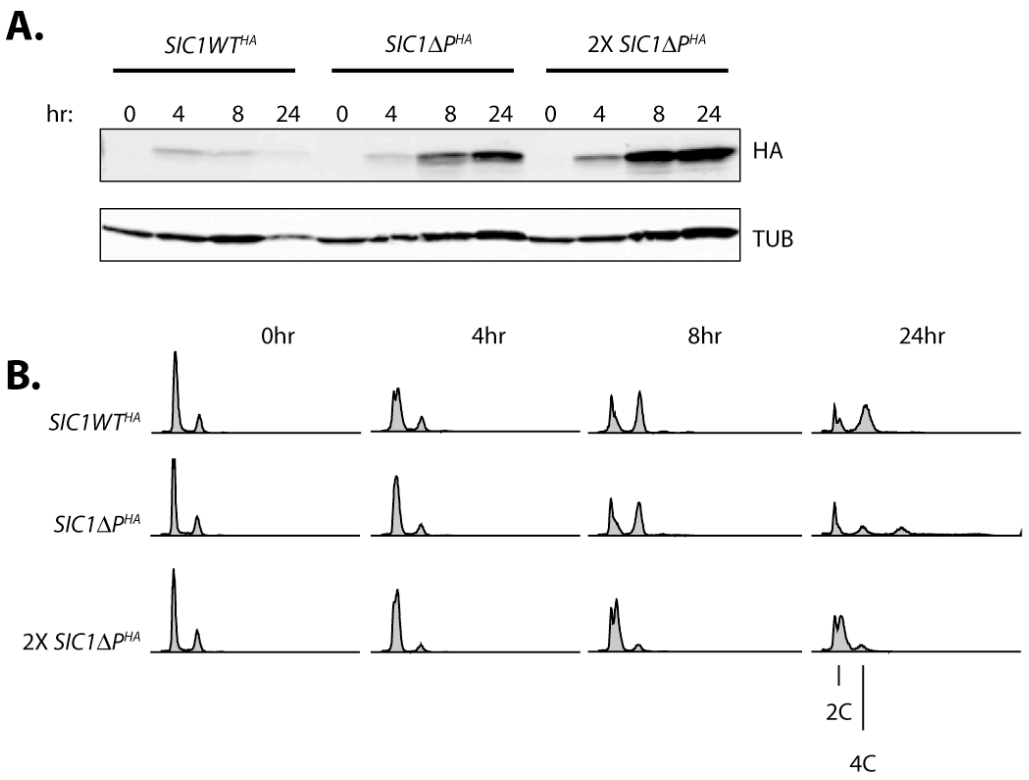


Figure 8. Two copies of *HOP1pr-SIC1ΔP^{HA}* prevent DNA replication and re-replication.
Strains *HOP1pr-SIC1WT^{HA}* (YGB613), *HOP1pr-SIC1ΔP^{HA}* (YGB495), and 2 copies (2X) *HOP1pr-SIC1ΔP^{HA}* (YGB617) were induced to enter meiosis and analyzed for various parameters over time. **A.** Protein analysis of a time course where HA tags were used for Sic1 detection and tubulin levels were analyzed as a loading control. **B.** DNA content was analyzed by flow cytometry. 2C signifies a population of cells prior to DNA replication, and 4C signifies cells that have completed one round DNA replication.

sporulation of *ndt80*Δ expressing Sic1ΔP^{HA} revealed that in the absence of Ndt80, cells were still able to undergo multiple rounds of DNA replication by analysis of DNA content (Figure 9). These data suggest that DNA re-replication is occurring prior to meiotic divisions.

We sought to investigate all other possibilities that could be responsible for the mechanism of meiotic DNA re-replication. Earlier, we mentioned origin dependent replication as a possibility. In this instance, DNA replication would initiate at regions of the DNA "licensed" for the replication machinery. There are many mechanisms defined in the cell cycle that prevent the "re-licensing" of these regions, and therefore would prevent DNA re-replication. For the next step in characterizing our DNA re-replication, we sought to determine whether the underlying mechanism was due to re-licensing of origins. We suspect that origins are involved because full genome equivalents are replicated in these cells. However, other mechanisms, such as break-induced replication, can also lead to extensive DNA replication. To further investigate this possibility, we performed comparative genomic hybridization (CGH), which is a method developed to monitor copy number changes on a genomic scale. One can distinguish at certain genomic locations if there is a relative increase in DNA copy number, since hybridization intensity is proportional to the relative copy number. Therefore, if origins were being re-fired, we hoped to detect a two-fold increase in DNA copy number in the origin regions relative to neighbors of the origins.

We examined the 24-hour samples of our re-replication strain, *HOP1pr-SIC1*Δ*P^{HA}* and our wild type version, *HOP1pr-SIC1WT^{HA}* (Figure 10). We

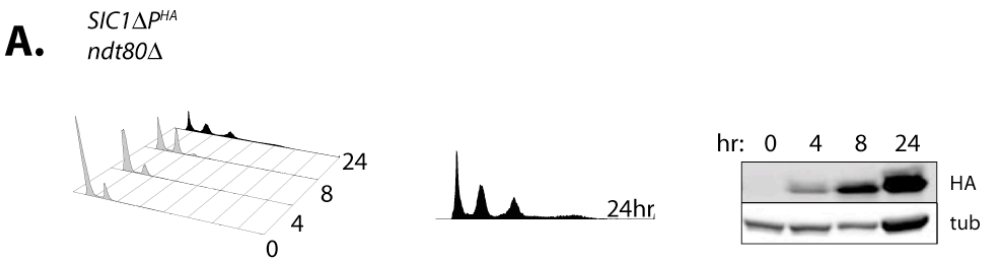


Figure 9. DNA re-replication does not require pachytene exit.
To investigate whether DNA re-replication occurs prior to pachytene, we generated a homozygote deletion of *NDT80* with *HOP1pr-SIC1*ΔP^{HA} at the *URA3* locus (YGB583). Synchronous sporulation was performed and aliquots were taken to analyze protein levels by SDS-PAGE and DNA content by flow cytometry. HA tag was used for Sic1 protein detection, and tubulin protein was analyzed as a loading control.

anticipated that certain regions near origins might be over-represented if origin dependent DNA re-replication were occurring. When comparing both microarrays, we found that there was no relative increase at any specific region of the DNA (Figure 10B). We could not detect origin enrichment because of the extent of DNA re-replication, and that perhaps we will need to use a S phase block to see such an effect.

Although we cannot deduce whether origins had been re-fired, our analysis was still able to provide evidence that DNA re-replication was likely genome-wide, which was also expected from the flow cytometry data. Since every gene and their neighbors were probably re-replicated, CGH did not detect any relative difference in gene copy number. To ensure that CGH could detect a two-fold increase, we compared our re-replication strain to GBY653, which is a strain from the laboratory of Dr. Grant Brown that contains 3 copies of the *SUL1* gene and deletion of the *MEC1* gene (Figure 10C). The software was able to recognize the differences in gene copy number for *SUL1* and *MEC1*. Therefore, the software is accurate in its analysis and DNA re-replication was not due to amplification of certain region of the *S.cerevisiae* genome.

Genome-wide re-replication could be due to re-licensing of origins or another possibility is break-induced replication (BIR), which mitotically requires the protein Pol32. In this case, DNA re-replication could initiate from DSBs. In meiosis, Spo11 catalyzes programmed DSBs to initiate meiotic recombination, but our data shows that a *spo11Δ* cell still undergoes re-replication (Sawarynski et al., 2009). Therefore, if BIR is the cause of DNA re-replication, the DSBs must

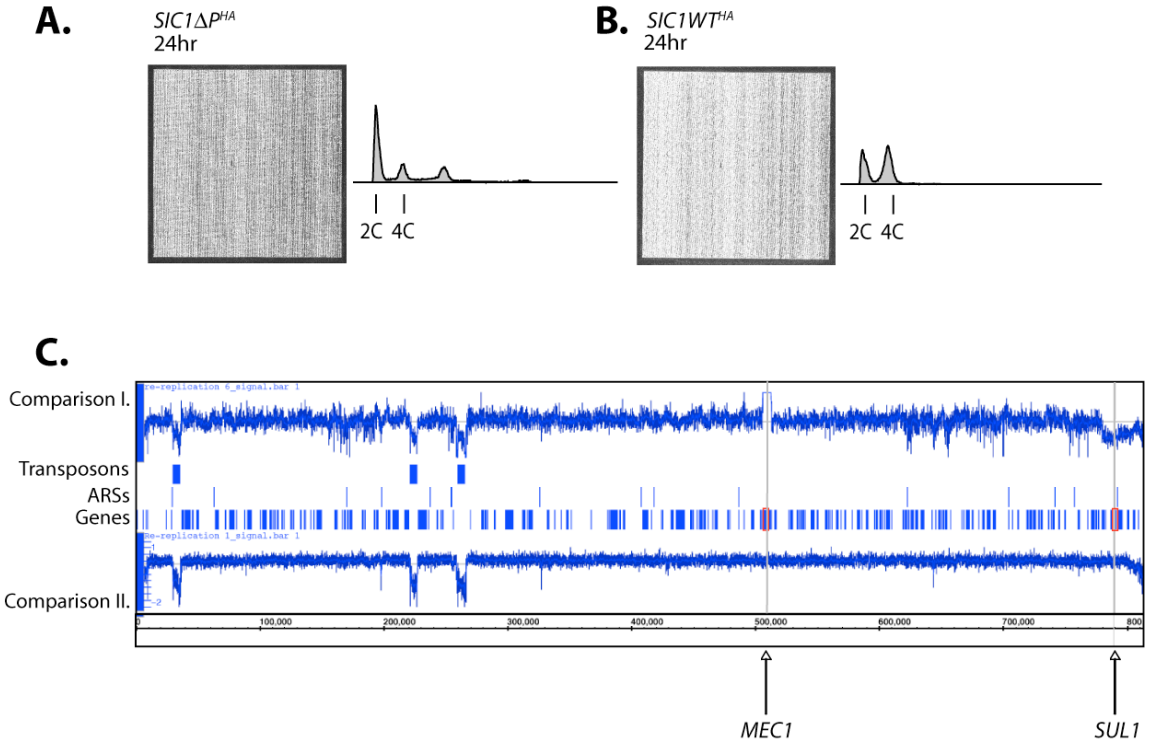


Figure 10. Comparative Genome Hybridization (CGH) reveals possible genome-wide amplification.
Snap shots of microarrays used in analysis of (**A.**) *HOP1pr-SIC1ΔP*^{HA} and (**B.**) *HOP1pr-SIC1WT*^{HA} with 24-hour flow data showing DNA content. **C.** Integrated Genome Browser (IGB) software analysis of wild type strain *SIC1WT*^{HA} vs. re-replication strain *SIC1ΔP*^{HA} (Comparison II). To display an example where CGH is can detect a two-fold change in gene copy number, analysis of a strain (GBY653-Dr. Grant Brown), which contained 3 copies of SUL1 and a deletion of MEC1, is depicted in Comparison I. Transposons are detectable by CGH, as seen in both Comparison I and II.

be formed by some other mechanism in our *HOP1pr-SIC1ΔP^{HA}* mutant. We reasoned that *HOP1pr-SIC1ΔP^{HA}* might have some sort of direct impact on the DNA that could cause damage. Since Pol32 is required and essential for mitotic BIR, we made a deletion mutant of this gene then analyzed the DNA content by flow cytometry and protein levels after the sporulation. If BIR were responsible for our DNA re-replication phenotype, then the *pol32Δ* mutant harboring *HOP1pr-SIC1ΔP^{HA}* should not re-replicate. Our results showed a homozygote deletion of *POL32* with *HOP1pr-SIC1ΔP^{HA}* went through more than one round of DNA replication (Figure 11). If Pol32 is also essential for meiotic BIR, then these data suggest BIR is not the mechanism for DNA re-replication. It is possible that Pol32 is not required for BIR in meiosis, and therefore it is still possible that BIR could account for our DNA re-replication phenotype. Arguably, there are other possible mechanisms that we have not investigated that could account for our DNA re-replication phenotype. However, the flow data exhibiting discrete 2^n DNA content peaks and the CGH data indicate genome-wide DNA re-replication, suggesting an origin dependent mechanism.

III. Discussion

By generating a non-degradable form of Sic1, we were able to investigate DNA re-replication in meiosis. The DNA was re-replicated due to our choice of promoter element, *HOP1pr*, and copy number of *SIC1ΔP^{HA}*. This suggested additional rounds of DNA replication can occur when levels of Cdk1-Clb5, -6 are reduced. This was interesting because previous studies showed that the same

