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The signal between the initiation of recombination and the first division of meiosis in Saccharomyces cervisiae

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THE SIGNAL BETWEEN THE INITIATION OF RECOMBINATION AND THE FIRST DIVISION OF MEIOSIS IN *SACCHAROMYCES CEREVISIAE*

by

Kelley Elizabeth Foreman

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Biology
in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Professor Robert E. Malone

ABSTRACT

Meiosis is the process by which diploid cells undergo DNA synthesis, homologous recombination and pairing, followed by the reductional division then the equational division. I present work in this PhD thesis which furthers the understanding of the coordination of the initiation of meiotic recombination and the reductional division. Ten genes are required to initiate recombination in *Saccharomyces cerevisiae*. The presence of a subset of recombination initiation proteins creates a Recombination Initiation Signal (RIS) that delays the start of MI in wild type cells. I present experiments demonstrating the first division kinetics of the two remaining recombination initiation genes that our lab had not yet studied. Rec107 is part of the RIS, while Ski8 is not. The RIS is conserved in a divergent *Saccharomyces* strain background. *rec102* and *rec104* SK1 strains both start the first division earlier that wild type SK1 strains. I present evidence that suggests that the RIS acts independently of the pathway that controls securin (PDS1) degradation.

The work in this thesis expands our knowledge of the mechanism by which the RIS delays the reductional division. In this thesis I present experiments showing that the DNA damage, spindle and S phase checkpoints do not transduce the RIS. I establish the meiosis-specific candidate Mek1 as a candidate for relaying the RIS. Lastly, experiments described in these chapters show that the transcriptional activator of Middle Meiosis, *NDT80*, is the target of the RIS. *NDT80* transcription and activity are both necessary and sufficient to affect an earlier reductional division, similar to the early MI seen in RIS mutants.

Abstract Approved: _____

Thesis Supervisor

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Title and Department

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Date

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Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

Kelley Elizabeth Foreman

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Biology at the May 2010 graduation.

Thesis Committee: _____
Robert E. Malone, Thesis Supervisor

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Marc Wold

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John Menninger

To Cam, I love you

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First and foremost, thank you to my wonderful husband Cam. Your love and encouragement have meant more to me than anything. Thank you for sacrificing and putting things on hold while I got through this. Your turn next-- I promise! Thank you to my Mom and Dad for your years of dedication to all of your children. Mom, I hope you enjoy having this thesis on your bookshelf. Thank you to my sister Heather for making me laugh. I am very grateful to my Uncle Mark Roth for believing in me and inspiring me to become a scientist. I have many wonderful friends who have cheered me on through this. In particular, thank you to Susan Dean for being there for me. Thank you to my wonderful friend, Lynn Bixler, for always rescuing me. Thank you to my advisor, Bob Malone, for the lessons you have taught me. I would also like to thank the members of my committee, John Logsdon, Marc Wold, John Menninger and George Stauffer for your service and advice. Jackie Segall, Orna Cohen-Fix and Scott Keeney very generously provided some of the strains and/or plasmids used in this thesis. I thank Greg Gingerich for his technical advice on Northern blotting. I have been fortunate to work with some incredibly talented people in the Malone lab. Thank you to Stuart Haring for being my friend during my early years in the Malone lab. Rachel Gast is both a wonderful friend and collaborator. She and I worked together on some of the work presented in Chapter 2. I have also been fortunate to work with some very intelligent Honor's undergraduates. It has been my privilege to be a mentor to Morgan Pansegrau, Logan Vidal, Ben Jorgensen and Heather De Bey. In particular, I am grateful to Logan and Morgan for contributing some of the work in this thesis. Thank you to my friend and lab-mate-for-life Demelza Koehn. We did it together! Last, but not least, I am grateful to the many students that I have taught. You have taught me as much as I have taught you. Thank you.

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PREFACE

Many of the experiments performed in this thesis are the result of collaboration. I have only presented data figures to which I have contributed. Where relevant, I discuss results obtained by many different members of the Malone lab. Though I have chosen not to show their data, I do provide significant discussion of it throughout the thesis to give my work better context. I have attributed these results to the original experimenter, and provided a reference, if the observation has been published.

At the end of the Introduction to Chapters 2-5, I have provided attribution to each collaborator who has contributed to the figures shown in this work in italics. I will also briefly list these contributions here. In Chapter 2, Morgan Pansegrau, an Honors undergraduate contributed significantly to the *rec107* experiments shown in Fig. 2-1. Also in this Chapter, Rachel Gast, a Masters student collaborated with me on the SK1 experiments shown in Fig. 2-3 and 2-4. In this chapter, our *rec107* (Fig. 2-1) and *ski8* (Fig. 2-3) experiments have been published in a 2004 paper in *Eukaryotic Cell* (Malone et al., 2004). The remainder of the experiments will be submitted in early 2010 to the journal *Genetics*. I am the primary author on this manuscript.

In Chapter 3, Sonja Smith, a R.A. II in the Malone lab made the heterozygous strains that I used in that chapter. This work has not yet been published and will be included in the aforementioned *Genetics* manuscript.

Logan Vidal, an Honors undergraduate, contributed significantly to the experiments shown in Fig. 4-5. Fig. 4-2 was included in our paper in Eukaryotic Cell. The remainder of the experiments will be included in our forthcoming manuscript to be sent to *Genetics*.

Morgan Pansegrau also contributed to work in Chapter 5. We collaborated on experiments shown in Fig. 5-3 and 4. Rachel Gast made the *mad3* deletion strain that I use in the Northern Analysis shown in Fig. 5-1. Although I do not show any of her work,

her initial observations of the meiotic phenotype of a *mad3* mutant were very influential in my work and are discussed throughout this thesis. Fig. 5-3 and 5-4 were published in our 2004 *Eukaryotic Cell* paper. The remainder of the experiments shown will be included in our *Genetics* manuscript.

In this thesis, I will discuss all protein and gene names using the standard nomenclature rules used in *Saccharomyces cerevisiae* research. Wild type gene names are italicized and capitalized (*e.g.*, *GEN1*). Mutant gene names are lowercase and italicized (*e.g.*, *gen1*). Protein names are un-italicized and have only the first letter of the protein name capitalized (*e.g.*, Gen1). Protein names are sometimes referred to in the literature followed by the letter "p" immediately after the name, but I have chosen not to use this convention.

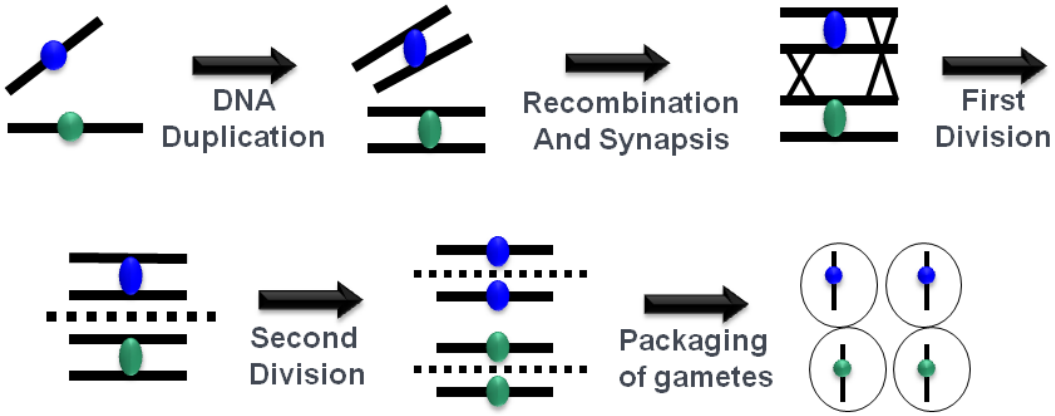
The references presented in this thesis are cited using the format used by the journal *Cell*.

CHAPTER 1

INTRODUCTION

Meiosis is the process by which a sexually-reproducing diploid organism reduces its chromosomal number by half to create haploid products. These haploid products are then reunited during fertilization or mating. In the hemi-ascomycete fungus *Saccharomyces cerevisiae* the end products of meiosis process are four haploid spores that are contained in a single ascus. The major chromosomal events of meiosis are largely conserved across eukaryotes. Chromosomes are first replicated during a period of pre- meiotic S- phase that is similar to S phase in mitosis, though in *S. cerevisiae*, longer in duration (Carballo and Cha, 2007). In *Saccharomyces* and most other eukaryotic cells, this stage is followed by recombination between homologous chromosomes (Fig. 1-1). This step creates chiasmata which physically hold together homologs. A protein structure known as the synaptonemal complex (SC) also assists this physical association. Homologous chromosomes then segregate during the first, or reductional, division (MI). This is immediately followed, without an intervening S phase, by the second, or equational, division (MII) where sister chromatids are segregated to opposite poles. Finally, in *Saccharomyces* and other related fungi, the meiotic products are packaged into four spores contained in an ascus. Errors in any of these stages can lead to a reduction in spore viability or, in the case of multicellular eukaryotes, decreased zygotic or embryonic viability after fertilization of gametes has occurred (Esposito and Esposito, 1975; Hochwagen and Amon, 2006). In particular, meiotic recombination is critical in most eukaryotes including *Saccharomyces.* A failure to initiate recombination leads to random segregation of homologous chromosomes during MI and the formation of aneuploid, inviable spores (Hochwagen and Amon, 2006; Keeney, 2001). Without pairing, the chance of a single pair of chromosomes segregating correctly into opposite cells is ½.

Figure 1-1: The major chromosomal events of meiosis [1]. Pre-meiotic DNA synthesis results in duplication of the genetic information. [2] Meiotic recombination and synaptonemal complex formation result in proper pairing of homologous chromosomes. [3] Segregation of the homologous chromosomes to opposite poles in the reductional division results in a halving of the genetic information. [4] Sister chromatids separate from each other during the equational division. [5] Finally, the chromatids are packaged into four meiotic products with 16 pairs of chromosomes in *Saccharomyces*, the chance that all chromosomes will segregate correctly is very small ($(1/2)^{16}$ = 1/65536 or 0.0015 % of meioses).


DNA
Duplication
Recombination
And Synapsis
First
Division
Second
Division
Packaging
of gametes

In addition to ensuring correct chromosomal segregation, recombination also creates genetic diversity along the length of a chromosome. The exchange of genetic material between homologs results in new combinations of alleles not seen in the original parent cell. Recombination of alleles can create more favorable combinations which can be acted upon by natural selection, thus changing allelic frequencies within populations. In addition, recombination can also purge lethal recessive alleles from a population.

Meiotic recombination initiates via double-strand DNA breaks

Mechanism of recombination: the DSB model

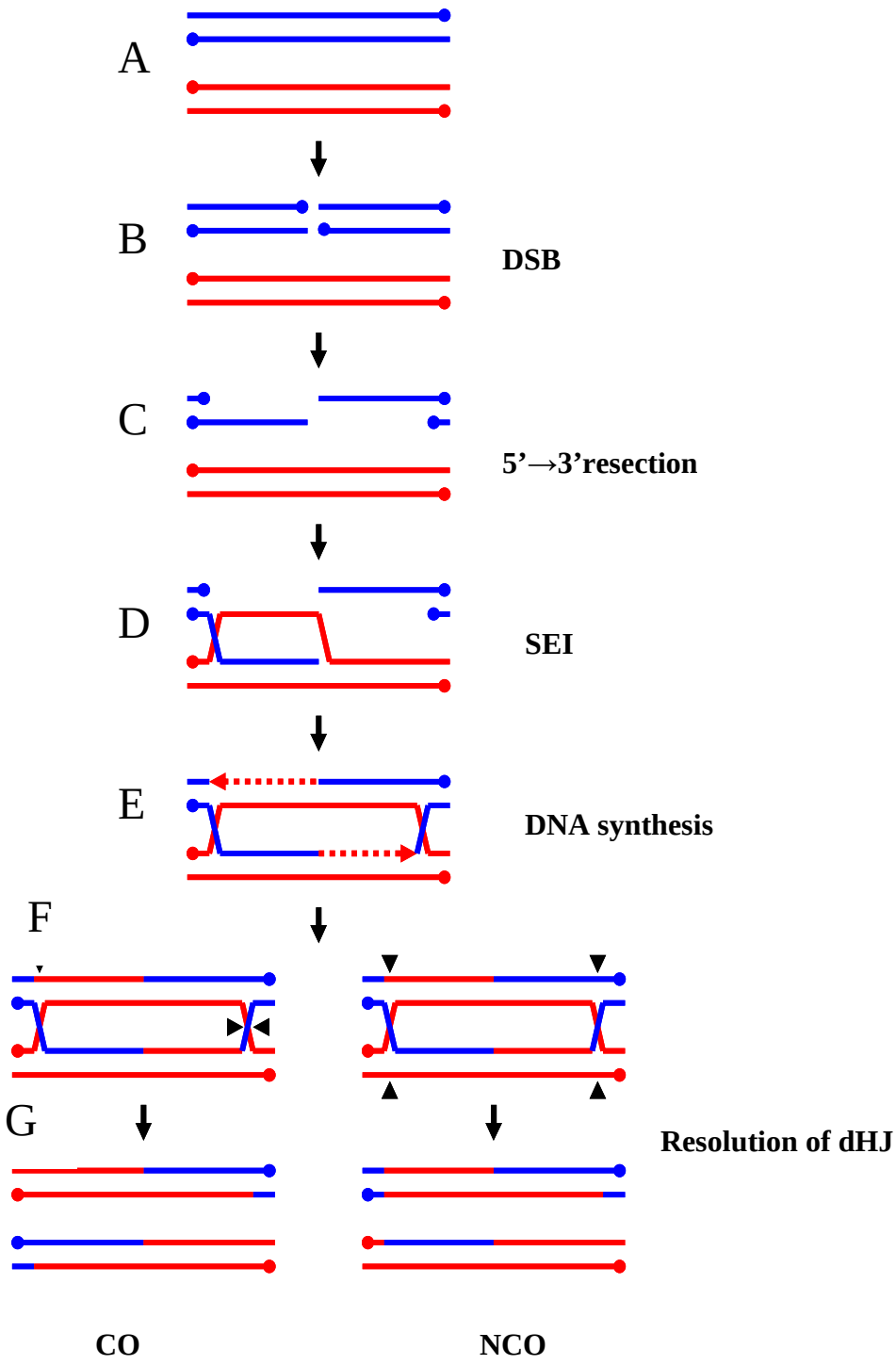
One aspect in which mitosis differs from meiosis is that levels of genetic recombination in meiosis are much higher (100-1000 fold) during meiosis than in mitosis. This difference is largely due to the programmed formation of DNA double-stranded breaks (DSBs) that are repaired using homologs. This homologous repair of DSBs can result in two types of recombination. One type is crossing over (reciprocal exchange); the other type is gene conversion (Fogel and Hurst, 1967; Fogel and Mortimer, 1971; Holliday, 1974; Stadler, 1959). In yeast crossing over can only be assayed using heterozygous loci. The outcome of crossovers between heterozygous loci is manifested as Mendelian 2:2 segregation of products. The second type of recombination is called gene conversion which results in nonreciprocal exchange or aberrant segregation (Ernst et al., 1981; Jinks-Robertson and Petes, 1985). In this case there are deviations from the expected 2:2 ratio of segregants. The result will be an aberrant (non-Mendelian) 3:1 (or 1:3) ratio. Gene conversion can occur with or without crossing over of surrounding loci.

Several models have been proposed to explain both crossing over and gene conversion. The first models of recombination (Holliday Model and Meselson-Radding Model) stipulated that recombination initiates via single-stranded DNA breaks (Holliday, 1964; Meselson and Radding 1975). The most accepted model for recombination today,

however, is the double strand break (DSB) repair model (Szostak et al., 1983). In 1981 it was observed that yeast transformation in mitotic cells is stimulated up to 3000-fold by introducing a DSB into a plasmid (Orr-Weaver et al., 1981). Based on these experiments the double-strand break repair model was proposed to be the actual method for meiotic recombination (Figure 1-2). These authors extrapolated from mitotic crossing over to explain meiotic events and predicted that meiotic chromosomes should display a high level of DSBs. The DSB model requires breaks to be created in both strands of DNA in one chromatid (Fig. 1-2 B). These breaks are then resected (exonuclease digested) in a 5' to 3' direction (Fig. 1-2 C). This will result in single stranded DNA with a free 3' end. This 3' recombinogenic end invades one chromatid on the homologous chromosome which results in displacement of one of the strands (Fig. 1-2 D). The displaced strand can then act as a template for DNA polymerase to fill in the gap left after the strand invasion (Fig. 1-2 E). Each strand is ligated to form double Holliday junctions which appear as cross bridges of single strands of DNA. Branch migration at these Holliday junctions can form creating regions of heteroduplex DNA that can have mismatches. Repair of these mismatches can lead to gene conversion. The Holliday junctions can be resolved in one of two ways to yield products either with or without reciprocal crossovers (Fig. 1-2 F and G).

A variation of the DSB model is called the synthesis-dependent strand annealing (SDSA) model (Allers and Lichten, 2001) (Fig. 1-3). This model was originally proposed to explain the wide variability in the frequency of crossover-associated gene conversion (18 to 66%) (Allers and Lichten, 2001). The DSB model posits that crossovers and noncrossovers occur with approximately equal frequency. Because of this variability, it seemed to Lichten's lab and to others that the DSB model alone (Fig. 1-2) could not be responsible for every recombination event. The initial steps of the two models are identical; the difference arises in how recombination events

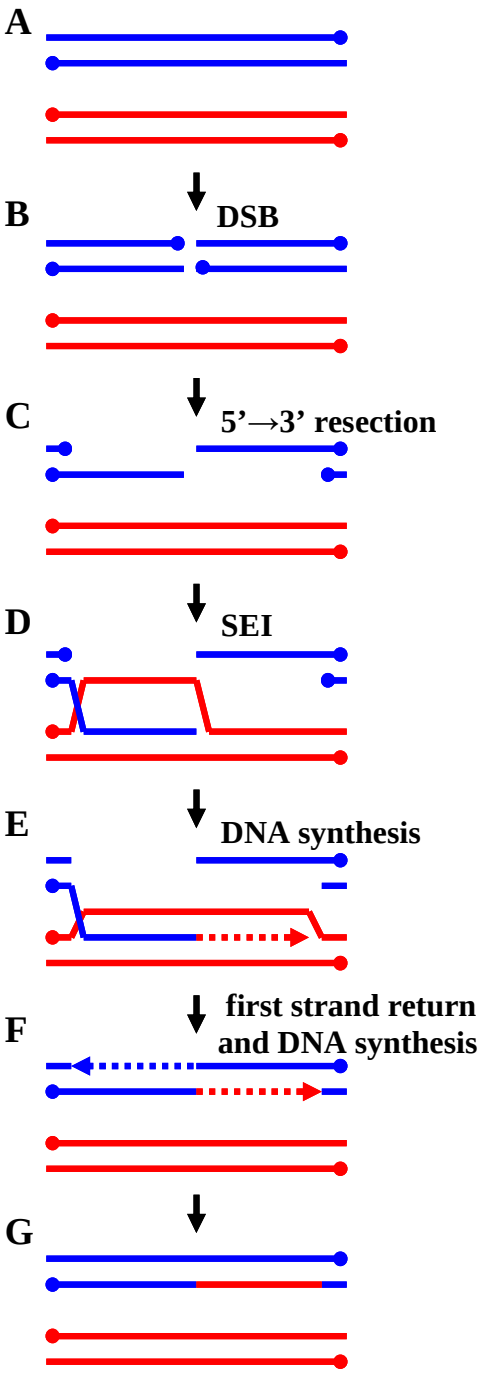
Figure 1-2: The double-strand break repair (DSBR) model for recombination. (Figure modified from Griffiths, A., *et al*. Introduction to Genetic Analysis, Seventh Edition, 2001) [A] The double-strand DNA of two (of four) homologous chromatids is shown. One homolog is designated in blue and the other in red. Solid dots represent the 5' ends of the DNA. [B] A lesion in one of the homologs is created by a double-strand break (DSB). [C] 5'→3' exonuclease digestion results in resected ends. [D] Single end invasion (SEI) involves one of the single-strand 3' ends invading the homologous DNA duplex. [E] Invasion and DNA synthesis displaces one strand of the homologous DNA. This displaced DNA can subsequently be "captured" by the originally broken and resected DNA molecule allowing for DNA synthesis from the noninvading 3' end. Asymmetric heteroduplex DNA surrounded by a double Holliday junction (dHJ) is the result. Branch migration of the Holliday junctions (HJ) can occur. [F] Before the reductional division, resolution into two individual chromatids must occur. This resolution can occur on different strands in both HJ to form a crossover (CO) product or on the same strands in both HJ to form a noncrossover (NCO) product. [G] The CO and NCO products are shown.


A
B
DSB
C
5’→3’resection
D
SEI
E
DNA synthesis
F
G
Resolution of dHJ
CO
NCO

become crossovers or noncrossovers. Instead of forming double Holliday junctions, the SDSA

A variation of the DSB model is called the synthesis-dependent strand annealing (SDSA) model (Allers and Lichten, 2001) (Fig. 1-3). This model was originally proposed to explain the wide variability in the frequency of crossover-associated gene conversion (18 to 66%) (Allers and Lichten, 2001). The DSB model posits that crossovers and noncrossovers occur with approximately equal frequency. Because of this variability, it seemed to Lichten's lab and to others that the DSB model alone (Fig. 1-2) could not be responsible for every recombination event. The initial steps of the two models are identical; the difference arises in how recombination events become crossovers or noncrossovers. Instead of forming double Holliday junctions, the SDSA model proposes that the initial invading strand synthesizes DNA for a small region. This synthesis causes the strand to dissociate with its homologous template and rejoin with its original template. The mechanism of strand dissociation is not clear. The SDSA model is supported by evidence from Allers and Lichten (2001) who examined heteroduplex DNA at different times during meiosis. They used gene specific probes to determine if the heteroduplex DNA was crossover associated or noncrossover associated and found that noncrossover associated gene conversion products were observed ~45 minutes earlier than crossover associated gene conversion products (Allers and Lichten, 2001). An alternative explanation is that resolution of crossover products simply takes 45 minutes longer than for non-crossover products. The best current evidence in yeast indicates the meiotic recombination initiates by a DSB, and that subsequent events can occur by one of two pathways: DSB recombination which can generate either crossover or gene conversion products, or the SDSA pathway which generates only gene conversion products.

Figure 1-3: The SDSA model for conversion without crossing over. [A] The double-strand DNA of two (of four) homologous chromatids is shown. One homolog is designated in blue and the other in red. Solid dots represent the 5' ends of the DNA. [B] A lesion in one of the homologs is created by a double-strand break (DSB). [C] 5'→3' exonuclease digestion results in resected ends. [D] Single end invasion (SEI) involves one of the single-strand 3' ends invading the homologous DNA duplex. [E] DNA synthesis occurs; however, unlike the DSBR model, the second strand is never captured. [F] The first (initially invading) strand returns to its original duplex, and the gap created by resection is synthesized from its original template. [G] The result is a noncrossover (NCO) between outer markers. Figure modified from Allers, T., and Lichten, M. (2001). Mol Cell *8*, 225-231.


A
B
DSB
C
5’→3’ resection
D
SEI
E
DNA synthesis
F
first strand return
and DNA synthesis
G

NON CO

Meiotic DSBs preferentially initiate at hotspots

Recombination in *S. cerevisiae* is not evenly distributed along chromosomes. Therefore, it is not surprising that the DSB events that initiate recombination are also not uniformLy distributed. Regions that experience high levels of recombination are known as hotspots and are associated with higher levels of DSB formation (Gerton et al., 2000; Goldway et al., 1993; Nicolas et al., 1989; Sun et al., 1989). Regions with low levels of recombination (coldspots) have a much lower frequency of DSBs (Petes, 2001). All of the factors that determine the location of hotspots are not yet known, but some features are common to many hotspots. Incidence of hotspots is correlated with promoters, or in other areas of open chromatin, such as those that are transcriptionally active and more sensitive to nucleases (Baudat and Nicolas, 1997; Blat et al., 2002; Petes, 2001; Wu and Lichten, 1994). Few hotspots are associated with regions surrounding centromeres, telomeres and other transcriptionally silent areas (Blat et al., 2002; Gerton et al., 2000; Klein et al., 1999; Lambie and Roeder, 1988; Petes, 2001). There is no known consensus sequence determining a hotspot, but they typically span an area of 70-250 base pairs (Baudat and Nicolas, 1997; Haring et al., 2003; Haring et al., 2004). A genome-wide microarray analysis suggested that hotspots are preferentially located in large chromosomal domains of higher GC content; of 177 identified hottest hotspots (defined as being in the top 12.5% of all ORFs examined in this genome-wide search) throughout the genome, 99 were associated with G-C content that was statistically above average (Gerton et al., 2000). If there were no correlation between hotspots and G-C content, random chance predicts that only 18 of the hotspots should overlap the areas of high G-C (Gerton et al., 2000). It has recently been reported that tri-methylation of lysine 4 on histone H3 is associated with many chromatin regions that are hotspots for DSB formation (Borde et al., 2009). However, this may simply be a reflection of the known association of hotspots with transcriptionally active regions (Petes, 2001).

Ten proteins are required for meiotic DSB formation

Several key proteins have been identified in *Saccharomyces* that catalyze the formation of DSBs. Mutations in any one of the ten recombination initiation genes confer similar phenotypes: complete elimination of DSB formation, a reduction in sporulation, inviable spore production, elimination of meiotic recombination, genetic epistasis to mutations in genes required later in the meiotic recombination process, and incomplete synaptonemal complex formation.

The four early recombination genes that function in both mitosis and meiosis are *RAD50, XRS2, MRE11*, and *SKI8* (also known as *REC103*) (Keeney, 2001). Three of the genes with roles in both mitosis and meiosis form a complex: *MRE11-RAD50-XRS2*. This complex is known as the MRX complex and plays a role in mitotic recombination repair as well as in several other mitotic DNA metabolic events (Ajimura et al., 1993; Alani et al., 1990; D'Amours and Jackson, 2001; Haber, 1998; Ivanov et al., 1994; Malone et al., 1985; Malone et al., 1990; Moreau et al., 1999). During mitosis the MRX complex is involved in repairing DSBs by two distinct mechanisms: homologous recombination and nonhomologous end joining (NHEJ). The three MRX proteins also function in telomere maintenance (Haber, 1998). The fourth gene with mitotic functions, *SKI8,* is involved in mRNA translation and stability (Brown et al., 2000; Searfoss and Wickner, 2000; Wickner, 1976). The products of the remaining six early recombination genes (*REC102, REC104, REC114, REC107* [also known *as MER2*], *MEI4,* and *SPO11*) function specifically in meiosis (Keeney, 2001; Malone et al., 1991; Malone and Esposito, 1981; Roeder, 1997; Weber and Byers, 1992). Mutations in any one of these ten genes completely abolish DSBs and meiotic recombination.