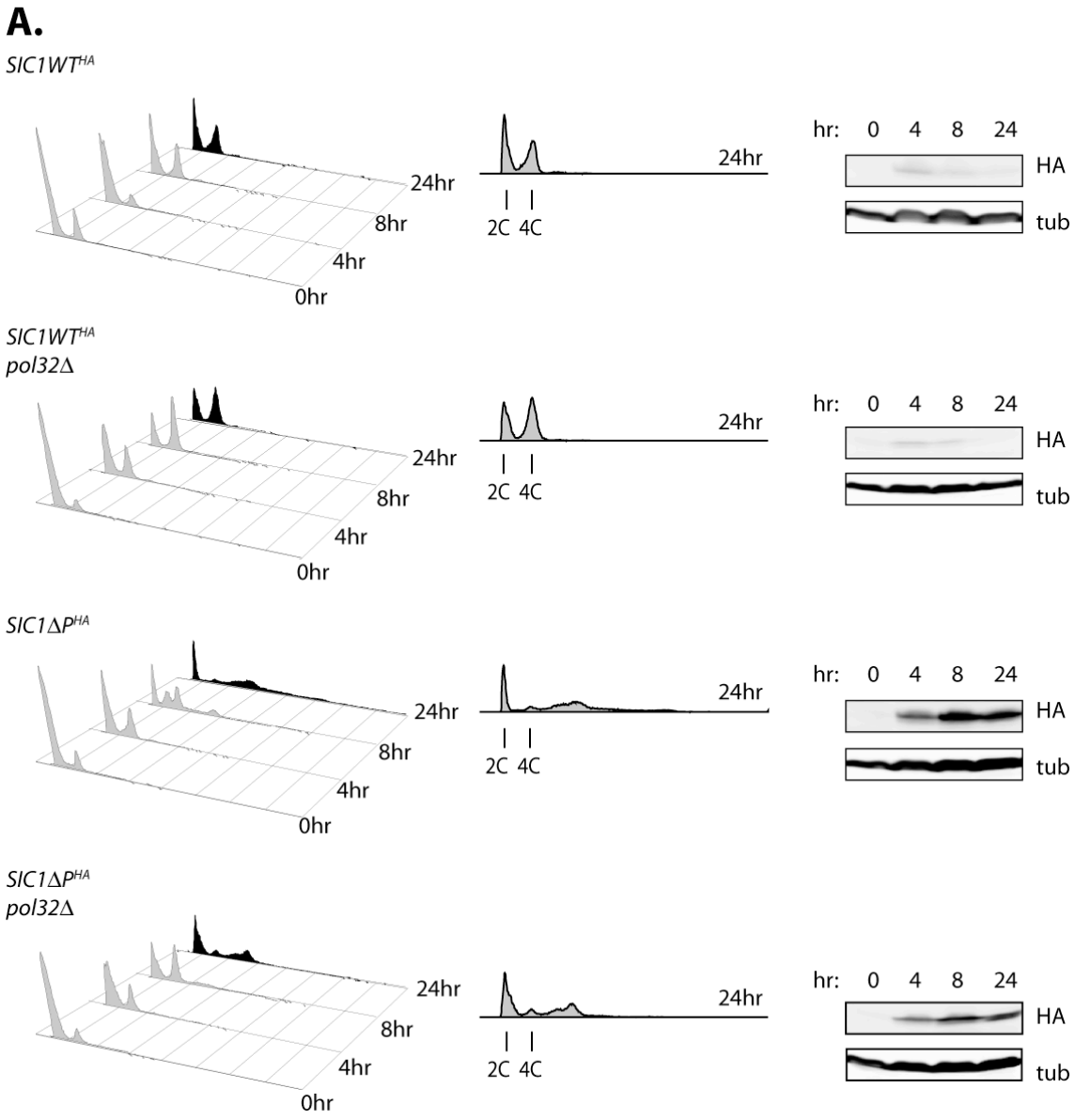


Figure 11. The absence of Pol32, an essential protein for BIR during the cell cycle, does not abolish Sic1ΔP^{HA}-induced meiotic DNA re-replication.
Cells were induced to enter meiosis and analyzed for various parameters over time. Comparisons were made between the *HOP1pr-SIC1WT^{HA}* (YGB613) *pol32Δ HOP1pr-SIC1WT^{HA}* (YGB678), *HOP1pr-SIC1ΔP^{HA}* (YGB495), and *pol32Δ HOP1pr-SIC1ΔP^{HA}* (YGB672). Synchronous sporulation was performed and aliquots were taken to analyze protein levels by SDS-PAGE and DNA content by flow cytometry. HA tags were used for Sic1 protein detection, and tubulin protein was analyzed as a loading control.

mutant form of Sic1 did not allow the cells to enter mitotic or meiotic S phase. This also suggested that the level of Cdk1 is important to prevent DNA re-replication. Our current hypothesis is that *HOP1pr-SIC1*ΔP^{HA} lowered Cdk1 levels to an amount that allowed re-initiation, and that if lowered further (2X *HOP1pr-SIC1*ΔP^{HA} or *IME2pr-SIC1*ΔP^{HA}) the cells would arrest and would not complete pre-meiotic S phase. Therefore, this suggests that prevention of DNA re-replication requires Cdk1 activity.

We were also able to show that DNA re-replication is occurring prior to meiotic divisions by the *ndt80*Δ mutant. These data suggest that prevention of DNA re-replication somehow involves Cdk1-Clb5, -6 complexes, since those are known to govern the mechanisms prior to meiotic divisions (i.e. DNA replication and recombination). In addition, based on our CGH and flow cytometry data, DNA re-replication is genome-wide and most likely due to origin re-firing. DNA re-replication does not occur as a form of BIR based on our experiments with Pol32. Pol32 is known to be essential for mitotic BIR, however, further investigations will be required to determine whether Pol32 is also essential for meiotic BIR. In summary, this evidence points to Cdk1-Clb5, -6 complexes being responsible for the prevention of re-licensing of DNA origins, which is similar to the prevention of mitotic DNA re-replication.

CHAPTER 4

LINKS BETWEEN DNA RE-REPLICATION AND THE RECOMBINATION CHECKPOINT

I. Introduction

In the organism *S.cerevisiae*, Cdk1 regulates the progression through the mitotic cell cycle and the meiotic program. To regulate meiotic S phase, Cdk1 is complexed with the B-type cyclins, Clb5 and Clb6, and a cyclin dependent kinase inhibitor, Sic1. Once Sic1 is phosphorylated, it is degraded through ubiquitin-dependent proteolysis, which activates the Cdk1-Clb5/Clb6 complexes and DNA synthesis initiates (Deshaies, 1997; Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997; Stuart and Wittenberg, 1998). During meiosis, the kinase responsible for catalyzing Sic1 phosphorylation is still in question, but evidence has pointed to the involvement of both Ime2 and Cdk1 (Sedgwick et al., 2006; Sawarynski et al., 2007).

Following S phase in meiosis, an important regulated and programmed process in prophase of MI is meiotic recombination, which ensures genetic diversity between the final haploid gametes and is required for proper chromosome segregation. Programmed recombination initiates from DSBs (Szostak et al., 1983; Sun et al., 1989). Many genes play a role in DSB formation, but the Spo11 transesterase has been proposed to be the direct protein involved in the generation of DSBs early in meiotic prophase (Keeney et

al., 1997). The DSBs are processed in a proteinaceous structure, the synaptonemal complex (SC), which forms along the meiotic chromosome. After the DSBs are formed, the recombinase Dmc1 plays a critical role in strand invasion of 3' tails into intact homologous non-sister chromatids (Collins and Newlon, 1994; Hunter and Kleckner, 2001). The crossovers that occur during recombination must take place in the SC (Engebrecht et al., 1990; Hollenberg et al., 1995).

If there are any aberrations in recombination or SC formation, a checkpoint will arrest the cells in the pachytene stage of prophase I. This checkpoint is known as the meiotic recombination checkpoint or the pachytene checkpoint. Specifically, defects in recombination by *dmc1Δ* (Bishop et al., 1992) (Leu et al., 1998; Gerton and DeRisi, 2002) or defects in SC formation by *zip1Δ* (Sym et al., 1993) cause arrest through the activation of the pachytene checkpoint, which stimulates a kinase cascade with members of this pathway described in Chapter 1. The main targets of this checkpoint are the meiosis-specific transcriptional activator Ndt80 (Chu and Herskowitz, 1998; Hepworth et al., 1998; Tung et al., 2000), the transcriptional reppressor Sum1 (Lindgren et al., 2000), and the Swe1 kinase (Leu and Roeder, 1999).

Ndt80 is a meiosis-specific transcription factor that activates a large set of middle sporulation genes. It binds to the middle sporulation element (MSE) located in promoter regions of the many genes required for exit of pachytene and progression through meiosis (Chu & herskowitz 1998, Chu S et al 1998-Science, Hepworth et al 1998). Sum1 is a transcriptional repressor of *NDT80* that binds to

the same MSEs (Xie J et al 1999-EMBO). Swe1 is a kinase that has been extensively studied as a main regulator of the G2 to M transition of mitotic cells. When activated in mitotic cells, it inhibits Cdk1 by phosphorylation at tyrosine 19 (Booher et al., 1993; Sia et al., 1998) most efficiently through Cdk1-Clb2 complexes, and has no effect on Cdk1-Clb5, -6 complexes (Hu and Aparicio, 2005). Genetic studies have shown that Clb2 is not expressed during meiosis (Grandin and Reed, 1993). Within context of the pachytene checkpoint, we have yet to understand which Cdk1-Clb complexes Swe1 inhibits to induce arrest.

We have shown that meiotic DNA re-replication can be induced by the production of a non-degradable form of the Cdk1 inhibitor Sic1 (*HOP1pr-SIC1ΔP*^{HA}) (Sawarynski et al., 2009). In addition, DNA re-replication induced by *HOP1pr-SIC1ΔP*^{HA} occurred prior the meiotic divisions, and activation of the pachytene checkpoint abolished DNA re-replication. Other groups have shown meiotic DNA re-replication with multispore phenotypes. Those phenotypes where induced by a homozygous deletion of *SWE1* or an activated allele of *CDK1* (Rice et al., 2005). Also, prior research shows that ectopic overexpression of *CLB1* or *CLB5* produced structures containing more then four spores (Strich et al., 2004). Although others have been able to show the induction of meiotic DNA re-replication, mechanisms that prevent re-replication have not been extensively studied in meiosis.

Here we show that the pachytene checkpoint can prevent re-replication, but not through the targets Swe1, Ndt80 or Sum1. Therefore, we suggest an

alternative branch or response of the checkpoint that prevents DNA re-replication.

II. **Results**

DNA re-replication can be inhibited by *dmc1*Δ-induced checkpoint arrest

Following meiotic DNA replication in S phase, programmed recombination occurs in prophase of meiosis I. When origins of replication have fired, a global signal prevents DSB formation at all potential sites. Once the replication fork passes, the inhibitory signal at potential DSB sites is abolished allowing for DSB formation and consequently programmed recombination (Hochwagen and Amon, 2006). To determine whether programmed recombination could affect the DNA re-replication phenotype associated with *HOP1pr-SIC1ΔP*^{HA}, we generated mutants defective in certain stages of the recombination pathway. We deleted genes encoding Spo11 (Sawarynski et al., 2009), a transesterase that catalyzes DNA DSBs (Keeney et al., 1997) and Dmc1, a meiosis-specific recombinase that catalyzes strand exchange (Bishop et al., 1992), in a strain containing *HOP1-SIC1ΔP*^{HA} (Figure 12A). The homozygous deletion of *SPO11* did not affect the DNA re-replication phenotype (Sawarynski et al., 2009), but strikingly the homozygous deletion of *DMC1* completely abolished the re-replication peaks seen by flow cytometry (Figure 12A). We also performed Western blot analysis of the deletion strains to ensure that the lack of DNA re-replication in the *dmc1Δ* strain was not due to the absence of Sic1ΔP^{HA} expression (Figure 12A).

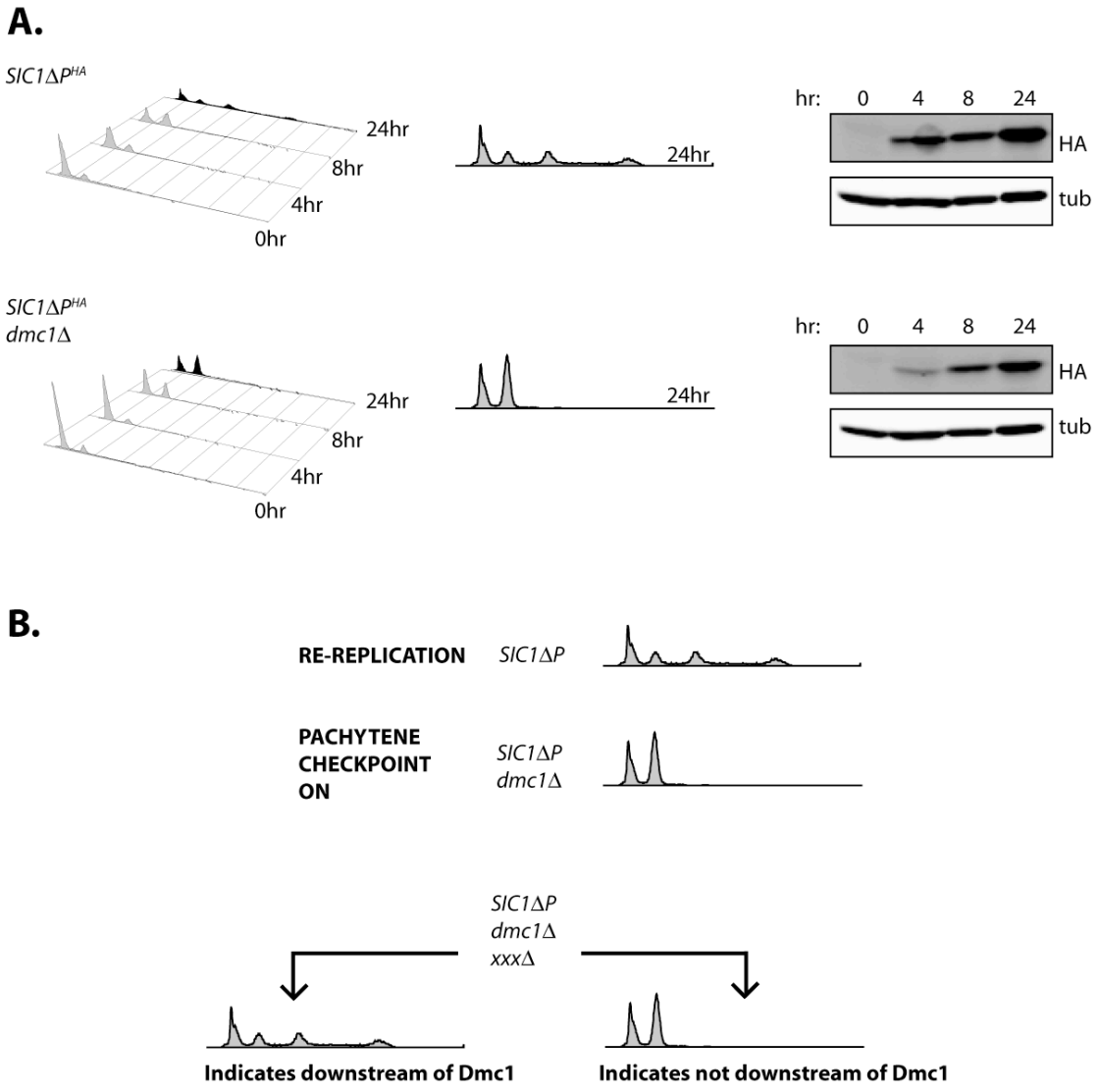


Figure 12. DNA re-replication can be inhibited by *dmc1*Δ-induced checkpoint arrest.
A. DNA content and protein were analyzed by flow cytometry and by SDS-PAGE of *HOP1pr-SIC1*Δ*P*^{HA} (YGB495) cells and *HOP1pr-SIC1*Δ*P*^{HA} with *dmc1*Δ (YGB604) cells, which were induced to undergo meiosis. HA tags were used to detect Sic1 protein. Tubulin protein was used as a loading control. 2C signifies a population of cells prior to DNA replication, and 4C signifies cells that have completed one round DNA replication. **B.** Methodology of dissecting the *dmc1*Δ-induced pachytene checkpoint responsible for preventing *SIC1*Δ*P*^{HA}-induced DNA re-replication. The genes of the corresponding proteins, which we considered might play a role in the *dmc1*Δ-induced abolishment of DNA re-replication are denoted by *xxx*Δ.