The MRX complex performs two roles in meiotic recombination

The recombination initiation gene Mre11 is homologous to *E. coli sbcD,* one component of the *SbcC/D* endo-exonuclease (Haber, 1998). Mre11 has single stranded DNA endonuclease and 3'-5' single-strand exonuclease activities (Cao et al., 1990; Nairz and Klein, 1997). Because the DSB model requires resection in the 5'-3' direction, the exonuclease activity of Mre11 is not involved in this process. Alleles of both *RAD50* and *MRE11* exist that allow the formation of DSBs but cannot process them; these alleles have been named "separation of function" or *rad50S* or *mre11S* (Nairz and Klein, 1997; Padmore et al., 1991). These alleles confirm that Rad50 and Mre11 are required not only to initiate recombination, but in later processing steps as well. The homolog of *XRS2* in higher eukaryotes is *NBS1* (D'Amours and Jackson, 2002) and is conserved in multi-cellular eukaryotes including mice (Bannister and Schimenti, 2004). It was difficult to identify Nbs1 as the homolog of Xrs2 because these proteins only share ~4% amino acid identity (Shima et al., 2005).

Mre11 (in combination with Rad50 and Xrs2) cleaves DNA to release Spo11 bound 5' ends of DNA at DSB sites (Borde et al., 2004; Neale et al., 2005). It was not initially clear whether Mre11 cuts the single strands of DNA adjacent to the Spo11 near the DSBs or if Mre11 involvement in 3' to 5' exonuclease processing causes Spo11 to be released or was Spo11 was directly hydrolyzed from DNA. However, in 2005, Neale *et al.* performed an experiment in meiotic cells of *S. cerevisiae* and found that the meiotic DSBs were processed by an endonuclease activity. This processing released and Spo11 attached to an oligonucleotide with a free 3'-OH (Neale et al., 2005; Smith et al., 2005). They demonstrated that Spo11 is released by MRX mediated single-stranded endonucleolytic cleavage rather than direct hydrolysis of the protein-DNA linkage or by exonuclease digestion. Rad50 can bind dsDNA (Raymond and Kleckner, 1993), and it contains a conserved zinc-coordinating "hook" motif potentially allowing interaction

between the MRX complexes present on two recombining DNA molecules (Wiltzius et al., 2005). The MRX complex functions in processing DSBs given the complex's role in mitotic DSB repair and as suggested by S mutations where DSBs are formed but not resected (Moreau et al., 1999; Padmore et al., 1991). I reiterate, however, null mutations in *RAD50, XRS2*, and *MRE11* are completely defective in DSB initiation. Taken together the data imply a dual role of DSB formation and processing for the MRX complex in *S. cerevisiae*.

While in *S. cerevisiae*, the MRX complex both creates and nucleolytically digests (resects) DSBs, only the latter role may be present in some other eukaryotes (Bannister and Schimenti, 2004; Borde, 2007). In *S. pombe*, null mutations in *MRE11* and *RAD32* (the *S. pombe* homolog of *RAD50*) can still form DSBs, though break levels are reduced. However, both genes are required for DSB processing (Young et al., 2004). In mice, deletion alleles of MRN complex proteins are embryonic lethal, so only hypomorphic alleles can be studied. Curiously, mice with mutations in positions homologous to the *Saccharomyces rad50S* mutation are still fertile and have few detectable defects in either ovaries and testes, though somatic defects have been found and the mutant mice are susceptible to cancer (Bender et al., 2002). This is quite different from *S. cerevisiae*; yeast *rad50S* strains barely sporulate (~1%) and produce inviable spores (Stuart Haring, personal comm. and Alani et al., 1990). It is also possible that *MRE11* or other factors could substitute for the DSB function of *RAD50* in these mice. In some organisms, it is possible that the Rad50 complex could be recruited after DSB formation. This appears to be the case in mice and also in *Arabidopsis thaliana* (Borde et al. 2007).

Spo11 is the proposed catalytic protein for DSB initiation.

Spo11 is the protein in the proposed initiation complex responsible for creating the actual break in each strand of the DNA (Neale et al., 2005). Spo11 is homologous to the A subunit of archaebacterial type II topoisomerase (topoisomerase VI) (Bergerat et

al., 1997). The tyrosine at amino acid site 135 is the presumed catalytic residue in *Saccharomyces* Spo11 (Keeney et al., 1997; Prieler et al., 2005). In some protists, this tyrosine is substituted by a phenylalanine residue (Malik et al., 2007; Ramesh et al., 2005). Tyrosine and phenylalanine are both aromatic ring-containing amino acid residues, and it has been proposed that this residue should perform the same biochemical activity as tyrosine. Yeast mutants that allow DSBs to form and accumulate (*rad50S* and *mre11S*) have Spo11 protein remains covalently attached to the 5' end of the DNA by Tyrosine 135 (Keeney and Kleckner, 1995; Smith et al., 2005). Although *SPO11* encodes a key protein for making the DSB, it requires all nine of the other recombination initiation proteins to do so (Pecina et al., 2002). Pecina et. al. (2002) tethered Spo11 to the Gal4 coldspot by fusing the DNA binding domain of *GAL4* with *SPO11* to test the hypothesis that most of the other nine initiation proteins were only required to recruit Spo11 to the DNA. The tethered Spo11 made DSBs at *GAL4* binding sites. However, each of the other nine recombination initiation genes were required for this DSB formation (Pecina et al., 2002). This indicates that there is additional importance for the other nine recombination initiation proteins in making DSBs beyond recruiting Spo11 to hotspots.

Spo11 is conserved throughout eukaryotes; homologs have been identified in a wide variety of plants, animals, fungi and protists (Malik et al., 2007; Ramesh et al., 2005). Spo11 has been shown to be indispensable for meiotic recombination in *Arabidopsis thaliana* (Grelon et al., 2001) , *Drosophila melanogaster* (McKim and Hayashi-Hagihara, 1998), *Caenorhabditis elegans* (Dernburg et al., 1998), *Mus musculus* (Baudat et al., 2000) and in a variety of fungi including *Neurospora crassa* (Bowring et al., 2006), *Sordaria macrospora* (Storlazzi et al., 2003) *Schizosaccharomyces pombe* (Lin and Smith, 1994) and *Coprinus cinereus* (Celerin et al., 2000; Merino et al., 2000). Mutating *SPO11* in these systems resulted in reduced recombination, sterility and/or reduced viability of gametes.

Ski8 protein

The role of Ski8 in mitotic RNA metabolism involves modulating 3' to 5' exonucleolytic degradation of damaged mRNAs that are not poly-adenylated (Frischmeyer et al., 2002; Searfoss and Wickner, 2000). Ski8 was originally characterized for its role in degrading "killer" (virus) dsRNAs. The loss of function mutation resulted in increased viral expression, and hence its name "superkiller". In meiosis, however, it has been proposed to function as a scaffold protein with a role in assembling the DSB initiation complex (Araki et al., 2001). Ski8 migrates from the cytosol to the nucleus during meiosis and it has been found to specifically localize to chromosomes during prophase I (Arora et al., 2004). If Ski8 acted as a scaffold required for Spo11-Rec102-Rec104 interactions, then one would predict that a *ski8* mutation would confer a phenotype similar to null *rec102, spo11* or *rec104* mutations.

It is puzzling how a protein required for mRNA degradation could be required for DSB formation in meiosis. During mitosis, Ski8 forms a heterotrimeric complex with Ski2 and Ski3 (Synowsky and Heck, 2008). Ski2 is a putative RNA helicase, Ski3 is a tetratricopeptide repeat (TPR) protein, and Ski8 contains five WD-40 (beta-transducin) repeats. The underlying common function of most WD-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions (Neer et al., 1994). Ski8 most likely facilitates protein-protein interactions between Ski2 and Ski3, and it is a reasonable hypothesis that Ski8 plays a similar role in meiosis. Demelza Koehn, a graduate student from our lab, has tethered Ski8 to the *GAL2* coldspot by fusing the *GAL4* DNA binding domain to the *SKI8* gene *(DB-SKI8)*. She found that *DB-SKI8* strains made DSBs at *GAL2*, although not as many as DB-Spo11 (7.3% in DB-Spo11 vs. 2.2% in DB-Ski8). This indicates that *SKI8* could recruit the other factors including *SPO11* to the DNA and form a functional complex, suggesting that Ski8 may serve a critical role in complex formation other than being

merely a scaffold protein that allows Spo11-Rec102-Rec104 association (Koehn et al., 2009).

Meiosis specific recombination initiation proteins

The roles of several of the other recombination initiation proteins are not as clear. Although they are absolutely required for DSB formation and almost certainly part of the putative initiation complex, their specific functions are unknown. This group includes Rec104, Rec102, Rec114, Mei4, and Rec107. Rec104 is phosphorylated during meiosis and Rec102 is necessary for this phosphorylation (Kee et al., 2004). Rec102 is phosphorylated but neither the kinase responsible nor the timing for this activation are presently known (Kee and Keeney, 2002).

During meiotic prophase Rec107 protein increases in abundance and is phosphorylated in a Cdc7/Dbf4-dependent manner on residues Ser11, Ser15, Ser19, Ser22, Ser29 of the N-terminal region of the Rec107 protein (Sasanuma et al., 2008). Cdc7/Dbf4 are the catalytic components of the DDK complex which, in addition to phosphorylating Rec107, also phosphorylate and activate proteins required for pre-replication complexes (Sclafani, 2000) Phosphorylation of all of these residues contributes to DSB formation, but phosphorylation of Ser 29 is essential (*i.e.*, Ser29 mutant strains make no DSBs while strains with mutations in Ser 11, 15, 19 or 22 make 3X fewer DSBs). Cdc28/Clb5 also directly phosphorylates Rec107 on Ser30 and Ser271 and is important for DSB formation but not for chromatin association (Henderson et al., 2006; Wan et al., 2008).

The five proteins in this last group have no known homologs in multicellular eukaryotes (John Logsdon, personal communication) though are conserved in some hemiascomycete fungi [(Henderson et al., 2006; Jiao et al., 2002) (Malone lab, unpublished results)]. Our lab has identified homologs of *REC102, REC104 and*

REC114 in the yeasts *Saccharomyces paradoxus* and *Saccharomyces pastorianus* [(Jiao et al., 2002; Nau et al., 1997) Doug Pittman, doctoral thesis).

During meiosis, the ten recombination initiation proteins act to create the DSBs necessary for recombination to occur and for subsequent chiasmata to form. Chromatin-immunoprecipitation experiments have shown that Spo11 is transiently associated with meiotic hotspots and that this association requires at least three other recombination initiation proteins, Rec102, Rec104, and Rec114 (Prieler et al., 2005). Rec102 and Rec104 also associate with the DNA on meiotic chromosomes and each requires the presence of the other for the interaction of the complete Rec102/Rec104 complex loading. The localization of Rec102 and Rec104 to chromatin also requires Spo11 and Ski8 (Kee et al., 2004). Unlike Spo11, Rec102 was found to associate with both DSB hot spot and DSB cold spot regions (Kee et al., 2004). It is possible that, although Rec102 has more non-specific interactions with the DNA, it is only active in recruiting Spo11 at the DSB hotspot regions. Chromatin-immunoprecipitation demonstrated that Rec102 and Rec104 are required for Spo11 association with meiotic hotspots (Prieler et al., 2005). As mentioned previously, the MRX complex is necessary for removal of Spo11; removal also requires Mei4 and Ndt80 (a meiosis specific transcriptional regulator of the middle meiotic genes). None of the other recombination initiation genes have been found to be necessary for the removal of Spo11 (Neale et al., 2005; Prieler et al., 2005). Rec114 and Mei4 can associate with hotspots in absence of Spo11 suggesting that they may be the first to bind to the DNA. Phosphorylation of Rec107 by Cdc7 is necessary for Rec114 and Mei4 binding (Sasanuma et al., 2008).

Beyond their essential role in forming meiotic DSBs, little biochemical information is known about Rec102, Rec104, Rec107, Rec114 and Mei4. Intact recombination initition complexes have yet to be purified, and there are no crystal structures for these proteins. Our lab has identified a putative leucine zipper in *REC102* suggesting that it may be involved in protein –protein tnteraction (Cool and Malone,

1992). No functional domains have been identified for Rec104, Rec107, Rec114, or Mei4. It is likely that Rec104, Rec107, Rec114, and Mei4are required for maturation of the SC, though there is not yet any direct evidence. *SPO11* is required for mature SC formation. *spo11* mutants cells form <1% mature SC (Bhuiyan and Schmekel, 2004). Suppressor analysis has suggested that that Hop1, a gene required for the axial elements of SC (discussed below) and Rec104 might interact and this interaction could be important for formation of mature SC (Hollingsworth and Johnson, 1993). There is some evidence to suggest that Rec114 may have role in attracting late recombination factors. Overexpresion of Rec114 can partially suppress the mononucleate arrest seen in cells with a *dmc1* mutation (Bishop et al., 1999). Dmc1 is a homolog of the *E. coli* protein RecA that is involved in strand invasion during the later events of recombination(Bishop et al., 1992) . *S. cerevisiae* has a second RecA homolog, Rad51 that is important for strand exchange during both mitotic and meiotic recombination. I hypothesize that Rec114 can interact with Rad51 and overproduction of Rec114 can attract sufficient Rad51 to overcome absent Dmc1 and allow strand transfer.

Evidence for a recombination initiation complex

Several experiments have shown specific interactions between certain proteins of the recombination initiation complex (Haber, 1998; Jiao et al., 2003; Johzuka and Ogawa, 1995; Kee et al., 2004; Li et al., 2006a, b; Prieler et al., 2005; Salem et al., 1999; Sasanuma et al., 2007). This has led to the hypothesis that sub-complexes exist within the overall recombination initiation structure. The exact stoichiometry of the initiation proteins and the order of complex assembly are areas that have not been as thoroughly studied. Several experiments have shown that a MRX complex exists and that this complex is not meiosis-specific (Chamankhah and Xiao, 1999; Tsubouchi and Ogawa, 1998; Usui et al., 1998). The MRX complex also functions during mitotic cell growth as part of the DNA repair pathway and several other functions. Johzuka and Ogawa (1995)

demonstrated with two-hybrid experiments that Mre11 interacts with itself and with Rad50 during mitosis. Additional two-hybrid experiments demonstrated that Mre11 interacts with Rad50 and with Xrs2 during meiosis (Usui et al., 1998). Mre11 can interact with Rad50 in the absence of Xrs2 and with Xrs2 in the absence of Rad50; however, Rad50 and Xrs2 cannot interact without Mre11 (Johzuka and Ogawa, 1995; Usui et al., 1998). This indicates that Mre11may be responsible for promoting MRX complex formation.