These results indicate that the recombination intermediates produced by a *dmc1Δ* mutant inhibit extra rounds of DNA re-replication associated with Sic1ΔP^{HA} expression. We also produced double homozygous gene deletions for *SPO11* and *DMC1* with *HOP1pr-SIC1ΔP^{HA}* background. Our reasoning was that if *dmc1Δ* were responsible for the inhibition of DNA re-replication, deletion of a gene upstream of Dmc1 should negate the *dmc1Δ* phenotype. We, therefore, expected the reconstitution of DNA replication in a *spo11Δ* and *dmc1Δ* mutant, and in fact this is what we observed (Sawarynski et al., 2009). We speculated that there were two possible mechanisms by which the *dmc1Δ* mutant could inhibit DNA re-replication. One possibility was that *dmc1Δ* in a *HOP1pr-SIC1ΔP^{HA}* background strain produced recombination intermediates that were physically impossible to replicate. Another possibility was that *dmc1*Δ-induced the pachytene checkpoint signal that inhibited DNA re-replication. To distinguish between these possibilities, we turned our attention to the protein Rad17, which is downstream of Dmc1 in the pachytene checkpoint. We generated double homozygous gene deletions of *RAD17* and *DMC1* with *HOP1pr-SIC1ΔP^{HA}* and found that DNA re-replication was restored. Originally, checkpoints were defined through mutations that allow a late event to occur without the completion of an early event (Hartwell and Weinert 1989). Our results indicate that Rad17 is a checkpoint protein involved in the *dmc1Δ*-mediated inhibition of the DNA re-replication phenotype, which would not normally occur after recombination (Sawarynski et al., 2009).

Our next sets of experiments were designed to elucidate the checkpoint pathway responsible for the inhibition of meiotic DNA re-replication. We employed the same strategy as described above for Rad17. We generated homozygote gene deletion mutants for genes encoding proteins we were interested in testing in a background that contained a homozygote deletion of *DMC1* with *HOP1-SIC1*ΔP^{HA} (Figure 12B).

Mec1 participates in checkpoint-induced abolishment of DNA re-replication

First we investigated at *MEC1*, which is an ortholog of mammalian ATR/ATM. In addition to its extensive role in the DNA damage checkpoint pathway, Mec1 is also required for meiotic arrest, along with Rad17 and Rad24, in the meiotic recombination checkpoint (Lydall et al., 1996). *MEC1* deletion mutants are lethal; however, an additional mutation in *SML1* will suppress the lethality. We found that the absence of Mec1 and Sml1 in a *dmc1*Δ and *HOP1pr-SIC1*ΔP^{HA} strain was able to re-constitute DNA re-replication (Figure 13A). We also performed Western blot analysis of the deletion strains to ensure the change in phenotype was not due absence of Sic1ΔP^{HA} expression. To ensure the deletion mutants did not have an effect on the DNA re-replication phenotype alone, we made control strains that contained homozygote deletions of *MEC1* and *SML1* but had wild type *DMC1* (*mec1*Δ *sml1*Δ *SIC1*ΔP^{HA}), and found that the deletions did not have an affect on our re-replication phenotype (Figure 8B). These data indicated that Mec1 participated in the checkpoint-induced abolishment of DNA re-replication.

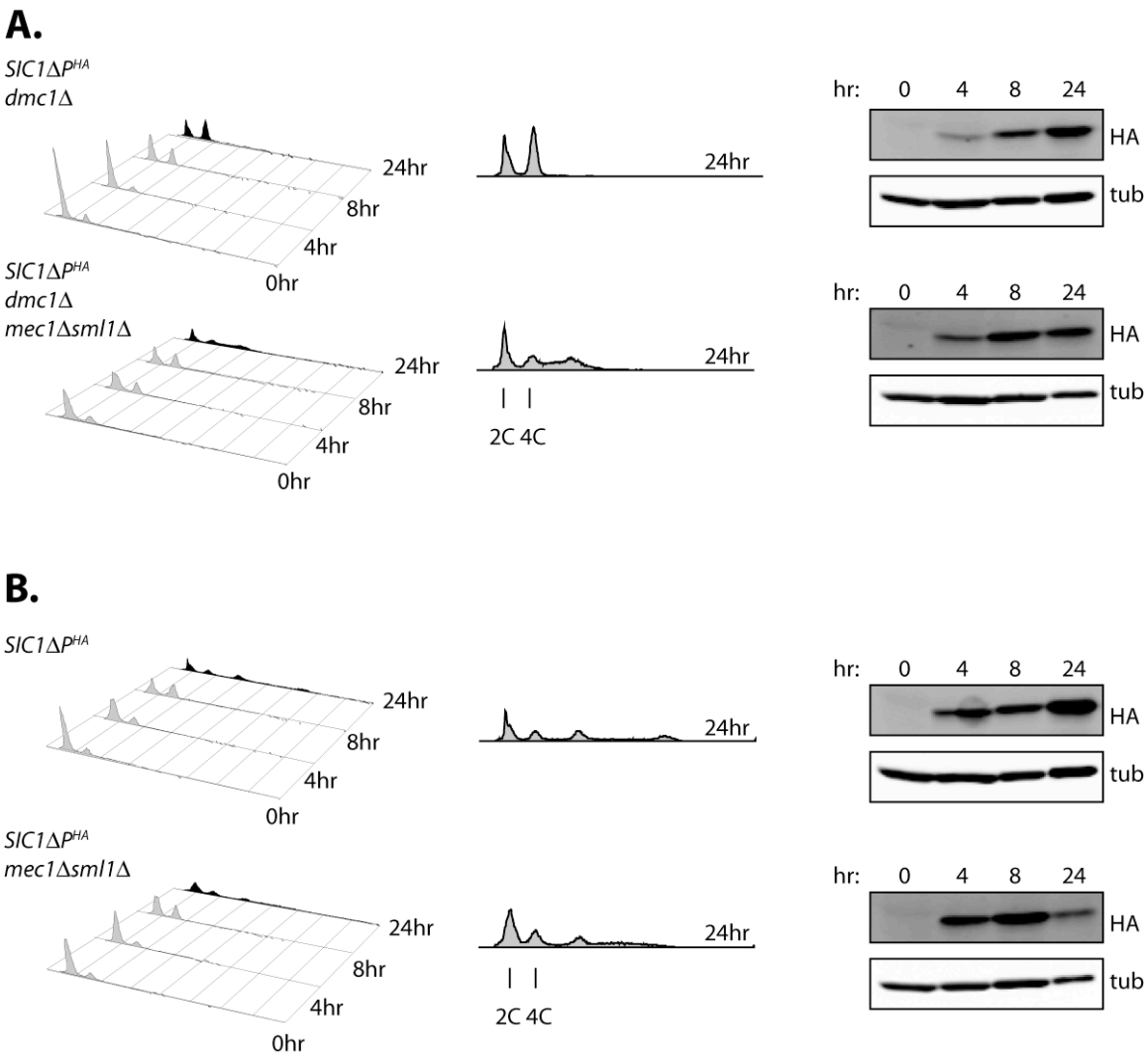


Figure 13. Mec1 participates in checkpoint-induced abolishment of DNA re-replication.
A. DNA content was analyzed by flow cytometry and protein was analyzed by SDS-PAGE of strains harboring *HOP1pr-SIC1*Δ*P*^{HA} with homozygous deletion of *DMC1* (YGB604) and strains with the additional gene deletions of *MEC1 and SML1* (YGB788) that were induced to undergo meiosis. **B.** A control strain containing *HOP1pr-SIC1*Δ*P*^{HA} with gene deletions *MEC1 and SML1* (YGB789) was generated to ensure gene deletions did not effect *HOP1pr-SIC1*Δ*P*^{HA}-induced DNA re-replication (YGB495). HA tags were used to detect Sic1 protein, and tubulin protein was used as a loading control.

Proteins Mek1, Red1, Hop1 function downstream of Dmc1 in the prevention of DNA re-replication induced by *SIC1*∆*P*^{HA}

Next, we decided to look at the proteins Mek1, Red1, and Hop1, which are the meiotic axial element (AE) proteins. These proteins are important in ensuring that crossovers occur between homologous chromosomes and not between sister-chromatids (Schwacha and Kleckner, 1995; Thompson and Stahl, 1999; Wan et al., 2004). Specifically, Mek1 kinase activity is required after DSB formation for preventing *DMC1*-independent DSB repair (Wan et al., 2004). It is thought that Hop1 binds to sites where DSBs will form, and recruits Red1. Then phosphorylated Red1 complexes with Mek1, which is then activated by phosphorylation and can further catalyze phosphorylation of other proteins to inhibit DSB repair using sister chromatids as substrates (Wan et al., 2004). To see whether these proteins also participated in the *dmc1*∆-induced DNA re-replication block, we made homozygote deletion mutants in diploid strains of the genes *MEK1, RED1,* and *HOP1*. We found that in the absence of Mek1, Red1, or Hop1 in strains with *dmc1*∆ and *HOP1pr-SIC1*∆*P*^{HA}*,* DNA re-replication was re-constituted when compared to *dmc1*∆ with *HOP1pr-SIC*∆*P*^{HA} alone (Figure 14). We also performed Western blot analysis of the deletion strains to ensure the change in phenotype was not due absence of Sic1∆P^{HA} expression (Figure 14). Therefore, these proteins also participate in the *dmc1*∆-induced abolishment of DNA re-replication. To ensure the deletion mutants did not have an effect on the DNA re-replication phenotype alone, we made control strains that were homozygote deletions for *MEK1, RED1, HOP1* with *HOP1pr-SIC1*∆*P*^{HA} (*mek1*∆

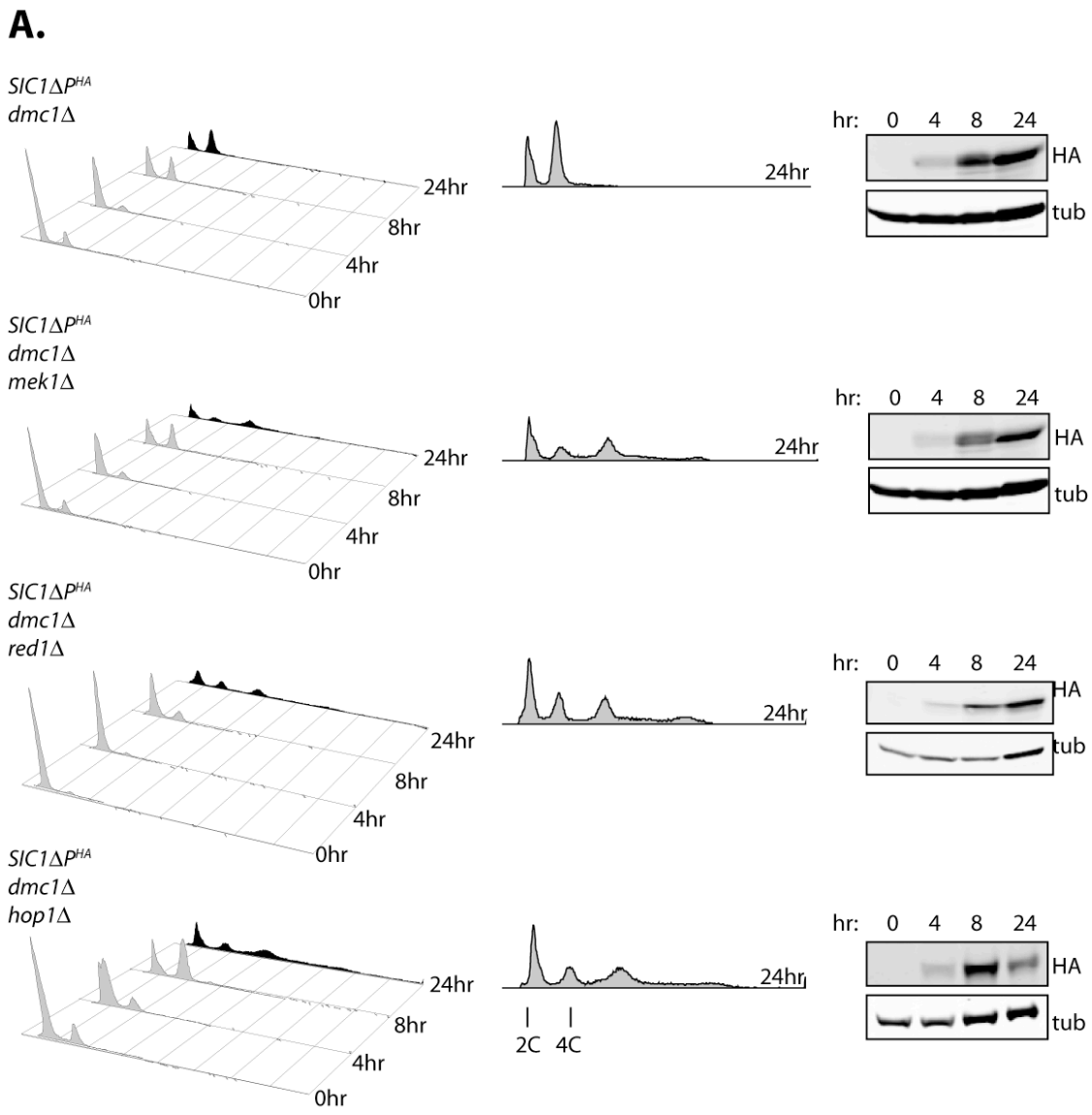


Figure 14. Proteins Mek1, Red1, Hop1 function downstream of Dmc1 in the prevention of DNA re-replication induced by *HOP1pr*::*SIC1*ΔP^{HA}.
A. Strains harboring *HOP1pr-SIC1*ΔP^{HA} with a homozygous gene deletion of *DMC1* (YGB604) and additional homozygous gene deletions of *MEK1* (YGB679), *RED1* (YGB722), and *HOP1* (YGB713) were induced to undergo meiosis. DNA content was analyzed by flow cytometry and protein was analyzed by SDS-PAGE. HA tags were used to detect Sic1 protein, and tubulin protein was used as a loading control.

SIC1ΔP^{HA}, *red1*Δ *SIC1*ΔP^{HA}, and *hop1*Δ *SIC1*ΔP^{HA}), and found that the deletion mutants did not have an affect on our re-replication phenotype (Figure 15).