Interactions between Rec102, Rec104, and Spo11 have been found using high copy suppression, allele specific suppression, co-immunoprecipitation (co-IP), yeast two-hybrid, and immunofluorescence microscopy (Jiao et al., 2003; Kee and Keeney, 2002; Kee et al., 2004; Li et al., 2006a; Salem et al., 1999; Wan et al., 2008).
Two-hybrid experiments performed in mitotic cells are particularly informative because the native copy of the initiation gene is not present and thus it is possible to demonstrate the existence of discrete interactions without the interference of other initiation proteins. Spo11 and Ski8 were found to interact in a global screen for two-hybrid interactions (Uetz et al., 2000). Later two-hybrid experiments using both mitotic and meiotic cells revealed interactions between Rec114 and Rec107, Mei4 and Rec104, Rec102 and Rad50, Spo11 and Rec104, Ski8 and Rec104, and Mei4 and Rec114 (Arora et al., 2004; Li et al., 2006a). In the Malone laboratory, Salem *et al*. (1999) demonstrated that overexpression of *REC102* suppresses a *rec104-8* mutation and Jiao *et al*. (2003) demonstrated that overexpression of *SPO11* suppresses specific *rec102* and *rec104* point mutations. Co-immunoprecipitation experiments demonstrated that Rec102 interacts with Spo11 and Rec104 (Jiao et al., 2003) and that Mei4, Rec114, and Rec107 all co-immunoprecipitate (Li et al., 2006a). Rec114 and Mei4 proteins can associate with the hotspot *YCR048w* in a *spo11* mutant suggesting that the Rec107/Rec114/Mei4 subcomplex precedes the binding of Spo11 to chromatin (Sasanuma et al., 2008). Taken together, these experiments demonstrate that each recombination initiation protein

interacts with at least one other initiation protein, and supports the hypothesis of a recombination initiation complex. ChIP experiments have shown that the association between Spo11 and recombination hotspots requires Rec102, Rec104, and Rec114. Mei4 may be involved with removing Spo11 from the DNA, suggesting that Mei4 may be the last factor required in the recombination initiation complex (Prieler et al., 2005). All current data is consistent with three major subgroups within the putative recombination initiation complex: Rad50-Mre11-Xrs2; Rec102-Rec104-Spo11-Ski8; Mei4-Rec107-Rec114.

Though the above evidence suggests an order of assembly of the recombination initiation complex, Demelza Koehn's work suggests that if this order of assembly occurs, then it is not absolutely essential for function (Koehn et al., 2009). She has shown that strains with DB fusions of *SPO11*, *REC104*, *REC114*, *REC102*, *REC107*, *MEI4* and *SKI8* can all make DSBs at *GAL2*, suggesting that each of these proteins are capable of recruiting and forming a functional recombination initiation complex, despite presumably altering the wild type order of assembly. Of the DB fusions that she tested, only a *DB-MRE11* strain could not make DSBs at GAL2. She did not test DB fusions of *XRS2* and could not test a *RAD50* fusion because the strain that she used in her studies contained a *rad50S* mutation.

Conflicting data exists regarding the interactions among the initiation proteins in different subcomplexes. Colocalization studies of Rec107 with proteins from different subcomplexes (namely, Mre11 and Rec102) reported no significant colocalization on meiotic chromosomes, suggesting that the three may not interact (Li et al., 2006b). Similarly, Rec102 did not co-immunoprecipitate with Rec107 (Li et al., 2006b). In a comprehensive two-hybrid study, interactions were not observed between Rec107 and Rec102 or Mre11 (Arora et al., 2004), which supports the results of Roeder and colleagues (Li et al., 2006b). In this same study, however, several interactions among other subcomplex components were identified. Some examples are Mei4 with Rec102

and Rec104, Rec114 with Rec104, Mre11 with Rec102, and a very strong interaction between Xrs2 and Rec107 (Arora et al., 2004; Henderson et al., 2006; Maleki et al., 2007). A conflicting observation is Mre11's association with hotspot DNA. A ChIP study by Borde *et al*., (2004) demonstrated that Mre11 does not precipitate hotspot DNA in the absence of Rec107. This result disagrees with the conclusions made by Li *et al*., (2006) and Arora *et al*., (2004) that Mre11 and Rec107 do not interact. Instead, it suggests that Mre11 and Rec107 do interact, at least indirectly. Further studies are required to resolve these paradoxes. Purification of intact recombination initiation complexes using TAP-tagging or other methods would be useful in determining the interactions and order of assembly of the recombination initiation complex.

Phosphorylation has been shown to be an important step in putative complex formation. ChIP experiments have shown that Cdc7-mediated phosphorylation of Rec107 is necessary to attract Rec114 and Mei4 to the hotspot *YCR048w* (Sasanuma et al., 2008). It is still unclear what if any role the phosphorylation of Rec104 or Rec102 plays in complex formation or chromatin association (Kee et al., 2004).

Meiosis is a transcriptionally regulated process

Sporulation in *Saccharomyces cerevisiae* involves not only the major nuclear events of meiosis, but also global cellular changes to the cytosol, organelles and cell wall. Approximately 500 genes are induced at least 2-fold during the sporulation program, while an almost equal number are repressed at least 2-fold (Chu et al., 1998). This transcriptional program can be divided into several discrete phases of gene expression: very early, early, middle, mid–late, and late phases (Chu et al., 1998; Primig et al., 2000). The transcriptional activator Ime1 is required for the initiation of meiosis. Middle meiosis is regulated by the transcriptional activator encoded by the gene *NDT80.*

Control of *IME1* transcription by mating type proteins

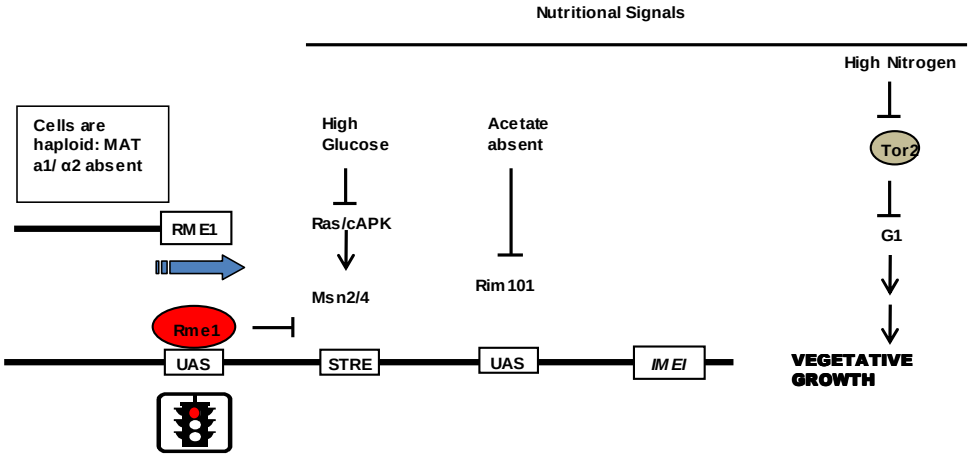
The sporulation transcriptional cascade is only initiated in response to both *MAT a1* and *MAT α2*-type proteins and starvation for carbon and nitrogen (Honigberg and Purnapatre, 2003; Mitchell, 1994; Vershon and Pierce, 2000) (Fig.1-4). These two types of signals converge to control the expression of the transcription factor Ime1. Ime 1 is necessary for transcription of most of the early meiotic genes including those necessary to create double stranded breaks and serves as the master regulatory switch for entry into sporulation (Kassir et al., 1988; Sagee et al., 1998). The regulation of transcription of *IME1* is a complex process that involves an unusually large 5' untranslated region (UTR) upstream of the *IME1* genes that is greater than 2 kb (Granot et al., 1989; Sagee et al., 1998). (For comparison, a typical 5'UTR in yeast is 100-200 bp (Tirosh et al., 2007)). In haploid cells, *IME1* transcription is repressed by Rme1, a haploid-specific negative transcriptional regulator which binds to Rme1 repressor elements (RREs) located about 2 kb upstream of the start site of *IME1* (Covitz et al., 1991; Covitz and Mitchell, 1993). Sin4 and Rgr1, members of the RNA polymerase mediator complex, bind with Rme1 and alter the surrounding chromatin into a structure that inhibits transcription (Covitz et al., 1994).

Nutritional regulation of *IME1* transcription

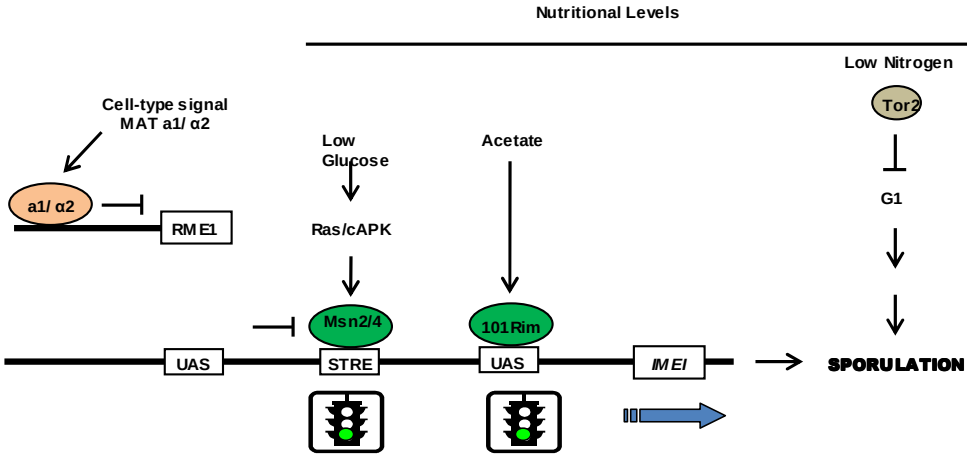
As opposed to the relatively simple mating type protein-presence requirement regulation of initiation of sporulation, the nutritional regulation of *IME1* transcription is more complex and less well understood (Honigberg and Purnapatre, 2003). Deletion and mutation studies of the *IME1* promoter region have shown at least ten distinct *cis* elements that respond to carbon or nitrogen levels (Sagee et al., 1998). The signaling pathways that regulate nutritional input for sporulation fall into three broad classes: 1) those that respond to nutrient starvation-induced arrest in G1, 2) those that respond positively to a non-fermentable carbon source (e.g., acetate), and 3) those that respond to glucose (Fig. 1-4).

Figure 1-4: Transcriptional regulation of *IME1*: Regulation of *IME1* involves both mating type and nutritional signals. A] When cells are haploid the yeast proteins MAT a1 or MAT α2 are absent (depending on mating type). When these proteins are absent, the transcriptional repressor RME1 is transcribed. Rme1 binds a UAS site and and represses trnascription of *IME1*. High glucose, absence of a non-fermentable carbon source and high nitrogen promote vegetative growth and do not promote transcription of IME1. B]. If cells are haploid, the presence of mating-type protens inhibits the expression of the repressor Rme1. Low glucose levels activate the Ras/cAPK pathway which causes Msn2/4 to bind to a STRE to promote transcription. Presence of a non-fermentable carbon source, such as acetate causes the Rim101 transcription factor to bind to a UAS to promote transcription of *IME1*. Repressed promoter elements are indicated by the red trafficlight icons. Activated promoters are indicated by green traffic light icons. Transcription of a gene is indicated by the presence of a blue arrow beneath the gene. Modified from Vershon, A.K., and Pierce, M. (2000). Curr Opin Cell Biol *12*, 334-339.

A. Vegetative Conditions



B. Sporulation Conditions



Glucose levels above a certain threshold in sporulation media (0.25%) are sufficient to prevent sporulation even if other necessary conditions have been met (Purnapatre and Honigberg, 2002). In response to low levels of glucose, Msn2 or Msn4 proteins bind to stress response elements (STREs) in the *IME1* promoter, to help activate *IME1* transcription (Fig. 1-4). Msn2 and Msn4 seem to be activated in response to signals from the RAS-cAPK pathway because mutations of proteins in this pathway, such as Ras2, Cyr1 and Cdc25, affect the regulation of *IME1*(Vershon and Pierce, 2000) (Fig. 1-4).

It is unclear whether starvation for nitrogen directly triggers sporulation or whether nitrogen starvation causes an arrest in G1 that is required for sporulation to initiate (Honigberg and Purnapatre, 2003). Some evidence favors the latter hypothesis, because meiosis can still occur in the presence of nitrogen when other essential nutrients such as phosphates are limiting (Freese et al., 1982). On the other hand, several nitrogen-sensory mechanisms do affect the timing of entry into meiosis. Tor2 is a PI-3 kinase localized in the cellular membrane that mediates the response to cell stresses, such as nitrogen starvation. The Tor2 pathway can be activated by the drug rapamycin, a drug which triggers G1 arrest and induces sporulation along with glycogen accumulation, autophagy and repressed rRNA transcription, even in the presence of nitrogen-containing homolog which phosphorylates many targets and is necessary for meiotic initiation and progression (Mandel et al., 1994; McDonald et al., 2009). Post-translational phosphorylation of Ime1 by Rim11 and Rim15 is also necessary for Ime1 binding to URS1 sites (Bowdish et al., 1994; Reinders et al., 1998). The Rim15 Ser/Thr kinase creates a stable association of Ume6/Ime1 at URS1 sites to promote transcription of early meiotic genes which can be destabilized by the presence of glucose in the media (Malathi et al., 1997, 1999) (Fig. 1-5) (Hardwick et al., 1999). However, microarray analysis indicates that activation of the Tor2 pathway by rapamycin does not directly induce *IME1*

transcription; instead Hardwick *et al*. (1999) propose that the Tor2 pathway directly controls several metabolic genes required for G1 arrest, though the mechanism of this remains unknown.

Though the nitrogen requirements of the initiation sporulation are unclear, it is known that starvation for nitrogen is required for the transcription of early meiotic genes (Kuhn et al., 2001). This will be discussed in the following section.

IME1 transcription normally requires respiratory metabolism of a non-fermentable carbon source such as acetate. Overexpression of Ime1 from a high-copy plasmid bypasses this requirement (Ohkuni and Yamashita, 2000). Respiration leads to the production of CO_2 and hence causes a change in the pH of the medium. This change in pH may contribute to meiotic initiation. For example, the C_2H_2 zinc-finger transcriptional activator Rim101 is required both for adaptation to an increase extracellular dissolved CO_2 and for *IME1* transcription through an unknown mechanism (Su and Mitchell, 1993) (Fig. 1-4)

Ime1 controls the transcription of early meiotic genes

Early meiotic genes transcribed by *IME1* contain conserved 9 base pair upstream regulatory sequence (URS1) in addition to the various upstream activating sequence (UAS) sequences (Buckingham et al., 1990). URS1 sites are always occupied by the zinc-finger protein Ume6, which is necessary for both repression of mitotic genes and activation of early meiotic genes (Anderson et al., 1995; Steber and Esposito, 1995; Strich et al., 1994). (Fig. 1-5) Sin3 and Rpd3 histone deacetylases bind to Ume6 during vegetative growth to create repressive chromatin (Kadosh and Struhl, 1997, 1998a, b). At the start of sporulation, phosphorylated Ime1 binds to Ume6 causing Sin3 and Rpd3 to release from Ume6 allowing transcription of early genes. Ime1 is required for the transcription of many of the early meiotic genes described in this thesis including the genes for recombination initiation and synapsis and the meiotic kinase *MEK1 (Kassir et*

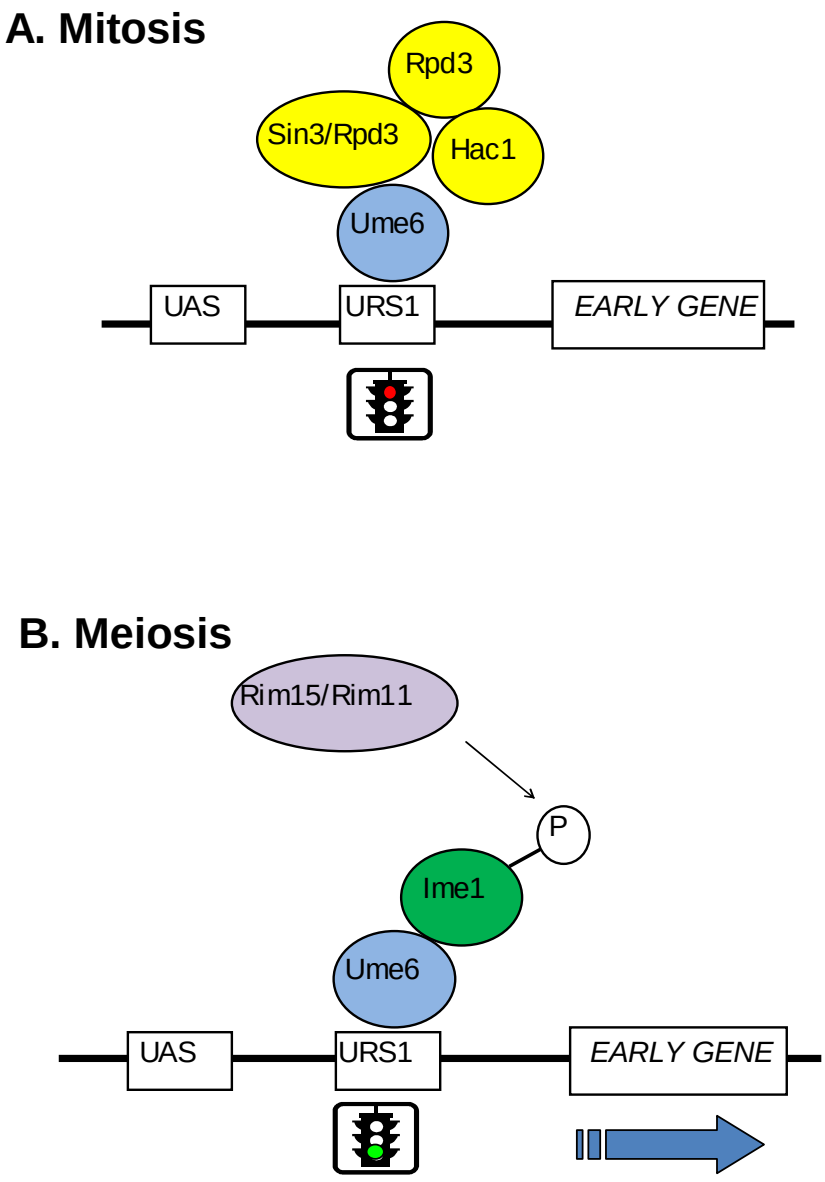
al., 1988; Kassir and Simchen, 1976; Malone, 1990). In addition, Ime1 is necessary for the transcription of *IME2*, a Ser/Thr regulatory kinase and Cdc28 (Cdk1).

Nitrogen levels are also important in influencing early gene expression. Hac1is a bZip family protein required for the unfolded protein response (UPR); the Hac1 pre-mRNA is only spliced when present when nitrogen levels are high (Kuhn et al., 2001). When nitrogen levels are low, an unspliced version of *HAC1* is translated, containing a premature stop codon. In 2004, Schroder *et al.* showed that spliced Hac1 binds at the URS1 site of a *REC104* promoter along with the Sin3/Rpd HDAC complex and Ume6 to suppress transcription during mitosis. Furthermore, a *hac1*Δ mutant induces *IME2* transcription during sporulation more rapidly than in wild type and overexpression of spliced *HAC1* delays transcription of *REC104* during meiosis (Kuhn et al., 2001). These data indicate that the presence of nitrogen could repress initiation of meiosis by affecting Ime1-mediated early gene transcription during growth in rich media (Lamb et al., 2001; Su and Mitchell, 1993).

Middle meiosis requires *NDT80* transcription

Approximately 200 middle meiotic genes (MMGs) are activated by the positive transcriptional activator Ndt80 (Chu et al., 1998). This transcription factor is essential for exit from pachytene. *ndt80* mutants arrest as mononucleate cells with homologs linked in close apposition by synaptonemal complexes. *ndt80* cells also lack MI spindles and duplicate, but do not separate spindle pole bodies (Hepworth et al., 1998; Xu et al., 1995). The initial transcription of *NDT80* is positively controlled by Ime1, but subsequent transcription is primarily autoregulatory [(Hepworth et al., 1998) (Fig. 1-6)]. The *NDT80* promoter contains both URS1 and Middle Sporulation Elements (MSEs) (Hepworth et al., 1998). Ime1 promotes a low level of transcription of *NDT80* by binding to the URS1 site (Pak and Segall, 2002a, b). This is followed by Ndt80 binding to MSE sites to

Figure 1-5: Regulation of early gene expression. URS1 sites are constitutively occupied by Ume6, which is required for both repression and activation of early meiotic promoters. During vegetative growth, promoters are repressed by Sin3and Rpd3 which are bound to Ume6. During early meiosis, Sin3/Rpd3 is absent; instead the positive transcriptional regulator Ime1 is bound to Ume6. Phosphorylation of Ime1 by Rim15 and Rim11 is required for early gene expression. Many genes contact additional activation sequences denoted here as UAS. The mechanism of that activation varies from gene to gene and for clarity is not shown in the figure. Modified from Vershon, A.K., and Pierce, M. (2000). Curr Opin Cell Biol *12*, 334-339


A. Mitosis
Rpd3
Sin3/Rpd3
Hac1
Ume6
UAS
URS1
EARLY GENE
B. Meiosis
Rim15/Rim11
P
Ime1
Ume6
UAS
URS1
EARLY GENE

promote high levels of transcription of *NDT80* (Pak and Segall, 2002a; Pierce et al., 2003). Initial transcription of *NDT80* from an Ime1-dependent promoter is minimal. High levels of *NDT80* transcription require Ndt80-mediated regulation as well as the Ime1-dependent protein kinase Ime2. Ime2 phosphorylates the MMG-repressor Sum1 and causes it to release from the MSE. *NDT80* transcription is negatively regulated by the repressor Sum1 (Xie et al., 1999) (Fig. 1-6).

As cells prepare to exit pachytene, Ndt80 competes with the transcriptional repressor Sum1 at MSEs (including the MSE in its own promoter) to activate transcription of MMGs (Pierce et al., 2003). Most MMGs (>70%) contain a MSE in their 5' UTRs which is bound by the repressor Sum1 during vegetative growth (Xie et al., 1999). Ime2 promotes derepression of middle meiotic promoters by phosphorylating Sum1 on Thr 306 at a consensus Pro-X-Ser/Thr site (Moore et al., 2007).

Sum1 represses the MSEs of many MMGs including the MSE found upstream of the *NDT80* gene. A Sir2 homolog transcriptional silencing protein Hst1 (a NAD+-dependent histone deacetylase), binds to Sum1 to assist repression of MSEs. Hst1 is recruited to MSEs by Rfm1 which interacts with both Sum1 and Hst1 and is required for the Sum1/Hst1 interaction (McCord et al., 2003). In order to transcribe MMGs, Ndt80 must be phosphorylated by Ime2 (Chu and Herskowitz, 1998; Hepworth et al., 1998) (Fig. 1-6). Not all Ndt80-controlled promoters are repressed during vegetative growth in a Sum1-dependent manner. For example *SPS4*, a middle meiotic gene whose transcription is entirely dependent on Ndt80 and is only expressed during meiosis, does not show Sum1-mediated repression during mitosis (Xie et al., 1999). An unknown mechanism is responsible for repressing *SPS4* transcription. Furthermore, not all MMGs are clearly regulated by Ndt80-controlled transcription. At least 60 known MMGs lack a true MSE, though *NDT80* may bind at non-canonical sites to promote transcription (Chu et al., 1998). The regulation of transcription of these genes is still unknown.

Approximately 60 meiotic genes are expressed later in sporulation and are mostly required for formation of the chitin/chitosan and dityrosene layers of the spore wall (Chu et al., 1998). Over half of these "late" genes contain MSEs in their promoter regions and are controlled by Ndt80 and repressed by Sum1. Late expression of these genes is achieved by additional repression mechanisms. These genes often contain negative regulatory elements (NREs) which are bound by the Ssn6-Tup1 co-repressor complex (Friesen et al., 1997). The mechanism of de-repression of these genes late in meiosis, as well as transcription of non-Ndt80-controlled late meiotic genes, is still unclear.

Repression of vegetative growth genes during sporulation is less-well understood than activation of sporulation-specific genes. Repression of many of these vegetative genes is likely due to an absence of specific nutrient signals or other co-factors necessary for transcriptional activators to function (Honigberg and Purnapatre, 2003; Vershon and Pierce, 2000).

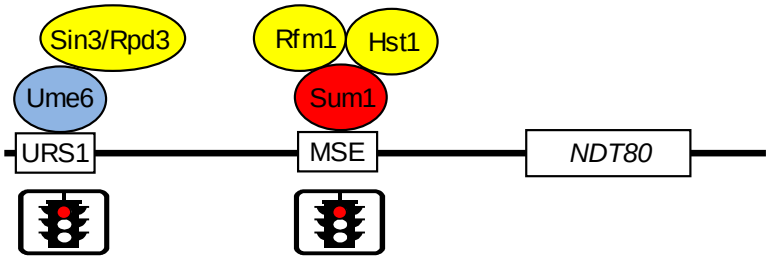
Proteins that assist in chromosomal association: the SC and cohesins

The SC assists in pairing of homologous chromosomes and promoting recombination

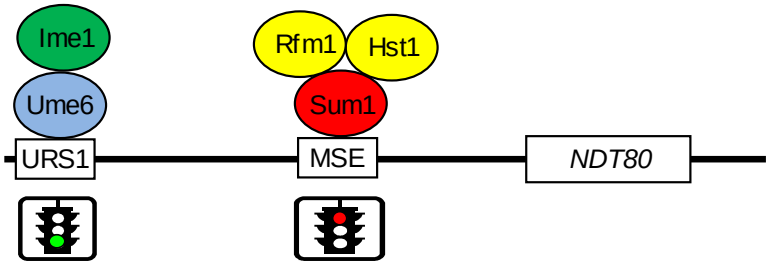
The synaptonemal complex (SC) is the proteinaceous structure that holds homologous chromosomes together prior to the reductional division (Bhalla and Dernburg, 2008; Lynn et al., 2007; Zickler and Kleckner, 1999) (Fig. 1-7). In *S. cerevisiae*, mature SC are composed of lateral elements (LE) containing the proteins Hop1 and Red1 and transverse elements containing Zip1-4 (de Carvalho and Colaiacovo, 2006; Roeder, 1997). Axial elements (AE) are immature LE containing Hop1 and Red1. AE first form in the axes of chromatin where recombination occurs during leptotene. These AE lengthen during zygotene to eventually form mature LE. Mature SC are

Figure 1-6: Regulation of transcription of *NDT80*. [A.] During vegetative growth, *NDT80* transcription is repressed. Sin3 and Rpd3 bound to Ume6 at an URS1 repress *NDT80* transcription along with Sum1 and Hst1 bound to a MSE. [B.] During early meiosis Ime1 is bound to Ume6 at URS1 causing low levels of *NDT80* transcription.[C.] During pre-middle meiosis, Ime2 kinase phosphorylates Sum1 causing it to become unbound from the MSE.[D.] During middle meiosis Ndt80 is bound to MSE sites and activates transcription auto-catalytically. Repressed promoter elements are indicated by red traffic-light icons. Activated promoter elements are indicated by green traffic light icons. Blue arrows below the *NDT80* gene indicate level of transcription. A thicker arrow denotes higher transcription levels. Modified from Pak, J., and Segall, J. (2002). Mol Cell Biol *22*, 6417-6429.