Pch2 participates in checkpoint-induced abolishment of DNA re-replication

Other proteins participate in the pachytene checkpoint, but their exact roles are yet to be defined. Pch2 is a part of a group of genes that encode chromatin-silencing factors, and was found to be essential for the pachytene checkpoint (San-Segundo and Roeder, 1999). Originally discovered as a protein that could bypass *zip1*Δ-induced pachytene arrest (San-Segundo and Roeder, 1999), it is thought that Pch2 keeps recombination complexes intact to maintain arrest (Borner et al., 2008). Research has shown that *pch2*Δ can also bypass *dmc1*Δ-induced pachytene arrest, but only partially (San-Segundo and Roeder, 1999). Therefore, we wanted to investigate whether Pch2 participated in the *dmc1*Δ-induced checkpoint that inhibits our DNA re-replication phenotype. We found that Sic1ΔP^{HA}-induced DNA re-replication re-appeared when Dmc1 and Pch2 were absent (Figure 16A). We also performed Western blot analysis of the deletion strains to ensure the change in phenotype was not due absence of Sic1ΔP^{HA} expression (Figure 16A). These data indicate Pch2 is downstream of Dmc1, and participating in the *dmc1*Δ-induced block to re-replication. To ensure the deletion mutant did not have an effect on the DNA re-replication phenotype alone we made a control strain that was a homozygote deletion for the *PCH2* gene with *HOP1pr-SIC1*ΔP^{HA}, and found that *pch2*Δ had a small affect on our

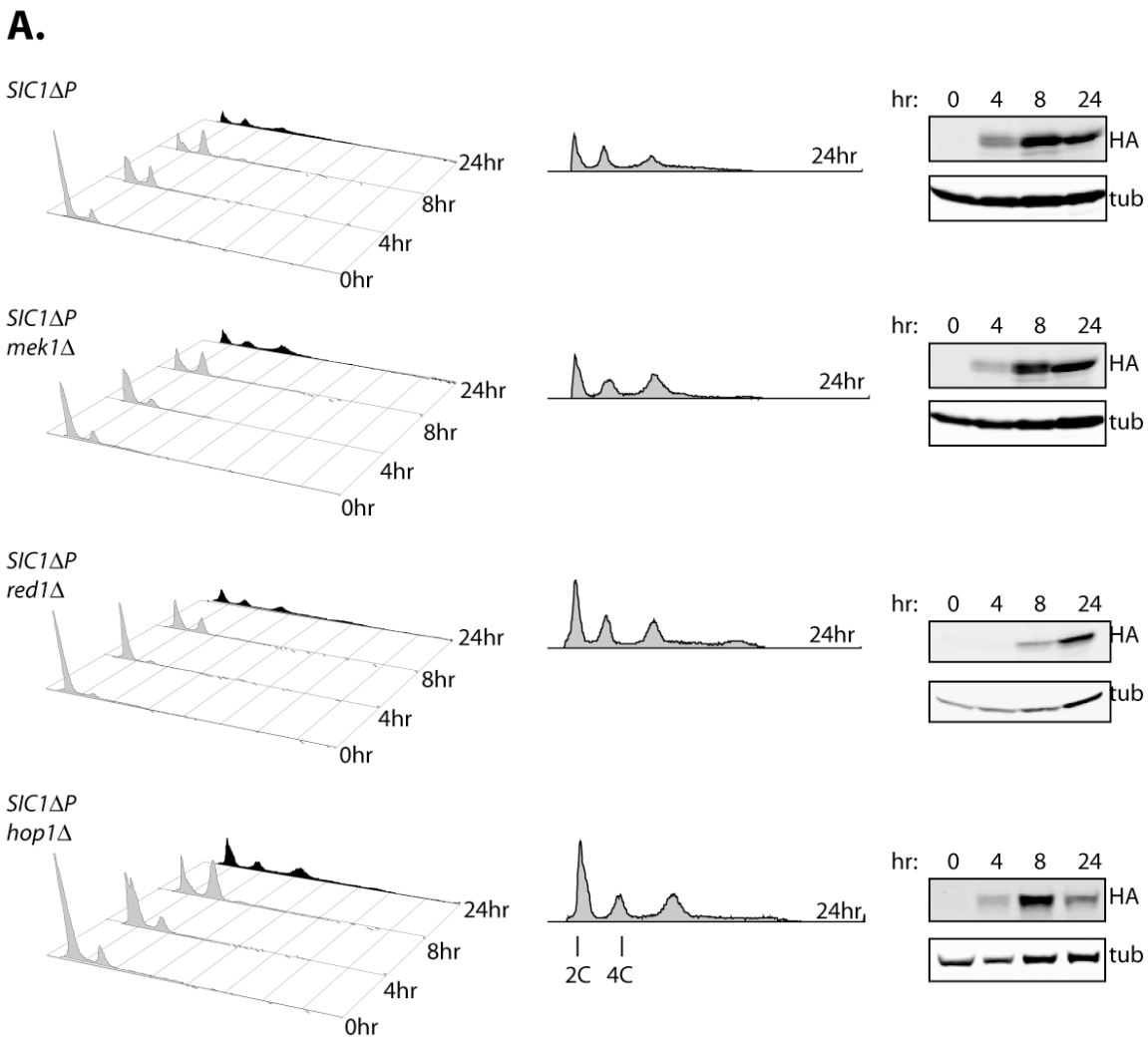


Figure 15. Gene deletions of *MEK1, RED1,* and *HOP1,* do not affect *HOP1pr*::*SIC1*ΔP^{HA}-induced DNA re-replication.

A. Control strains were generated containing *HOP1pr-SIC1*ΔP^{HA} with homozygous gene deletions of *MEK1* (YGB673), *RED1* (YGB721) and *HOP1* (YGB712) to ensure deletion mutants did not affect DNA re-replication induced by Sic1ΔP^{HA} (YGB495) expression. Strains were induced to undergo meiosis. DNA content was analyzed by flow cytometry and protein was analyzed by SDS-PAGE. HA tags detected Sic1 protein levels and tubulin protein was used as a loading control.

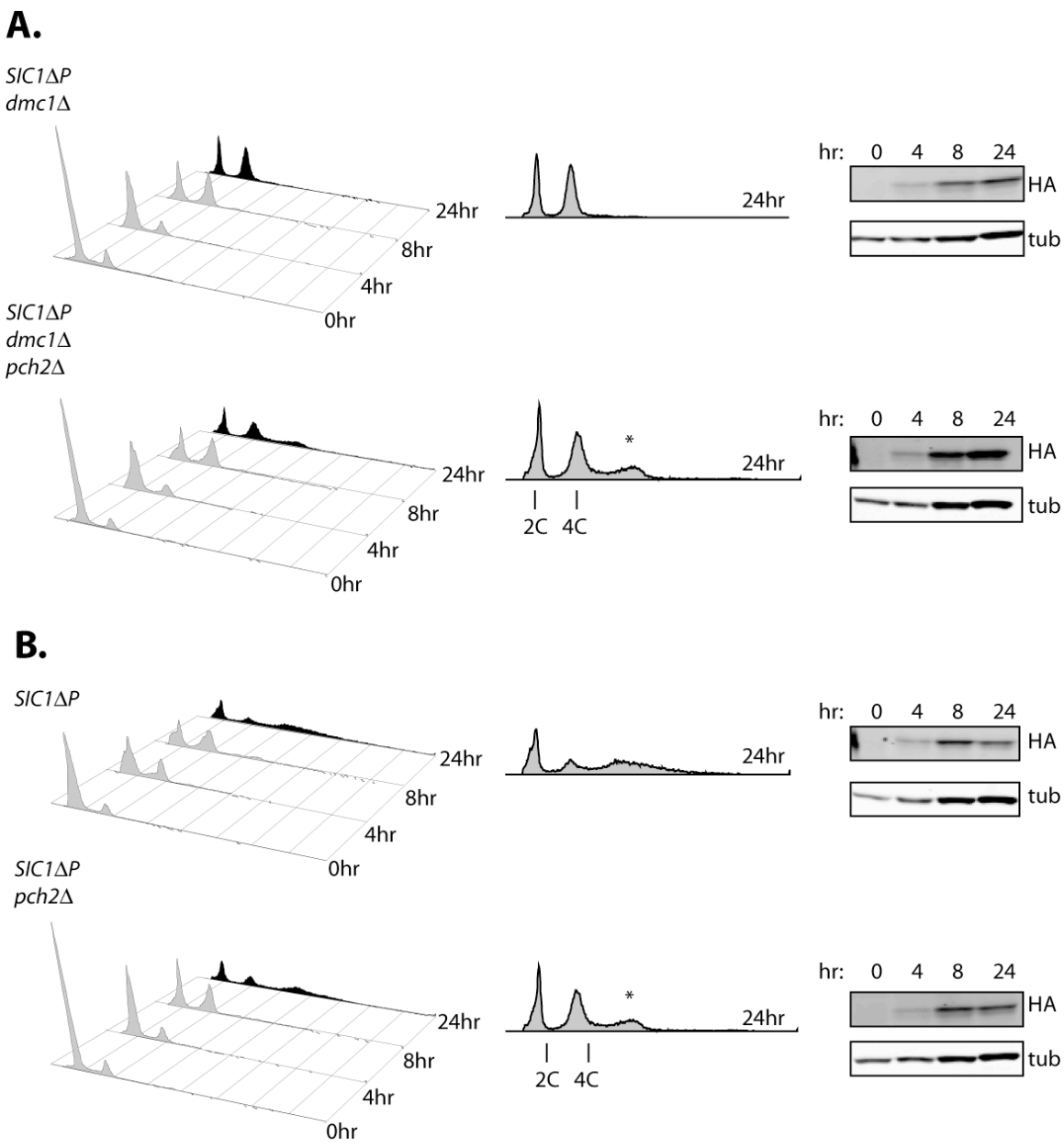


Figure 16. Pch2 participates in checkpoint-induced abolishment of DNA re-replication.

A. DNA content was analyzed by flow cytometry and protein was analyzed by SDS-PAGE of strains containing *HOP1pr-SIC1*Δ*P*^{HA} with a homozygous gene deletion of *DMC1* (YGB604) and an additional homozygous gene deletion of *PCH2* (YGB700). **B.** A control strain was generated containing *HOP1pr-SIC1*Δ*P*^{HA} with homozygous gene deletion of *PCH2* (YGB703) to investigate whether the gene deletion of *PCH2* affected Sic1ΔP^{HA}-induced DNA re-replication (YGB495). Asterisk (*) denotes effect of *pch2*Δ on DNA re-replication phenotype. HA tags were used to detect Sic1 protein, and tubulin detection was used as a loading control.

DNA re-replication phenotype (Figure 16B). The degree of DNA re-replication tends to be variable amongst our experiments, but we noticed that *PCH2* gene deletions had an affect on extent of DNA re-replication by 24hr (Figure 16A and 16B). Although a small affect was noted, DNA re-replication in the absence of Pch2 with *dmc1*Δ and *HOP1pr-SIC1*ΔP^{HA} was still re-constituted. Therefore, the data indicate the involvement of Pch2 in the *dmc1*Δ-induced block to DNA re-replication, but further investigations will divulge whether *pch2*Δ mutants affect a biological mechanism that is connected to DNA re-replication.

Main downstream targets of the meiotic recombination checkpoint are not involved in the *dmc1*Δ-induced checkpoint that can inhibit DNA re-replication

Three known targets of the pachytene checkpoint are Ndt80, Sum1, and Swe1. The DNA re-replication phenotype we have observed is caused by the expression of a mutant Cdk1 inhibitor (*HOP1pr-SIC1*ΔP^{HA}) that theoretically leads to lowered B-type cyclin-CDK activity. Because this type of CDK is required for the meiotic divisions, we suspected that DNA re-replication in our system did not require pachytene exit. To better understand if this was the case, we turned our attention to Ndt80. Normally, this transcription factor promotes the activation of Clb/Cdk1 complexes, which would promote pachytene exit. We deleted the gene *NDT80* with *HOP1pr-SIC1*ΔP^{HA} to investigate whether absence of Ndt80, and therefore absence of potential pachytene exit, would affect our re-replication phenotype and uncover if re-replication was occurring prior to the meiotic

divisions (discussed in Chapter 3). In the absence of Ndt80 with Sic1ΔP^{HA} expression (Figure 9), we found that DNA re-replication still occurred, indicating that our phenotype did not require pachytene exit. These data were the first piece of evidence that suggested a possible novel branch of the pachytene checkpoint pathway could inhibit our DNA re-replication phenotype.

To further investigate whether this was specific to Ndt80 or whether a novel branch does, in actuality, exist, we turned our attention to the other downstream targets of the pachytene checkpoint: Sum1 and Swe1. Ndt80 is a transcriptional activator of middle sporulation genes, while Sum1 is the transcriptional repressor of those same genes (Xie et al., 1999; Pak and Segall, 2002). Research has shown that *sum1*Δ can bypass *dmc1*Δ-induced arrest, which suggests signals are directed to the Sum1 protein in the pachytene checkpoint (Lindgren et al., 2000; Pak and Segall, 2002). In terms of its function in the checkpoint, research has suggested that Sum1 is upregulated, which would cause a vast repression of MSGs and, hence, cause pachytene arrest. Therefore, if Sum1 were to function in the pachytene checkpoint that prevents DNA re-replication, we should see a re-instatement of DNA re-replication when Sum1 is absent in a strain that contains *dmc1*Δ and *HOP1pr- SIC1*Δ*P*^{HA} (Figure 12B). We constructed a homozygous deletion of *SUM1* in a diploid strain that was also a homozygous deletion for the gene *DMC1* expressing Sic1ΔP^{HA}. We tested Sum1 function as for other potential checkpoint proteins (see above), and did not observe rescue of *dmc1*Δ-induced DNA re-replication block (Figure 17A). As before, to ensure the *SUM1* deletion mutant did not have an effect on the

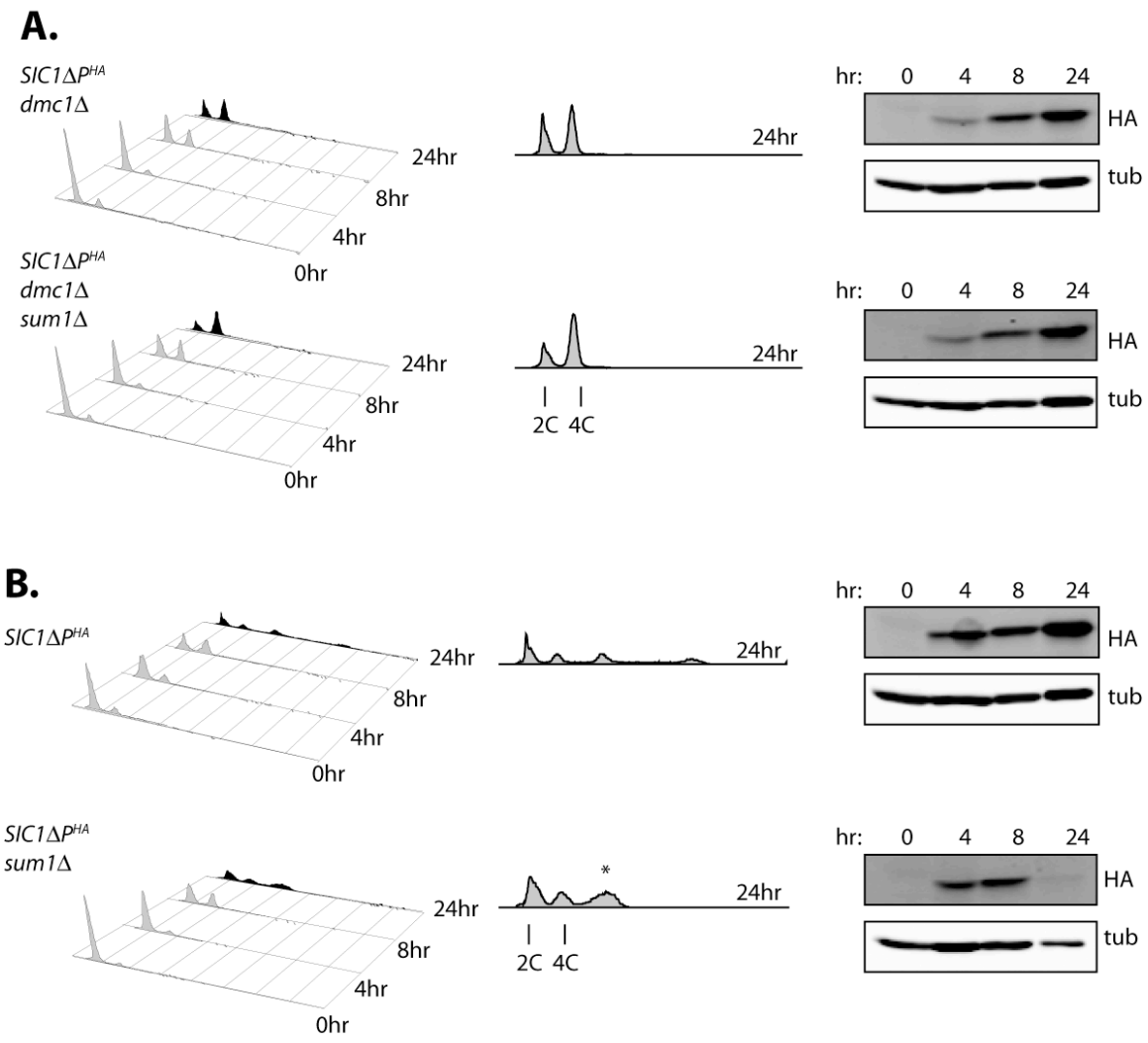


Figure 17. Transcriptional repressor of middle sporulation genes, Sum1, does not participate in *dmc1*Δ-induced block to DNA re-replication.
A. DNA content was analyzed by flow cytometry and protein was analyzed by SDS-PAGE of strains harboring *HOP1pr-SIC1*ΔP^{HA} with a homozygous gene deletion of *DMC1* (YGB604) and additional homozygous gene deletion of *SUM1* (YGB786) **B.** A control strain was generated containing *HOP1pr-SIC1*ΔP^{HA} with the gene deletion of *SUM1* (YGB785) to evaluate its effect on Sic1ΔP^{HA}-induced DNA re-replication (YGB495). Asterisk (*) denotes effect of *sum1*Δ on DNA re-replication phenotype. HA tags detected Sic1 levels, and tubulin detection was used as a loading control.

DNA re-replication phenotype alone, we made a control strain that was a homozygote deletion for *SUM1* alone (*sum1*Δ *SIC1*Δ*P*^{HA}), and found that, similar to the Pch2 experiments, there was an effect on our re-replication phenotype (Figure 17B). Nonetheless, DNA re-replication did occur. Further studies need to be completed to investigate how Sum1 affects our DNA re-replication phenotype. Therefore, these data indicate that, along with Ndt80, Sum1 does not appear to participate in the pachytene checkpoint pathway that blocks DNA re-replication.

Up to this point, our data has suggested that known meiotic recombination checkpoint downstream targets do not participate in the prevention of DNA re-replication in our system. We finally turned our attention to the third downstream target, Swe1. Defects in meiotic recombination, which activate the pachytene checkpoint, cause Swe1 accumulation and phosphorylation. This phosphorylated form of Swe1 will further inhibit Cdk1 by phosphorylation of the kinase at tyrosine 19 (Booher et al., 1993) and in turn prevent pachytene exit. We deleted *SWE1* and *DMC1* and found that Sic1ΔP^{HA}-induced DNA re-replication was not restored (Figure 18A). As before, to ensure that the homozygous *SWE1* gene deletion mutant did not have an effect on the DNA re-replication phenotype alone, we constructed a control strain that contained a homozygous gene deletion of *SWE1* with *HOP1pr-SIC1*Δ*P*^{HA}, and found that there was no effect on our re-replication phenotype (Figure 18B). We also performed Western blot analysis of the deletion strains, and found there was no obvious change in Sic1ΔP^{HA} expression that could explain the inability of a *SWE1* homozygous gene deletion to re-constitute DNA re-replication in a strain containing a homozygous gene deletion of *DMC1*

and *HOP1pr-SIC1ΔP*^{HA} (Figure 18A and 18B). These data suggested that the downstream target Swe1 was not involved in the *dmc1Δ*-mediated inhibition of the DNA re-replication phenotype associated with Sic1ΔP^{HA} expression.

Homozygous deletion of *SWE1* does not induce meiotic DNA re-replication

We were also interested in investigating whether the absence of Swe1 was able to induce meiotic DNA re-replication, which was previously reported (Rice et al., 2005). These experiments showed that diploid cells lacking Swe1 were able to complete meiosis and exhibited a "multispore" phenotype. We examined a *swe1*Δ homozygous deletion mutant and did not observe any "multispores" by flow cytometry or DAPI staining (Figure 19). Our current hypothesis is that differences in protocol explain the disparate results. Through personal communications, Dr. Josef Loidl has suggested that the previous phenotype could reflect aberrations in pre-meiotic growth.

***swe1*Δ and *sum1*Δ do not bypass *dmc1*Δ-induced arrest in W303 yeast strain**

When Swe1 and Sum1 were discovered as part of the pachytene checkpoint, experiments showed their ability to bypass *dmc1*Δ-induced arrest by DAPI staining. Arrest in pachytene prevents further progression into the meiotic divisions. Since *dmc1*Δ causes pachytene arrest, staining of the nuclei can indicate whether cells have been able to complete the meiotic divisions with 4 staining nuclei, versus cells that have not completed the meiotic divisions. We

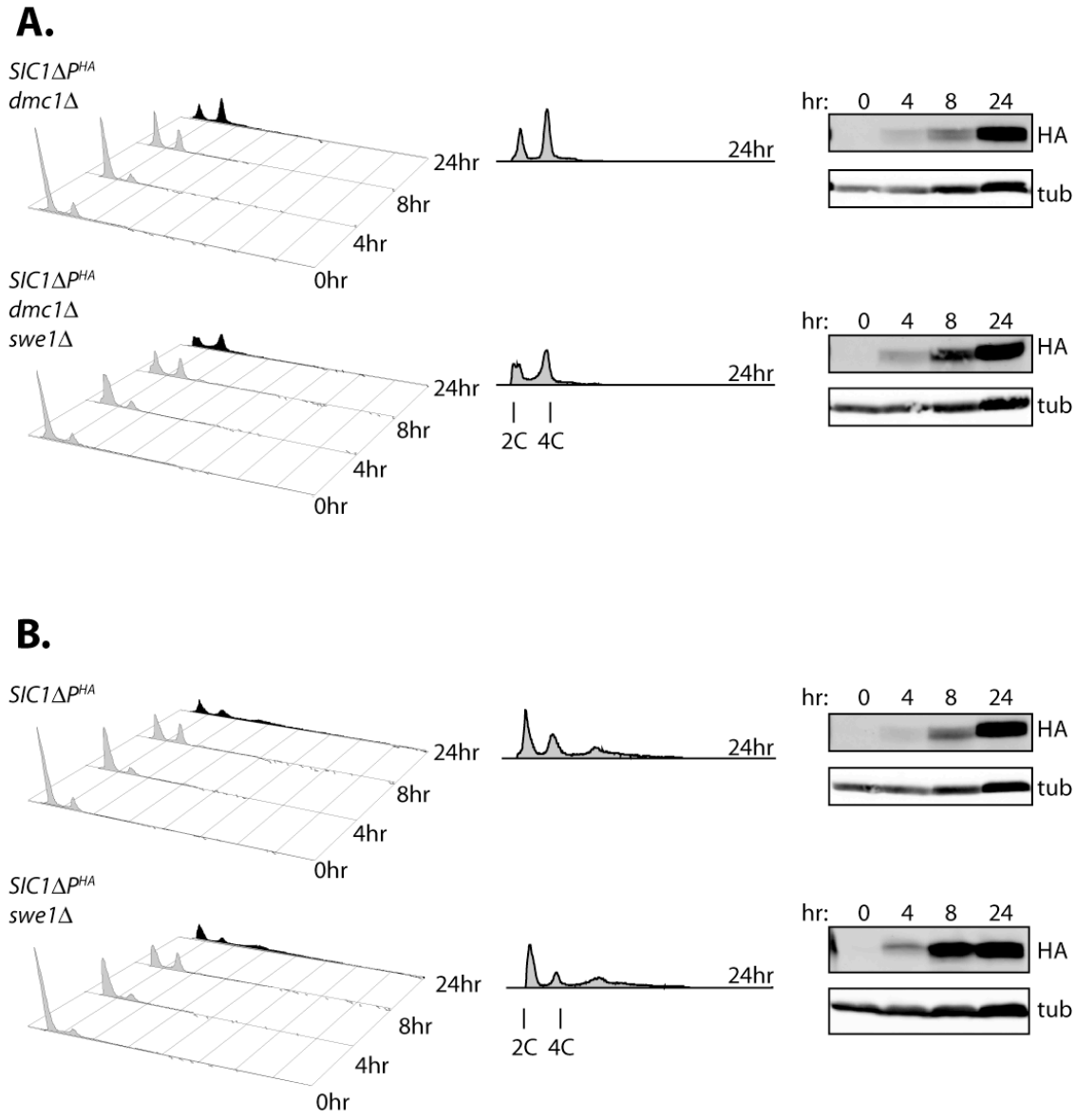


Figure 18. Downstream target of the pachytene checkpoint Swe1 does not participate in *dmc1*Δ-induced block to DNA re-replication.
A. Strains harboring *HOP1pr-SIC1*ΔP^{HA} with a homozygous gene deletion of *DMC1* (YGB604) and an additional homozygous gene deletion of *SWE1* (YGB697) **B.** A control strain containing *HOP1pr-SIC1*ΔP^{HA} with the gene deletion of *SWE1* (YGB689) was generated to test whether *swe1*Δ effected Sic1ΔP^{HA}-induced DNA re-replication (YGB495). DNA content was analyzed by flow cytometry and protein was analyzed by SDS-PAGE. HA tags were used to detect Sic1 protein, and tubulin detection was used as a loading control.

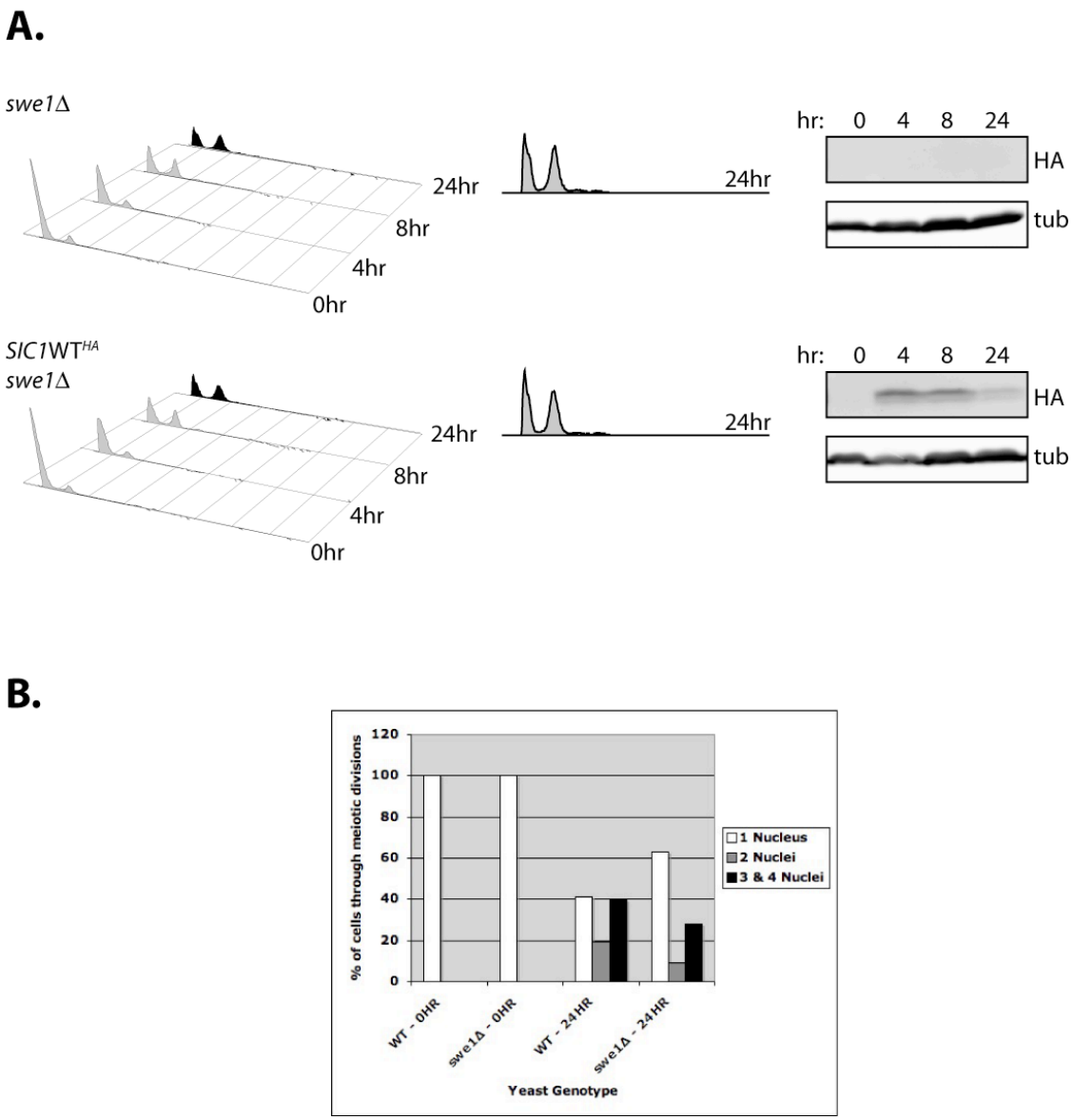


Figure 19. Deletion of *SWE1* does not induce meiotic DNA re-replication. **A.** Strains were generated harboring the homozygous gene deletion of *SWE1* (YGB687) and *HOP1pr-SIC1WT*^{HA} with a homozygous deletion of *SWE1* (YGB688). Meiosis was induced, DNA content was analyzed by flow cytometry, and Sic1 protein was analyzed by SDS-PAGE. HA tags were use to detect Sic1 levels, and tubulin detection was used as a loading control. **B.** Wild type strain (YGB138) and homozygous *swe1*Δ (YGB687) were analyzed by DAPI staining. Cells were counted (200 total) for 1, 2, or 3 & 4 DAPI staining bodies to show the percentage of cells progressing through the meiotic divisions.