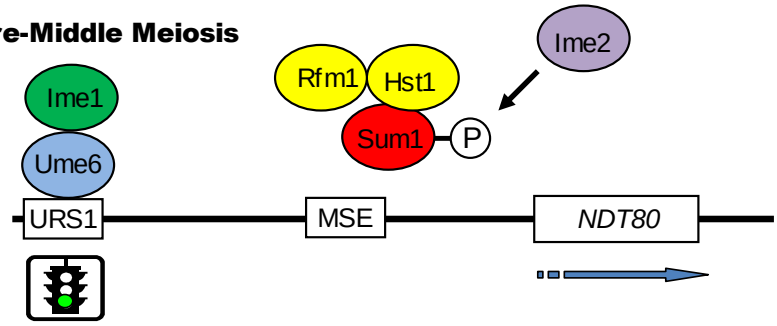
A. Vegetative Growth



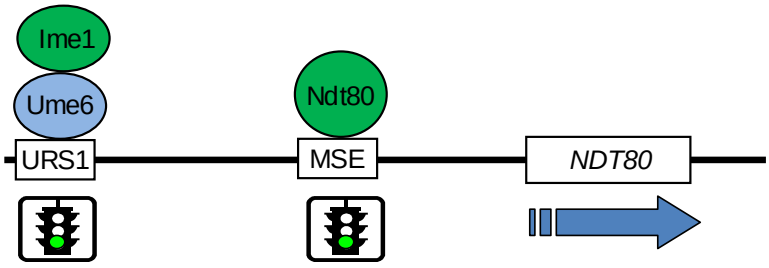
B. Early Meiosis



C. Pre-Middle Meiosis



D. Middle Meiosis



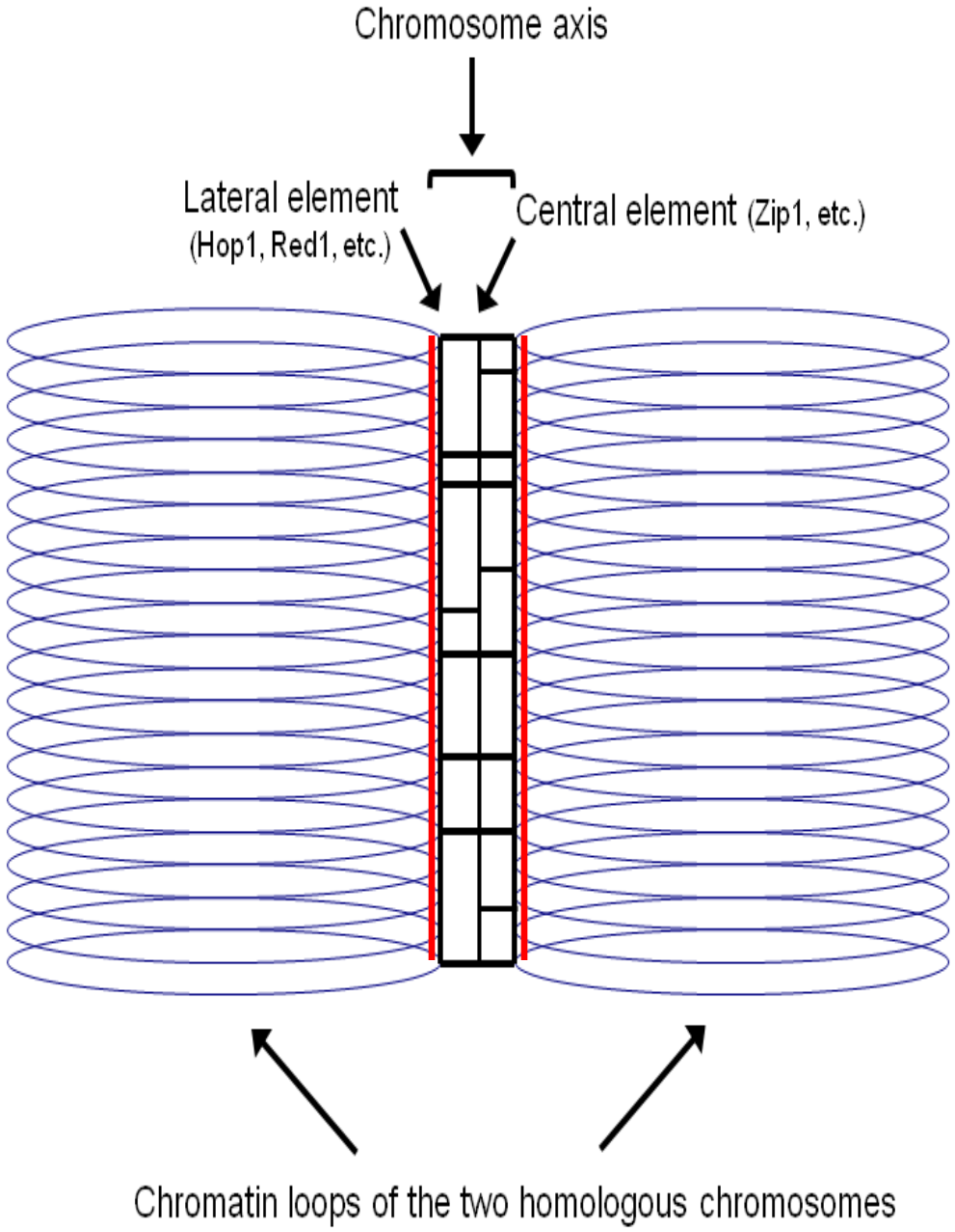
present by pachytene. Red1 has been shown to associate with chromosomes before Hop1 (Smith and Roeder, 1997).

Hop1 and Red1 are necessary for wild type levels of homologous recombination (Hollingsworth and Byers, 1989; Mao-Draayer et al., 1996; Rockmill and Roeder, 1990; Zickler and Kleckner, 1999). In *hop1* and *red1* mutants DSBs are still made but the level of homologous recombination is reduced to ~10% of wild type levels with a concomitant reduction in sporulation and spore viability Therefore, Hop1 and Red1 are not absolutely required for DSB formation, although they do play some sort of role since levels of DSB formation are reduced 10-fold if Hop1 and Red1 are absent. Our lab has shown a 20-fold reduction in DSB formation at the *HIS2* hotspot in *hop1* and *red1* mutants (Mao-Draayer et al., 1996), while Kleckner's lab has shown a 4-fold reduction in DSB formation at the *his4::LEU2* hotspot in *red1* mutants (Mao-Draayer et al., 1996). This suggests that there is likely some variability in the necessity of the SC to promote recombination at different loci.

There is significant evidence in *Saccharomyces* and in some other eukaryotes that formation of the SC depends on recombination initiation proteins. Mutations in recombination initiation genes prevent mature SC from forming (Alani et al., 1990; Bhargava et al., 1992; Giroux et al., 1989; Loidl et al., 1994; Menees et al., 1992; Rockmill et al., 1995). Henderson and Keeney (2004) have shown that some strains containing point mutations in *SPO11* have a reduction in DSB formation that is correlated with a similar reduction in mature SC.

In addition to recombination initiation proteins, several other factors are required for the formation of mature SC. Zip1 is a coiled-coil protein with two terminal globular domains (Sym et al., 1993). This structure allows Zip1 to polymerize along the length of homologous chromosomes to form the transverse elements of mature SC (Fig. 1-7). The SUMO-ligase Zip3 along with Zip2 and Zip4 are accessory factors that assist in the polymerization of Zip1 (Bhalla and Dernburg, 2008; Lynn et al., 2007). Prior to

Figure 1-7: Simplified schematic of the mature synaptonemal complex (SC). Homologous chromosomes are held together by the SC. The axial elements, which become the lateral elements in mature SC, are comprised of Red1 and Hop1 proteins, and possibly others. The location of the axial elements is shown be the red lines. Zip1 is a component of the central element. The chromosome axis is composed of the lateral elements and central element of the SC. Only the structural elements of the SC are shown. Zip2-4, factors that are important for central element assembly, are not shown. This figure was re-created from Zickler, D., and Kleckner, N. (1998). Annu Rev Genet *32*, 619-697.


Chromosome axis
Lateral element
(Hop1, Red1, etc.)
Central element (Zip1, etc.)
Chromatin loops of the two homologous chromosomes

synapsis, Zip2-4 co-localize to foci along the axes of chromosomes(Agarwal and Roeder, 2000) Zip1 is a SUMOylated protein and it has been shown that mutating *UBC9*, a member of the SUMO (Small Ubiquitin-related Modifier) conjugation pathway leads to decreased SC formation (Cheng et al., 2006; Hooker and Roeder, 2006). SUMO has been implicated in protein–protein and protein–DNA interactions and is involved in regulating a variety of cellular processes such as nuclear transport, signal transduction, stress response, and cell cycle progression (Lynn et al., 2007). Interestingly, while ubiquitination usually targets substrates for degradation via the 26S proteosome, the addition of SUMO conjugates appears to promote stability of protein-protein interactions (de Carvalho and Colaiacovo, 2006). The observation that Zip1 is SUMOylated has led to the hypothesis that the SUMO ligase function of Zip3 is required for Zip1 polymerization; however the direct targets of Zip3 are currently unknown.

Cohesin destruction is necessary for chromosome segregation

The faithful segregation of genetic material is crucial for the propagation of organisms during mitotic growth. If improper segregation occurs, aneuploidy results leading to inviable products. To facilitate proper mitotic segregation, eukaryotes have evolved a system that holds sister chromatids together that can be disassembled in a careful and controlled manner. Cohesins are a large ringed structure of proteins comprised of the subunits Smc1, Smc3, the kleisin subunit Scc1/Mcd1 and Scc3 that encircle sister chromatids and hold them together in MI (Haering and Nasmyth, 2003; Nasmyth, 2002; Nasmyth and Haering, 2005). Microtubules in the mitotic spindle can then attach to the kinetochores and align the chromosomes along the metaphase plate. Once spindle attachments are formed, the kleisin subunit (Scc1/Mcd1) of the cohesin ring is cleaved by the cysteine protease separase (Esp1 in *S. cerevisiae*), severing the cohesin

ring allowing for sister chromatids to segregate due to the tension created by the pull of the spindle fibers (Nasmyth and Haering, 2005).

Prior to correct spindle attachment, separase is sequestered by the inhibitory chaperone protein securin (Pds1, in *Saccharomyces*). At the metaphase to anaphase transition, securin is degraded by the anaphase promoting complex (APC) which includes Cdc20, an E1 ubiquitin ligase. During mitosis, the APC is only activated when each chromosome is correctly attached to spindle fibers extending from both faces of the kinetochore to the spindle pole bodies (SPBs) at opposite poles (bipolar attachment) as opposed to the same pole (monopolar attachment) (Nasmyth and Haering, 2005). When correct attachment is achieved, the APC ubiquitinates securin and targets it for destruction by the 26S proteosome. This allows separase (Esp1)to become active and the kleisin subunit of cohesins to be cleaved (Cooper et al., 2009; Nasmyth and Haering, 2005).

Cohesin assembly and disassembly is somewhat different in meiosis than in mitosis. Scc1/Mcd1 is substituted by the meiosis-specific kleisin subunit Rec8 (Molnar et al., 1995). Spindle attachment is monopolar in Meiosis I meaning that spindle fibers radiate from the kinetochore of a homolog to an SPB at only one pole of the cell. During Meiosis II, spindle attachment is bipolar, similar to the spindle attachment found in mitosis. Rec8 is removed in a step-wise manner, first from the length of the chromosome arms during Meiosis I allowing the segregation of homologs, then from the centromeres in Meiosis II allowing sister chromatids to segregate (Lee et al., 2002; Shonn et al., 2002). In *S. cerevisiae* this protection of centromeric cohesin is mediated by the meiosis-specific protein Spo13 which protects Rec8 at centromeres from being cleaved by Esp1 (separase) during Meiosis I.

The role of cyclins, CDKs and DDK in meiosis

Cyclins and CDKs

Cyclins and cyclin-dependent kinases (CDKs) are master regulators of mitotic cell cycle progression. CDKs are expressed continuously throughout the cell cycle but are only active when bound to an appropriate cyclin. As the name suggests, cyclin protein levels fluctuate during the cell cycle, thus allowing the cell to coordinate different events at different times (Evans et al., 1983; Goldbeter, 1991). Cyclins regulate the major transitions of the mitotic cycle (*e.g.*, G2/M transition). A single CDK can have different functions depending on which cyclin it is bound to. In contrast to most other eukaryotes (Doonan and Kitsios, 2009), *Saccharomyces* has only one CDK, Cdc28 (Hartwell et al., 1973). Specificity of cell cycle regulation occurs because several different cyclins are expressed at different times throughout the cell cycle (Honigberg, 2004)]. For example, Cln2 is the cyclin primarily responsible for the G1/S transition (Colomina et al., 1999; Purnapatre et al., 2002), while Cln1, Cln2, Clb5, and Clb6 are required for the initiation of mitotic S phase. Four B-type cyclins (Clbs1-4) are responsible for promoting the G2/M transition in mitotic cells (Blondel and Mann, 1996; Fitch et al., 1992; Honigberg, 2004).