generated diploids containing homozygous gene deletions for *SWE1* and *DMC1* as well as *SUM1* and *DMC1.* We analyzed these strains to determine whether the absence of Swe1 or Sum1 could bypass *dmc1*Δ-induced arrest. We found that *dmc1*Δ-induced arrest had approximately led to 98% of the cells staining for one nucleus. When we turned our attention to the *dmc1*Δ mutants containing an additional homozygous deletion of *SWE1* or *SUM1*, we found again that approximately 98% of the cells stained for one nucleus (Figure 20A and 20B). The mutants were unable to bypass *dmc1*Δ-induced arrest as examined by DAPI staining, so we considered two possibilities. First, it is possible that Sum1 and Swe1 do not function as downstream targets of the pachytene checkpoint in the yeast strain W303. Rather, Ndt80 may be the main downstream target. The data indicated Swe1 and Sum1 were unable to bypass pachytene arrest, and an explanation might be that, in the absence of Swe1 and Sum1, *dmc1*Δ is still signaling to inhibit Ndt80, which prevents transcription of genes required for pachytene exit. When we reviewed the literature, we found that Swe1 function in the pachytene checkpoint appears to be strain specific. In the strain SK1, only 10-30% of *swe1*Δ *dmc1*Δ cells were able to bypass *dmc1*Δ-induced arrest (Pak and Segall, 2002). In the strain YAB36, *swe1*Δ *dmc1*Δ entered meiotic divisions almost as efficiently as wild type cells (Leu et al., 1998). An alternative explanation, not exclusive from the first, is that a phenotype is apparent only when more than one target is missing.

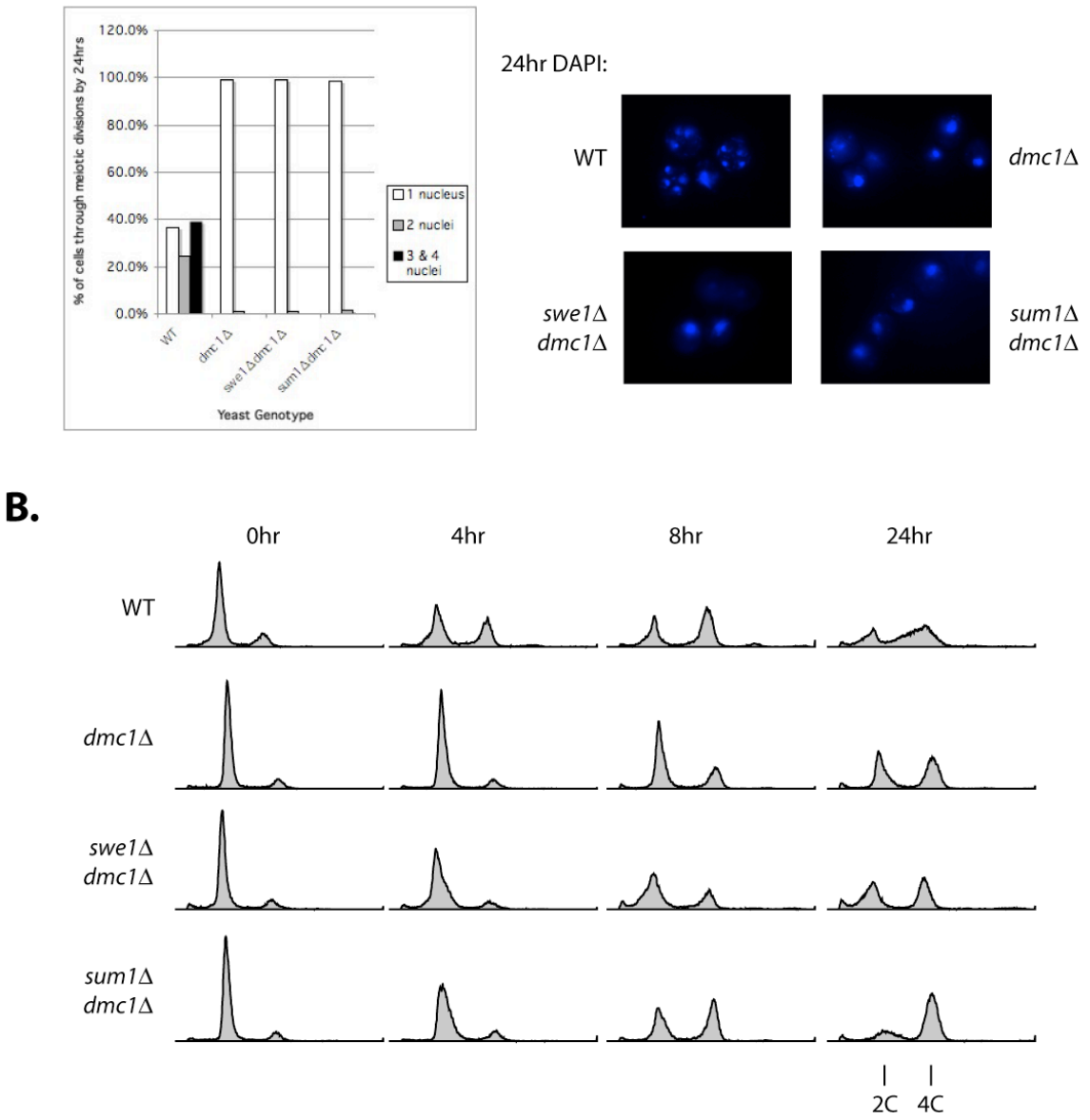


Figure 20. Mutations in Swe1 and Sum1 do not bypass *dmc1*Δ-induced arrest by DAPI staining.
A. Strains were generated to investigate whether the homozygous gene deletion of *DMC1* with the absence of Swe1 (YGB765) or the absence of Sum1 (YGB792) are able to bypass arrest induced by deletion of *DMC1* (YGB764). Meiosis was induced and the 24 hour time point was analyzed by DAPI staining. Cells were counted (200 total) for DAPI staining bodies in each strain. Percentages are plotted by bar graph (left), and corresponding images were taken (right). **B.** DNA content was analyzed by flow cytometry of wild type (YGB138), homozygous gene deletion of *DMC1* (YGB764), and an additional homozygous gene deletion of *SWE1* (YGB765) or *SUM1* (YGB792).

DNA damage checkpoint is not the alternative branch to the pachytene checkpoint that can inhibit DNA re-replication

Up to this point, our data suggests complementation by the other targets when one is missing or a novel alternative branch of the pachytene checkpoint, which can inhibit our DNA re-replication phenotype. We turned our attention to the G1-S DNA damage checkpoint as a possible alternative branch. The G1-S DNA damage checkpoint has been extensively studied in mitosis. During mitosis this checkpoint pathway is thought to inhibit Cdk1 and S phase entry by inhibiting Sic1. Lydall et al., 1996 have found that DNA damage checkpoint proteins Rad9 and Rad53 can sense mitotic DSBs, but cannot control MI progression in response to programmed DSBs in meiosis. In addition, Rad53 is not phosphorylated/active when meiosis specific DSBs occur, suggesting meiotic DSBs are hidden from the DNA damage checkpoint (Cartagena-Lirola et al., 2008). Although it seems unlikely that Rad53 could participate in sensing meiotic DSBs, when unrepaired DSBs escape the recombination checkpoint in *sae2*Δ cells, Rad53 phosphorylation is triggered and activation results in a delay of MII (Cartagena-Lirola et al., 2008).

We elected to examine levels of Sic1 protein by the C-terminal tag 13-myc::kanMX6 in our DNA re-replication strain. First to ensure that the 13myc tag we used to detect Sic1 did not functionally interfere with Sic1 function, we examined its behavior mitotically. We synchronized mitotically growing yeast cells into G1 by the addition of the yeast pheromone alpha factor. When the pheromone is washed off the cells, they progress through the cell cycle with

relative speed. We took time points every 15 minutes and examined protein levels and DNA content level by flow cytometry (Figure 21A and 21B). It is known that during mitosis, Sic1 protein levels decrease at the time of S phase entry, and subsequently increase when S phase is complete. We observed this pattern with our *SIC1*^{13myc} strain (Figure 21B). These data suggested the functionality of Sic1 was unaffected by the 13myc tag.

Next we constructed heterozygote *SIC1*/*SIC1*^{13myc}::*kanMX6* strains for examination during meiosis. We genomically tagged endogenous *SIC1* in strains that harbored *HOP1pr-SIC1WT*^{HA}, *HOP1pr-SIC1*ΔP^{HA}, *HOP1pr-SIC1*ΔP^{HA} with *dmc1*Δ*,* and a parental strain, and examined Sic1 levels (Figure 22). We found no significant difference in Sic1 levels in the three strains. Also, during the meiotic time course we did not see a drop in Sic1^{13myc} expression at S phase, as we did mitotically. This result is likely due to meiotic cells not being as synchronous mitotic cells.

Even though we noted Sic1^{13myc} was able to functional normally mitotically, our assumption that it also functions normally meiotically required evaluation. One possible explanation for why we saw relatively no change in Sic1^{13myc} expression could be due the presence of the untagged *SIC1* allele. Therefore, we generated homozygous *SIC1*^{13myc}*::kanMX6* strains. We tagged *SIC1* at each allele in wild type, *HOP1pr-SIC1*ΔP^{HA}, and *HOP1pr-SIC1*ΔP^{HA} with *dmc1*Δ cells*.* We found that by 24 hours there was no notable change in Sic1^{13myc} expression (Figure 23). Therefore, since the heterozygote Sic1^{13myc} appears similar to the homozygote Sic1^{13myc}, it is unlikely that the 13myc tag can suppress

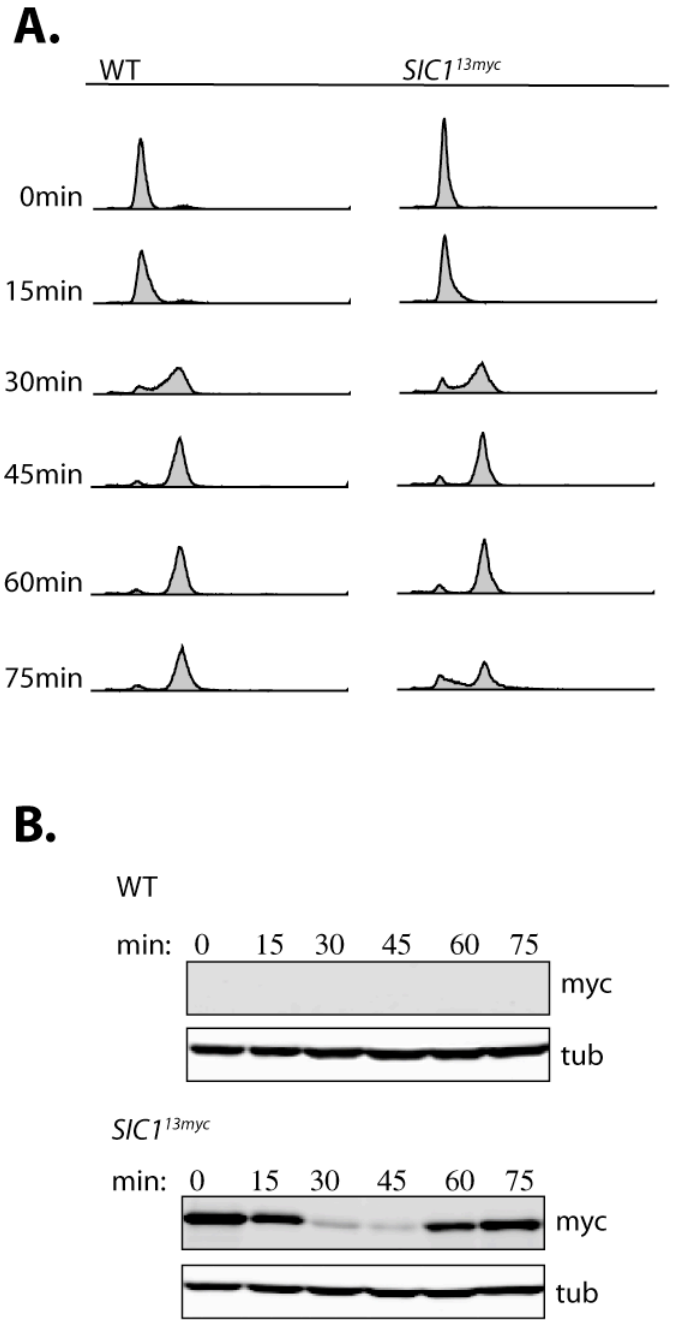


Figure 21. Decreased expression of Sic^{13myc} around the time of mitotic S phase.
Haploid strains wild type (W303) and *SIC1*^{13myc} (YGB502) were synchronized by the addition of alpha factor. Time points were taken upon removal of alpha factor. **A.** DNA content of wild type and *SIC1*^{13myc} were examined and show similar progression through the cell cycle. **B.** Protein was analyzed by SDS-PAGE. Myc tag was used for endogenous Sic1 protein detection, which decreases around 30 minutes (the approximate time of S phase). Tubulin was used as a loading control.