In addition to the mitotic cell cycle, cyclins and CDK are also involved in meiotic progression. Clb5 and Clb6 are both required for pre-meiotic DNA synthesis (Benjamin et al., 2003; Dirick et al., 1998; Stuart and Wittenberg, 1998). Clb5 mutants are not only defective in meiotic DNA synthesis, but are also unable to initiate recombination (Smith et al., 2001); this phenotype is likely a consequence of the lack of replication (see below). Recently, Cdc28 has been shown to be required for recombination. Cdc28 phosphorylates the recombination initiation protein Rec107 on Ser30 and Ser271 (Henderson et al., 2006). It has been recently demonstrated that most of the cyclins that function during mitosis also function throughout meiosis, but the specificity of their function differs

between the two processes (Carlile and Amon, 2008). In particular, they showed that Clb3 activity is restricted to MII even though it is transcribed during MI. They found that the 5'UTR of the *CLB3* mRNA was important for limiting its translation to MII.

DDK is required for meiotic progression

Cdc7 is a the catalytic component of the DDK (Dbf4-dependent Cdc7 kinase) complex which phosphorylates and activates pre-replication components such as MCM helicase proteins during mitosis and meiosis (Sclafani, 2000). This activity is required for licensing the origins of replication (licensing origins refers to activating origins of replication to allow replication to initiate) (Sclafani, 2000). Analog sensitive mutations have been useful in studying Cdc7 and other essential kinases (Bishop et al., 2001). Analog sensitive mutants contain kinase domain mutations that are completely functional under normal conditions, but are inactivated by presence of the drug 1-NM-PP1. This drug has no effect on wild type kinases. An analog-sensitive mutant of Cdc7 lacks DSBs in the presence of 1-NM-PP1, at least partially due to their inability to phosphorylate Rec107 (Sasanuma et al., 2008; Wan et al., 2008). A recent study by Matos *et al*., (2008) further showed a link between DDK function, recombination initiation and MI (Matos et al., 2008). Mutants expressing only 15% of WT levels of DDK were unable to produce DSBs (or recombinant products), but produced live, diploid, dyad spores (Matos et al., 2008). Dyad spores were produced because the cells divided equationally. Phenotypes similar to this have been observed in *spo11 spo13* and *rec104 spo13* double mutant cells (Klapholz and Esposito, 1980a, b; Malone et al., 1991). This suggests DDK has another function after pre-meiotic replication to enable the formation of DSBs and to establish the monopolar spindle attachment required for the reductional division (Matos et al., 2008; Rabitsch et al., 2003; Tóth et al., 2000). These experiments suggest that there is coordination between meiotic S-phase and recombination, but the exact mechanism which coordinates these two critical events is still not understood.

The Recombination Initiation Signal

The initiation of recombination must occur precisely between replication of chromosomal DNA and the reductional division. Our lab has studied the properties of the 10 genes required to form DSBs. A challenge in studying these genes has been that strains with mutations in any one of the ten recombination initiation genes show similar phenotypes: inviable spores, reduced sporulation, no DSBs and no meiotic recombination. These strains do, however, complete both MI and MII. Anne Galbraith, a graduate student from the Malone lab, investigated when a recombination initiation mutant started the first and second divisions of meiosis compared to wild type cells. She and her colleagues found that *rec104, rec102* and *rec114* mutants start the first division about 1-1.5 hours earlier than wild type cultures (Galbraith et al. 1997). These results led Galbraith and Malone to hypothesize that the presence of these proteins creates a signal that delays the start of the reductional division in a wild type cell. We refer to the signal that creates the normal delay of the first meiotic division as the Recombination Initiation Signal or RIS (Malone et al., 2004).

Other members of the Malone lab found that other recombination initiation mutants also started the first division of meiosis earlier than wild type. Kai Jiao, a graduate student, and Sonja Smith, a research assistant, have shown that *rad50* and *xrs2* mutants also start MI earlier than wild type, respectively (Jiao et al., 1999) (Malone lab, unpublished). Logan Vidal, an honor's undergraduate in our lab, has shown that *mre11* mutants begin the reductional division earlier than in wild type.Members of our lab showed that null mutations in *SPO11* also start MI early (Malone et al., 2004). This observation was also observed by Kee and Keeney (2002). We have published that *rec102* and *rad50* mutations start MI about an hour earlier than *rec104* strains (about 2-2.5 hours earlier than wild type), however, we have not been able to explain this phenomenon yet. Work presented in Chapter 2 suggests that the difference in MI timing between different recombination initiation mutants *(e.g., rec102 vs. rec104)* may not be

conserved across different *S. cerevisiae* strain backgrounds though not all combinations of initiation mutants have been tested in other strain backgrounds (*e.g., rec104 vs. rad50, spo11 vs. mre11*, etc.) (Malone lab, unpublished and Chapter 3).

Two synaptonemal complex genes have also been shown to be required for the timing of the start of the reductional division. Lindsay Carpp showed that *hop1* and *red1* mutants start the first division of meiosis earlier than wild type, similar to a *rec104* mutant (Malone et al., 2004). Sarah Nord Zanders, an Honors undergraduate, has shown that mutating *ZIP1* results in extremely reduced and delayed MI and MII divisions, suggesting that Zip1 does not coordinate the timing of the reductional division (Malone lab, unpublished results). This result is consistent with results observed by the Roeder lab (Tung et al., 2000). We have yet to investigate the role of other SC genes in coordinating the timing of the first division. It is unclear whether Hop1 and Red1 are a part of the RIS or are downstream sensors of the RIS. This idea will be explored in the final chapter.

Unlike the Pachytene checkpoint (discussed below and in Chapter 5), which is activated in cells with some late recombination defects (*e.g.,* cells with mutations in genes required for strand invasion, such as *DMC1),* the recombination initiation signal is normal and it regulates the timing of a normal MI division We propose that the RIS delays the start of the reductional division until recombination has been completed. The recombination initiation proteins included in the RIS are present during meiosis in wild type cells but when even one RIS gene is absent, the RIS signal is not present to delay the reductional division (Galbraith et al., 1997; Malone et al., 2004).

Galbraith and Malone originally hypothesized that the initiation of DSBs could be the signal monitored to coordinate recombination and the reductional division. Mitotic cells can detect the presence of even one unrepaired DSB and arrest (Elledge, 1996; Fogel and Mortimer, 1971; Hartwell and Weinert, 1989; Hurst and Fogel, 1964). The mitotic DNA damage checkpoint system results in an arrest of all cells with that break

(discussed in detail below and in chapter 5). However, the early segregation of chromosomes in mutants lacking the recombination initiation signal cannot be due to the absence of DSBs alone, because *mei4* and *ski8* (see Chapter 2) mutants strains still begin the reductional division at the normal time, even though these mutants completely eliminate DSBs (Galbraith et al., 1997; Jiao et al., 1999; Malone et al., 2004). *rec104, spo11, rec102,* and *rad50* mutations are epistatic to *mei4* mutations (*i.e*., a *rec104 mei4* double mutant starts the first division early like in a *rec104* mutant, rather than at the same time as wild type, as seen in a *mei4* mutant) (Galbraith et al., 1997; Jiao et al., 1999). Furthermore, adding an artificial double strand break to a *rec104* mutant was not sufficient to restore the normal timing of the first division indicating that a DSB is not sufficient to restore timing in the absence of recombination (Jiao et al., 1999). Jiao concluded that the presence of a subset of initiation proteins, but not DSBs, creates the signal that coordinates the initiation of recombination with the start of the reductional division.

Our initial finding led us to propose that the presence of RIS proteins is required for the signal to delay MI. This was a reasonable hypothesis because the mutations that we have studied to characterize the RIS have all been complete deletions of the coding region. However, work done by Nick Lyons, an Honors undergraduate, conflicts with this hypothesis. Lyons examined the MI timing of a strain containing a mutation in the proposed catalytic domain of SPO11 (*spo11-Y135F*). It has been previously shown that Spo11-Y135F protein can localize to meiotic chromatin, even though DSBs are made (Prieler, et al., 2005). Lyons showed that *spo11-Y135F* cells start the reductional division at a time indistinguishable from *spo11* null cells. Thus, our initial hypothesis that the presence of the members of the RIS is required for the RIS may not be true. However, it is formally possible that the Y135F mutation disrupts the binding of one or more of the RIS proteins. To test this, it would be necessary to determine that each of the RIS

proteins were recruited to hotspots in *spo11-Y135F* cells using chromatin immunoprecipitation, or other methods.

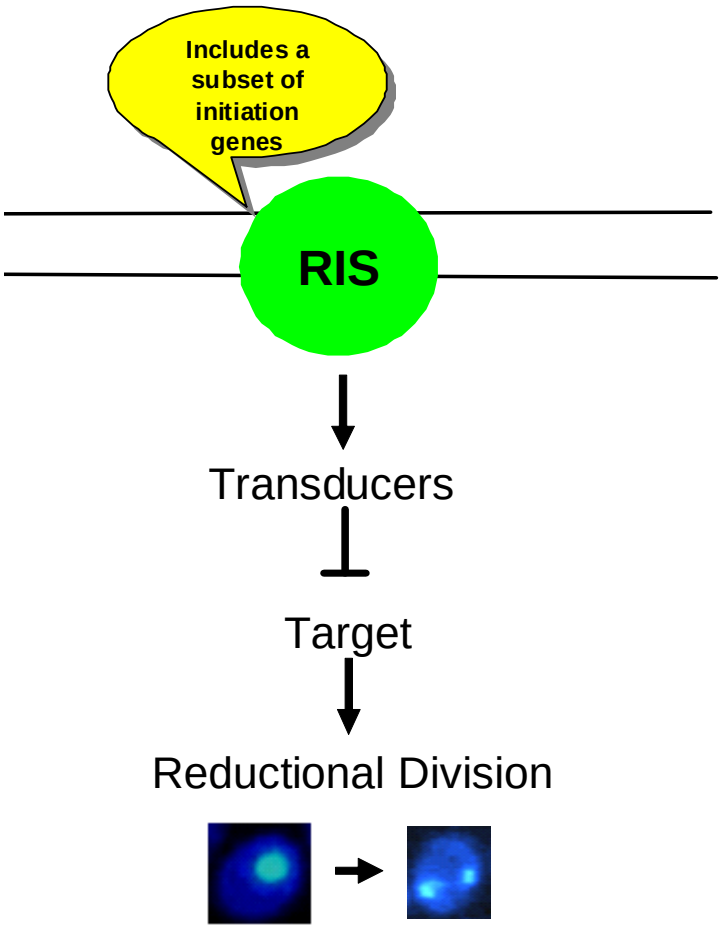
The RIS must be a transient signal in order to the reductional division to occur. Termination of the RIS is not something that our work in the Malone lab has yet addressed. I will speculate on a mechanism for the termination of the RIS in the last chapter.

A major goal of the Malone lab has been to determine the mechanism by which the RIS delays the reductional division. The remainder of this chapter will present background relevant to our experiments that elucidate this mechanism. Specifically, we have asked what is the ultimate target of the RIS and how is the RIS transduced to this target (Fig. 1-8).

Signal transduction of the RIS

No signal transduction role has been ascribed to the proteins of the RIS. Signal transduction is often carried out by kinases or phosphatases which can interact with a wide variety of targets to propagate a biological signal (Hartwell et al., 1989, Pasero et al., 2003). We therefore hypothesized that additional factors are required to transduce the signal that delays the start of meiosis. Our first candidates for the transduction of the RIS were mitotic checkpoint proteins known to be important for monitoring chromosomal events during mitosis in *Saccharomyces*. Many of these transducing proteins are kinases that have been shown to have numerous important targets for mitotic progression (Pasero et al., 2003). Several Malone lab members and I have evaluated proteins involved in mitotic checkpoints in their potential role in transducing the RIS. Before discussing the role of known checkpoint mutants in meiosis, I will review the role of checkpoints in mitosis.

Figure 1-8: Overview of the mechanism of the RIS. The RIS consists of a subset of recombination initiation proteins. These proteins delay the start of the reductional division in a wild type cell. In this thesis I will present experiments investigating the role of the signal transduction and the target of the RIS.


Includes a subset of initiation genes
RIS
Transducers
Target
Reductional Division

Checkpoints in mitosis

There are at least three main checkpoints important in monitoring chromosomal behavior during mitosis: the DNA damage checkpoint, the S phase checkpoint, and the spindle checkpoint (Harrison and Haber, 2006; Lew and Burke, 2003; Sclafani and Holzen, 2007). There is significant overlap between some of the components of these checkpoints and they can impinge on similar targets. However, the three checkpoints differ in signal input (Pasero et al., 2003). The DNA damage checkpoint monitors DSBs and other DNA lesions, the S phase checkpoint monitors replication fork progression and structure and helps to ensure that all origins of replication fire only once and the spindle checkpoint monitors correct attachment of spindle fibers between spindle pole bodies (SPBs) and kinetochores and mitotic exit.