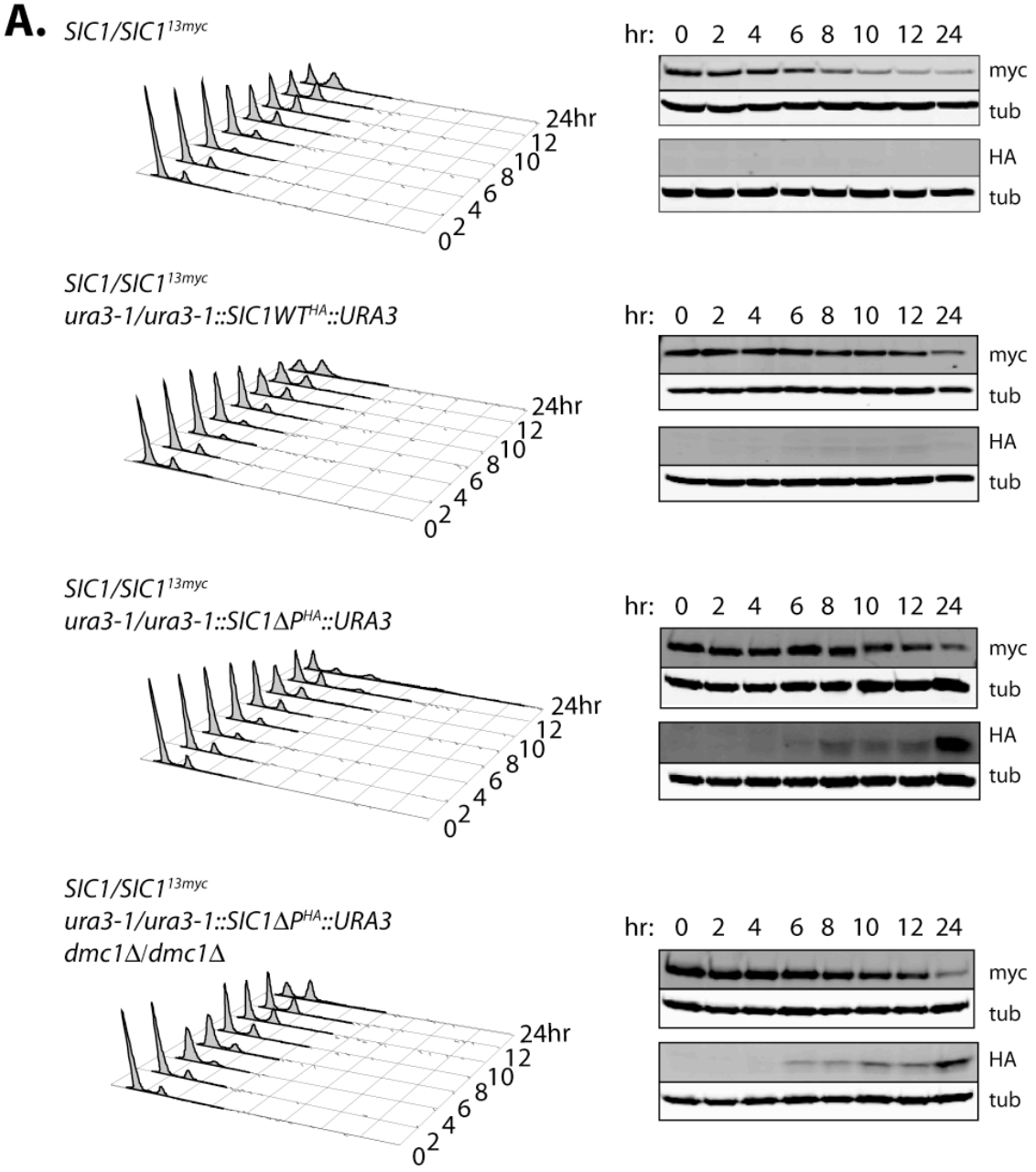


Figure 22. Heterozygote *SIC1*^{13myc} reveals no change in endogenous Sic1 protein level between *HOP1pr*::*SIC*Δ*P*^{HA} and *HOP1pr*::*SIC*Δ*P*^{HA} with *dmc1*Δ.
A. Heterozygote strains of *SIC1*^{13myc} were constructed in wild type, (YGB513) *HOP1pr*-driven *SIC1*WT^{HA} (YGB514), *HOP1pr*-driven *SIC1*Δ*P*^{HA} (YGB515), and *HOP1pr*-driven *SIC1*Δ*P*^{HA} with a homozygous deletion of *DMC1* (YGB757). All strains were induced to undergo meiosis and DNA content was analyzed by flow cytometry. Protein was examined by SDS-PAGE. HA tags were used for detection of Sic1 constructs at *URA3* locus. Myc tag was used for detection of endogenous Sic1 protein. Tubulin was used as a loading control.

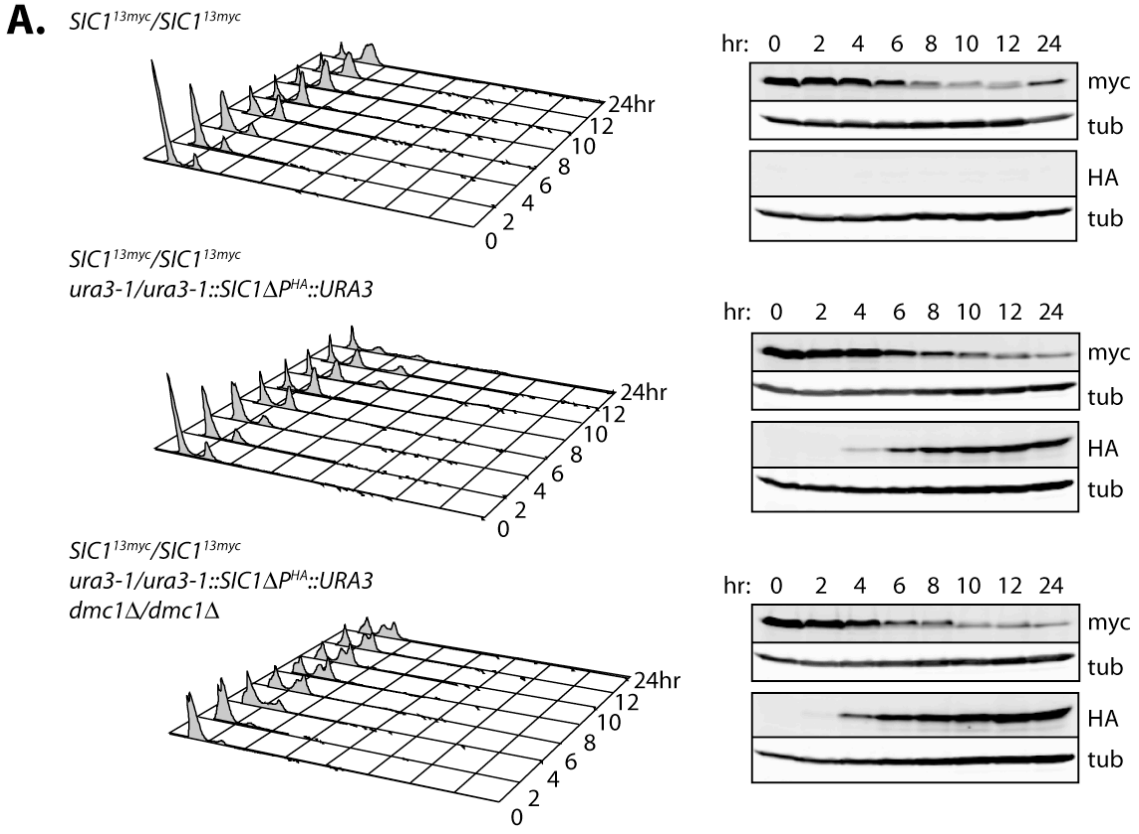


Figure 23. Homozygote *SIC1*^{13myc} reveals no change in endogenous Sic1 protein level between *HOP1pr*::*SIC*Δ*P*^{HA} and *HOP1pr*::*SIC*Δ*P*^{HA} with *dmc1*Δ. **A.** Both *SIC1* alleles were tagged with *13MYC* in wild type (YGB807), *HOP1pr-SIC1*Δ*P*^{HA} (YGB808), and *HOP1pr-SIC1*Δ*P*^{HA} with homozygous gene deletion of *DMC1* (YGB809). All strains were induced to undergo meiosis and DNA content was analyzed by flow cytometry. Protein was examined by SDS-PAGE. HA tags were used for detection of Sic1 constructs at *URA3* locus. Myc tag was used for detection of endogenous Sic1 protein. Tubulin was used as a loading control.

Sic1. Rather the likely explanation is that difference in phenotype between *HOP1pr-SIC1*ΔP^{HA} and *HOP1pr-SIC1*ΔP^{HA} with *dmc1*Δ is not due to a change in expression of endogenous Sic1.

We also turned our attention to the protein Rad9, which is required for the G1-S DNA damage checkpoint. We found that homozygous deletion of *RAD9* with *HOP1pr-SIC1*ΔP^{HA} did not affect our DNA re-replication phenotype, and homozygous deletions of *RAD9* and *DMC1* with *HOP1pr-SIC1*ΔP^{HA} did not bypass the *dmc1*Δ-induced inhibition of DNA re-replication (Figure 24A). We also performed Western blot analysis of the deletion strains, and found there was no substantial change in Sic1ΔP^{HA} expression that could have explained the lack of effect (Figure 24B). These data suggested that the pachytene checkpoint branch capable of inhibiting DNA re-replication was not associated with the known G1-S DNA damage checkpoint.

III. Discussion

We have shown DNA re-replication by expression of Sic1ΔP^{HA} can be abolished by *dmc1*Δ-induced arrest. We have also shown many of the proteins known to function downstream of Dmc1 (*i.e.* Mec1, Mek1, Red1, Hop1, and Pch2) participate in the checkpoint induced inhibition of DNA re-replication. However, we also found that the three downstream targets, Ndt80, Sum, and Swe1, did not. These data implied Ndt80, Sum1 and Swe1 were unable to prevent DNA re-replication when the checkpoint was induced by *dmc1*Δ.

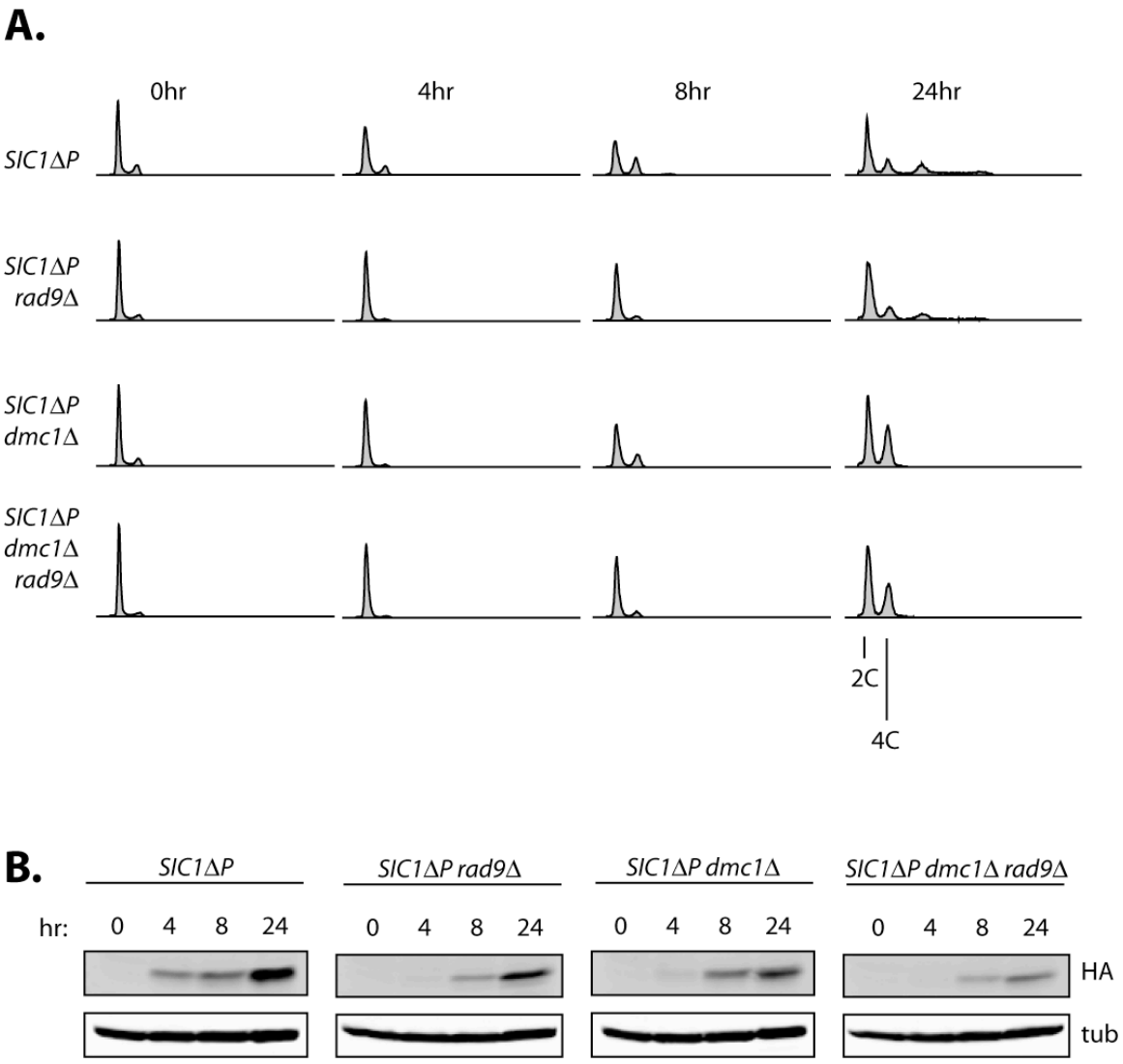


Figure 24. Mitotic G1-S DNA damage checkpoint protein Rad9 does not participate in the *dmc1*Δ-induced block of meiotic DNA re-replication.
A. DNA content was analyzed by flow cytometry of strains containing *HOP1pr-SIC1*Δ*P*^{HA} (YGB495), with a homozygous gene deletion of *RAD9* (YGB758), with a homozygous gene deletion of *DMC1* (YGB604), and with homozygous gene deletions of *RAD9* and *DMC1* (YGB759). **B.** Protein was analyzed by SDS-PAGE of strains containing *HOP1pr-SIC1*Δ*P*^{HA} with homozygous gene deletion of *RAD9* (YGB758) and with homozygous gene deletions of *RAD9* and *DMC1* (YGB759). HA tags were used to detect Sic1 protein, and tubulin was used as a loading control.

Perhaps, this is not as surprising since the downstream targets are all involved with the progression through the meiotic divisions.

Our data indicated that Sum1 and Pch2 had an effect on meiotic DNA re-replication, regardless of the pachytene checkpoint. These data will need to be further investigated, but these proteins could allow us to better understand the mechanisms that would normally prevent meiotic DNA re-replication. We questioned whether an alternative branch of the pachytene checkpoint might be responsible for inhibition of DNA re-replication. We turned our attention to the G1-S DNA damage checkpoint and found that there was no change in endogenous $Sic1^{13myc}$ expression whether in a heterozygote or homozygote tagged strain. We also constructed *rad9*Δ mutants to investigate whether that G1-S DNA damage checkpoint protein participated in the *dmc1*Δ-induced arrest, and found that the deletion mutant did not rescue the abolishment of DNA re-replication.

The data indicated that the G1-S DNA damage checkpoint was unlikely to be the alternative branch of the pachytene checkpoint, which can inhibit DNA re-replication induced by expression of Hop1pr-Sic1ΔP^{HA}. Therefore, we further investigated whether Sum1 and Swe1 functioned as the downstream targets of the pachytene checkpoint in the yeast strain W303, regardless of our DNA re-replication phenotype. What we uncovered by nuclear staining was that the absence of Sum1 or Swe1 could not bypass *dmc1*Δ-induced arrest.

In summary, an alternative pathway is able to inhibit meiotic DNA re-replication induced by Sic1ΔP^{HA} expression. We have hypothesized that the

pachytene checkpoint, which is able to inhibit DNA synthesis, might have a direct effect on the replication machinery. Further experiments will be required to resolve this question.

CHAPTER 5

DISSCUSSION

During the cell cycle and meiosis, tightly regulated events control the entry into S phase to ensure one round of DNA replication. By expression of a mutated form of the cyclin dependent kinase inhibitor Sic1 (Sic1ΔP^{HA}), we disabled a meiotic mechanism that would normally prevent DNA re-replication from occurring. In addition, we identified a recombination (pachytene) checkpoint pathway that can prevent DNA re-replication. Through these dissertation studies, we have used the pachytene checkpoint to better understand the controls of re-replication, while simultaneously using DNA re-replication as a tool to better understand the pachytene checkpoint.

Cdk1/Clb activity promotes the entry of S phase and prevents DNA re-replication during mitosis (Schwob and Nasmyth, 1993; Dahmann and Futcher, 1995). Since altering Cdk1/Clb activity by expression of Sic1ΔP^{HA} can allow for the initiation of DNA re-replication, we assumed that it must somehow be involved in the prevention of DNA re-replication. Our data suggest that the mechanisms that prevent mitotic DNA re-replication are similar in mitosis and meiosis. The meiotic events and transitions between phases require specific levels of Cdk1 activity to allow for normal progression. According to current models regarding the cell cycle, one threshold is required for initiation of DNA replication and prevention of re-initiation, while a higher threshold is required for

chromosome segregation (Stuart and Wittenberg, 1998). We have shown that the meiosis-specific induction of Sic1ΔP^{HA} allowed for DNA replication without preventing DNA re-replication. Our hypothesis is that *HOP1pr*-driven *SIC1ΔP^{HA}* lowered Cdk1 activity and subsequent fluctuations in this lowered Cdk1 activity allowed for pre-RC formation and a second round of origin firing. Increasing the expression of Sic1ΔP^{HA} inhibited DNA replication altogether, most likely by further decreasing Cdk1 activity to a level that was below the threshold required meiotic S phase. Ideally in a wild type cell, Cdk1 activity reaches the appropriate amounts to allow for smooth progression through meiosis.

The mechanisms that are known to prevent mitotic DNA re-replication are all associated with Cdk1-mediated inhibition of proteins required for replication-origin licensing. It will be important to specifically investigate these mechanisms in our yeast strain harboring *HOP1pr-SIC1ΔP^{HA}*. Specifically, the behavior of proteins such as Cdc6, Ccdt1, the MCM complex, and ORC during normal meiotic progression and under conditions of DNA re-replication will be informative.

Although our data suggest that the mechanisms that inhibit meiotic DNA re-replication are similar to those in mitosis, it is quite possible there is another mechanism involved connected with the prevention of meiotic DNA re-replication, possibly through Ime2. In mitosis, Cdc6 degradation is one mechanism that prevents DNA re-replication. It has been shown that Cdc6 degradation in meiosis is independent of Cdk1, and by two-hybrid/co-immunoprecipitation experiments a physical interaction between Cdc6 and Ime2 was revealed (Ofir et al., 2004).

Also, mitotic cells harboring an analog sensitive Cdk1 mutant (Cdk1-as1), arrested after S phase by nocodazole and treated with the analog 1-NM-PP1 to inhibit Cdk1, were able to participate in multiple round of DNA replication (Holt et al., 2007). In addition, it has been shown that ectopically expressed Ime2 can prevent DNA re-replication induced by lowered Cdk1 in the mitotic cell cycle. In this scenario, Ime2 prevented nuclear accumulation of Mcm7, which is a normal function of Cdk1 (Holt et al., 2007). Therefore, while we induced meiotic DNA re-replication by lowering Cdk1 activity, it is possible that Ime2 is also involved.

Others have shown mitotic and meiotic DNA re-replication in *S. cerevisiae*. Sic1 overexpression in the mitotic cell cycle can induce DNA re-replication, but only when the level of Sic1 expression is precisely controlled. By increasing the expression of Sic1, 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 and Futcher, 1995). In our case, we did not have to alter levels of Sic1ΔP^{HA} expression. Rather, the simple use of a meiotic inducible promoter, *HOP1pr,* allowed us to achieve a level of Cdk1 activity that allowed for meiotic DNA re-replication. As indicated above, we speculate that natural fluctuations in this Cdk1 activity allowed for DNA re-replication to occur. DNA re-replication through the overexpression of Clb1 or Clb5 (Strich et al. 2004) or the deregulation of Cdk1 (Rice et al 2005) has also been observed. These data conflict with our findings, since we have shown that a decrease in Cdk1/Clb activity can induce meiotic DNA re-replication. The nature of DNA re-replication observed by these two groups display phenotypes vastly different from ours,

which could explain our conflicting results. Namely, both groups have shown the production of large asci with multiple DAPI staining nuclei. We speculate that their DNA re-replication occurred prior to meiosis and resulted in cells containing two or more nuclei. Subsequently, each nucleus would undergo normal meiosis and result in an asci with numerous haploid spores (Josef Loidl, personal communication).

By testing different recombination mutants, we found that the loss of Dmc1 could abolish our DNA re-replication phenotype induced by the expression of Sic1ΔP^{HA}. There were three reasons that could explain why the loss of Dmc1 would abolish meiotic DNA re-replication. The first was that the Dmc1 recombinase itself was somehow required for DNA re-replication. The second was that the loss of Dmc1 led to DNA structure physically impossible to replicate. The third was that loss of Dmc1 turned on a checkpoint that inhibited DNA re-replication. By making additional gene deletions to the yeast strain harboring a homozygous deletion of *DMC1* with *HOP1pr-SIC1*Δ*P*^{HA}, we were able to uncover a recombination checkpoint that inhibited DNA re-replication. The proteins participating in this checkpoint include Rad17, Mec1, Mek1, Red1, Hop1, and Pch2.

Surprisingly, the downstream targets of the pachytene checkpoint, Ndt80, Sum1, and Swe1, did not seem to function in the pachytene checkpoint-induced block of DNA re-replication. Although Swe1 can affect multiple Cdk1-Clb complexes, it is known that Swe1 prefers Cdk1-Clb2 complexes for inhibitory phosphorylation, and does not recognize Cdk1-Clb5, -6 complexes (Hu and

Aparicio, 2005). Clb2 is not expressed during meiosis (Grandin and Reed, 1993; Dahmann and Futcher, 1995), Therefore, the inability of Swe1 to participate in the *dmc1*Δ-induced block to DNA re-replication might be explained by the absence of its preferred Cdk1-cyclin complex and the inability to recognize Cdk1-cyclin complexes present.

The gene deletion of *DMC1* has been shown to induce pachytene arrest (Bishop et al., 1992; Gerton and DeRisi, 2002; Leu et al., 1998) and an additional mutation in Swe1 or Sum1 bypasses the arrest and allows for progression through the meiotic divisions (Leu and Roeder, 1999; Lindgren et al., 2000; Pak and Segall, 2002). Because we did not observe an effect of gene deletions *SUM1* or *SWE1* on *dmc1*Δ-induced inhibition of DNA re-replication, we wanted to further investigate the role of these proteins in the checkpoint that prevents meiotic divisions. Regardless of Sic1ΔP^{HA} expression, we found that *swe1*Δ and *sum1*Δ were unable to bypass arrest induced by *DMC1* gene deletion. An explanation might be that in the absence of Swe1 and Sum1, *dmc1*Δ is still signaling to inhibit Ndt80, which prevents transcription of genes required for pachytene exit. Since Ndt80 is inhibited by the *dmc1*Δ-induced arrest, the construction of a deletion mutant would not give similar insight. Further experiments will reveal whether Ndt80 is the sole target of the pachytene checkpoint in the yeast strain W303.

In terms of the ability of the pachytene checkpoint to inhibit DNA re-replication, we propose 1 of 3 possible mechanisms that could explain how a *dmc1*Δ mutant can prevent Sic1ΔP^{HA}-induced meiotic DNA re-replication:

Our first hypothesis is, that the pachytene checkpoint effects Cdk1 through the targets Ndt80, Sum1, and Swe1, but in the yeast strain W303 the absence of one target is compensated by the other two. This can be easily answered by the construction of double deletion mutants to (1) see if *swe1*Δ *sum1*Δ can rescue *dmc1*Δ-induced arrest, and (2) see if *swe1*Δ *sum1*Δ participates in the *dmc1*Δ-induced block of DNA re-replication.

Another possibility could be that the pachytene checkpoint has a direct effect on the replication machinery preventing DNA re-replication. It has been shown that G1-S DNA damage checkpoint protein Rad53 can catalyze phosphorylation of the kinase Ddk and inhibit its activity (Weinreich and Stillman, 1999). Ddk and Cdk1 are required for proper assembly of the pre-RC at the origins (Figure 3). It is important to note that G1-S DNA damage checkpoint proteins are unlikely to participate in our *dmc1*Δ-induced block to DNA re-replication, as indicated by the Rad9 experiments (Figure 24). Instead we suggest the target of the *dmc1*Δ-induced block to DNA re-replication might be directed towards the origins. Evidence has shown that the DNA damage checkpoint can directly affect replication machinery (Weinreich and Stillman, 1999), and it is, therefore, possible that the *dmc1*Δ-induced block to DNA re-replication might function in the same manner.

A third scenario is the pachytene checkpoint prevents meiotic DNA re-replication through Cdk1 and a meiosis-specific protein such as Ime2. Earlier we discussed that Ime2 is likely responsible for the degradation of Cdc6 in meiosis (Ofir et al., 2004), and it is known that kinase activity of Cdk1 performs the same

function in mitosis (Drury et al., 1997; Jallepalli and Kelly, 1997; Elsasser et al., 1999). This example provides evidence in that Ime2 can replace some Cdk1 mitotic functions. In addition, the two kinases have been shown to both be required for certain meiotic events. While Cdk1 is required for the exit from pachytene (Shuster and Byers, 1989), Ime2 is also required pachytene exit by activating the transcription factor Ndt80, which activates middle sporulation genes including all five *CLB* genes (Chu and Herskowitz, 1998; Hepworth et al., 1998). Ime2 does not require cyclin binding partners for its activation, and it has been shown that phosphorylation of Ime2 activates and regulates the protein kinase through different phases of meiosis (Schindler and Winter, 2006). It is then possible that Ime2 is activated by Cdk1 phosphorylation in the *dmc1*Δ-induced block to DNA re-replication. Therefore, based on this proposal we suggest that, although we are manipulating Cdk1 levels to induce meiotic DNA re-replication, it is possible that mechanism that prevents DNA re-replication is not solely through the kinase Cdk1. Whether Ime2 replaces Cdk1, Ime2 functions downstream of Cdk1, or the kinases function together in the *dmc1*Δ-mediated block of meiotic DNA re-replication, further experiments will address the involvement of Ime2.

Our studies have provided the first piece evidence that DNA re-replication is prevented similarly in mitotic and meiotic cells, and that the meiotic recombination checkpoint can influence DNA synthesis. Further studies will be required to more precisely define the mechanisms by which Sic1ΔP^{HA} induces

meiotic DNA re-replication and the ability of the pachytene checkpoint to prevent its occurrence.

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ABSTRACT

MEIOTIC DNA RE-REPLICATION AND THE RECOMBINATION CHECKPOINT

by

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Progression through meiosis occurs through a strict sequence of events, so that one round of DNA replication precedes programmed recombination and two nuclear divisions. Cyclin dependent kinase 1 (Cdk1) is required for meiosis, and any disruption in its activity leads to meiotic defects. The Cdk1 inhibitor, Sic1, regulates the G1-S transition in the mitotic cell cycle and the analogous transition in meiosis. We have employed a form of Sic1, Sic1ΔP^{HA}, that is mutated at multiple phosphorylation sites and resistant to degradation. Meiosis specific expression of Sic1ΔP^{HA} disrupts Cdk1 activity and leads to significant accumulation of over replicated DNA. These data suggested that Cdk1 is required to prevent inappropriate re-initiation of DNA synthesis during meiosis, as it is during mitosis. In addition, deletion of the gene *DMC1,* which encodes a recombinase required for meiotic recombination, prevented DNA re-replication.

However, the additional deletions of *RAD17, MEC1, MEK1, RED1, HOP1,* or *PCH2* restored the re-replication phenotype. These proteins are all individually required for the meiotic recombination checkpoint. Therefore, indicating that induction of the pachytene checkpoint by *dmc1*Δ was responsible for meiotic DNA re-replication. The downstream targets of the meiotic recombination checkpoint, Ndt80, Sum1, and Swe1, which function to maintain arrest in the pachytene stage of prophase of MI, were unable to inhibit meiotic DNA re-replication induced by Sic1ΔP^{HA} expression. Therefore, it appears that a separate branch of the pachytene checkpoint exists that has the ability to prevent extra rounds of meiotic DNA replication. We investigated whether the G1-S DNA damage checkpoint as defined in the mitotic cell cycle might be implicated, and found that this checkpoint was not involved. In summary these dissertation studies discuss the implications of lowering Cdk1 activity to induce meiotic DNA re-replication, as well as the interplay of the pachytene checkpoint. Our results provide strong evidence that the control of DNA replication is likely to be similar in mitosis and meiosis. In addition, our results are the first to show the ability of the pachytene checkpoint to monitor meiotic DNA replication.

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