The DNA damage checkpoint

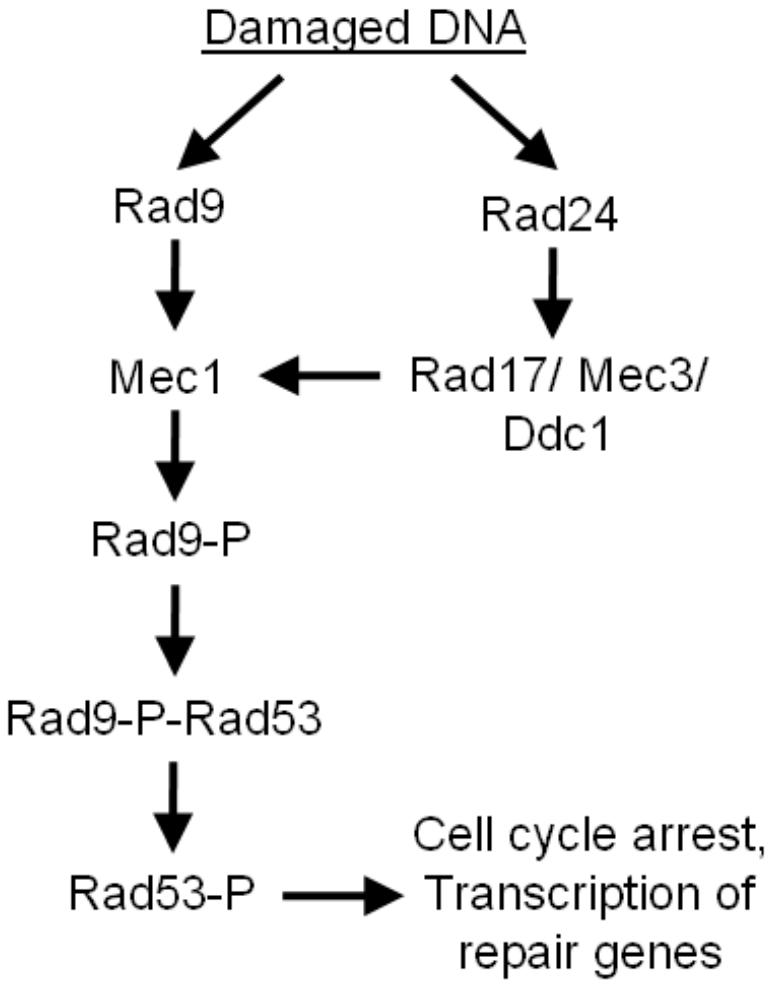
The DNA damage checkpoint functions throughout the cell cycle to sense damage to DNA that can occur through such means as ionizing radiation, UV damage, free-radical damage and base adducts (Harrison and Haber, 2006; Paulovich et al., 1997). During mitosis DNA damage that causes DSBs is primarily carried out by homologous recombination using the sister chromatid as a template. Mutations in DNA damage checkpoint genes are sensitive to genotoxic agents such as methyl methane sulfonate (MMS) or to radiation. The DNA damage checkpoint is comprised of two separate branches. Rad9 functions in one branch of the DNA damage protein and Rad24 functions in the other branch (Friedel et al., 2009). These proteins function distinctly from one another but both ultimately converge on the PI 3-kinase family protein Rad53 (a homolog of mammalian Chk2) (de la Torre-Ruiz et al., 1998). Rad53 contains homology to PI 3- kinases, but additionally contains two FHA domains which can interact with phosphoproteins (Harrison and Haber, 2006; Liao et al., 1999; Liao et al., 2000). The DNA damage checkpoint is summarized in Fig. 1-9.

Rad24 is a replication factor C (RFC)-like protein which acts as clamp loader to load a PCNA-like complex of Rad17, Ddc1 and Mec3 onto the DNA at the sites of DNA damage (Zhou and Elledge, 2000). In other organisms, such as S. pombe, this is known as the "9-1-1" complex (Harrison and Haber, 2006). Loading this clamp is necessary for activation of the kinase Mec1 which phosphorylates downstream targets for transcriptional activation of genes necessary for DSB repair (Rouse and Jackson, 2002a, b). Rad9 is an adaptor protein which becomes hyper-phosphorylated by the PI 3-kinase family protein Mec1 (a homolog of mammalian ATR) in response to DNA damage (Friedel et al., 2009; Soulier and Lowndes, 1999).

The role of Rad9 is complex because it is both an upstream sensor of DNA damage and a downstream protein (adaptor) that interacts with the effector kinase Rad53 which phosphorylates downstream targets for repair (Friedel et al., 2009). A mutation in either the Rad9 or Rad24 branch of the DNA damage checkpoint pathways results in only a partial loss of checkpoint function consistent with the view that there are two upstream redundant damage sensing pathways (de la Torre-Ruiz et al., 1998) (Fig. 1-9). Disabling both branches of the pathway (*rad9 rad24* double mutants) is required to completely deactivate the DNA damage checkpoint ((de la Torre-Ruiz et al., 1998) and Fig. 1-9).

Like Mec1, Tel1 (a homolog of mammalian ATM) is a PI 3-kinase family protein important the DNA damage checkpoint. Tel1 was originally identified for its role in telomere length regulation (Greenwell et al., 1995). Mec1 and Tel1 are somewhat functionally redundant in mitotic growth. While Mec1 is necessary in response to wide variety of DNA lesions, Tel1, is activated principally in response to DSBs (Cimprich and Cortez, 2008; Friedel et al., 2009). Mec1 and Tel1 also act different biochemically. During the mitotic cell cycle, Tel1 appears to bind unresected (undigested) DSBs via the Mre11-Rad50-Xrs2 (MRX) complex and the signaling activity of Tel1 is disrupted when DSB termini are resected (Mantiero et al., 2007; Nakada et al., 2003). In contrast, Mec1

Figure 1-9: **The DNA damage checkpoint** is composed of two branches that converge on the Mec1 and Rad53 protein kinases. In one branch of the pathway, Rad9 and Mec1 recognize damaged DNA. Mec1 phosphorylates Rad9 (represented by "Rad9-P"), which promotes an interaction between Rad9 and Rad53 (represented by Rad9-P-Rad53). Rad53 becomes activated through autophosphorylation and phosphorylates its targets to activate or inhibit effectors to cause cell cycle arrest and transcription of repair genes. In the second branch of the pathway, Rad24 recognizes damaged DNA and loads the Rad17, Mec3, and Ddc1 complex. This is required for full activation of Mec1 and Rad53. This figure is modified from (de la Torre-Ruiz et al., 1998; Hochwagen and Amon, 2006; Meier and Ahmed, 2001; Roeder and Bailis, 2000).


Damaged DNA
Rad9
Rad24
Mec1
Rad17/ Mec3/
Ddc1
Rad9-P
Rad9-P-Rad53
Rad53-P
Cell cycle arrest,
Transcription of
repair genes

is thought to recognize ssDNA regions in conjunction with Rad24 and Rad9 that arise after DSB resection. Mec1 also recognizes and binds to long stretches of ssDNA that have been coated with replication protein A in response to nucleolytically processed DNA found when replication forks have stalled (Friedel et al., 2009). Mec1 and Tel1 have a wide variety of downstream targets including the effector kinase Rad53 (de la Torre-Ruiz et al., 1998; Hochwagen and Amon, 2006). Mec1 and Tel1 also phosphorylate *S. cerevisiae* histone H2A (an ortholog of the mammalian histone variant histone H2AX) over a 50-kilobase region surrounding the DSB to form gamma-H2A(X) (Keogh et al., 2006; Unal et al., 2004). This chromatin modification is important for recruiting numerous DSB-recognition and repair factors to the regions surrounding DSBs, including chromatin remodellers and cohesins.

mec1 and *rad53* deletion mutants are lethal. In order to study these mutants, either *ts* mutants can be used or a strain background containing the *smL1* (*suppressor of mec1 lethality*). The lethality of *rad53* mutations is also repressed by a *smL1* mutation. *SML1* encodes an inhibitor of ribonucleotide reductase (Rnr1) that is dispensable for cell growth (Zhao et al., 2001; Zhao et al., 2000). Rnr1 is required for synthesizing nucleotides. Removing an inhibitor of Rnr1 ensures that nucleotide pools will be high. SmL1 is phosphorylated and concomitantly degraded by Rad53 and Mec1 in response to S phase or DNA damage. *rad9*, *rad24* and *tel1* mutants are all viable during vegetative growth (Sclafani and Holzen, 2007).

The S phase checkpoint

During S phase unreplicated DNA or stalled replication forks can be sensed by some of the factors necessary for replication itself (Friedel et al., 2009; Sclafani and Holzen, 2007) and Fig. 1-10). Pol2 and Dbp11, subunits of DNA polymerase epsilon and Rfc5 (a subunit of replication factor C which recruits the clamp loader for lagging strand synthesis) have been implicated in S phase checkpoint function. Strains with mutations in

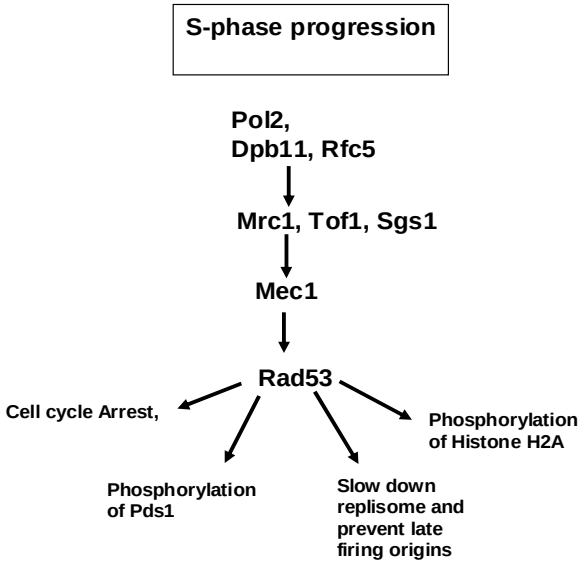
these genes are sensitive to hydroxyurea (HU), an inhibitor of Rnr1 (Araki et al., 1995; Longhese et al., 2003; Navas et al., 1995). Inhibiting Rnr1 function depletes dNTP pools required for replication fork progression (Saka and Yanagida, 1993). The heterotrimeric complex of Tof1, Mrc2 and Csm3 interacts with stalled polymerases (Nyberg et al., 2002). In addition to its checkpoint signal role, the Tof1/Mrc2/Csm3 complex has been implicated in promoting sister chromatid cohesion after DNA damage, facilitating gap repair of damaged DNA; and interacting with the MCM (Minichromosome Maintenance) helicase (Fig. 1-10). Sgs1 is a DNA helicase of the RecQ family which stabilizes replication forks in response to DNA damage and which acts as an upstream signal for the S phase checkpoint (Versini et al., 2003).

Many of the downstream components of the DNA damage checkpoint are also required for the S phase checkpoint. Stalled replication forks result in the accumulation of RPA- coated ssDNA which causes the Tof1/Mrc2/Csm3 complex to signal to the downstream effector kinases Rad53 and Mec1(Friedel et al., 2009; Hochwagen and Amon, 2006). Mec1 is subsequently activated, leading to phosphorylation of various downstream targets including the adaptor proteins Mrc1 (a protein that stabilizes Pol2 at stalled replication forks during stress) and Rad9, which contribute to the activation of the downstream effector kinases Rad53 and Chk1 is a serine/threonine kinase that mediates cell cycle arrest via phosphorylation and stabilization of Pds1 (securin, reviewed in chapter 3) (Friedel et al. 2009). Rad53 has been shown to play a crucial role in stabilizing the replisome, preventing late origins from firing, preventing repair from homologous chromosomes.

The spindle checkpoint

The spindle checkpoint has two branches (Fig. 1-11). Bub1, Bub3, Mad1, Mad2 and Mad3 are part of the pathway that senses unattached kinetochores and comprises the spindle-assembly checkpoint (SAC) complex (Cahill et al., 1999; Li et al., 1997).

Figure 1-10: The S phase checkpoint. Model of a moving and stalled replication fork in *S. cerevisiae*. [A] At an unperturbed replication fork MCM helicases unwind the parental DNA double strand. Polymerase (Pol ε) is responsible for leading strand synthesis, while polymerase α (Pol α/prim) initiates Okazaki-fragment-synthesis at the lagging strand that is completed by polymerase δ (Pol δ). [B] At a stalled replication fork single stranded DNA coated by RPA (RPA-ssDNA) accumulates. This triggers an S phase-specific checkpoint response, where Rad17 and Rad24 and the checkpoint kinase Mec1-Ddc2 are recruited independently to RPA-ssDNA. Subsequently, Mec1-Ddc2 is activated, leading to phosphorylation of various downstream targets including the mediator proteins Mrc1 or Rad9, which contribute to the activation of the downstream effector kinases Rad53 and Chk1. Rad53 was shown to play a critical role in stabilizing the replisome, preventing late origins from firing, preventing homologous recombination (HR), and mediating DNA repair. Modified from Friedel, A.M., Pike, B.L., and Gasser, S.M. (2009). ATR/Mec1: coordinating fork stability and repair. Curr Opin Cell Biol *21,*


S-phase progression
Pol2,
Dpb11, Rfc5
Mrc1, Tof1, Sgs1
Mec1
Rad53
Cell cycle Arrest,
Phosphorylation
of Histone H2A
Phosphorylation
of Pds1
Slow down
replisome and
prevent late
firing origins

Several of these proteins co-localize with the kinetochore. This branch of the spindle checkpoint has been proposed to be the component that senses when chromosomes are aligned and correctly attached (see below), Bub2 is part of a pathway that detects spindle attachment at SPBs and is a mitotic exit network (MEN) regulator (Fraschini et al., 2006; Fraschini et al., 1999). It forms a GTPase-activating complex with Bfa1 and Tem1 and binds to spindle pole bodies to block cell cycle progression in response to spindle and kinetochore damage (Hu et al., 2001) (Fig. 1-11)

Bipolar attachment of spindle fibers (microtubules) radiating from SPBs to kinetochores is necessary to segregate sister chromatids correctly into daughter cells during mitosis (Lew and Burke, 2003; Pinsky and Biggins, 2005). Two spindle fibers extend from the kinetochores on each of the sister chromatids to opposite poles (Fig. 1-12). This attachment creates tension which pulls chromosomes into alignment at the metaphase plate and allows for chromosome segregation when cohesins are degraded at the beginning of anaphase (Lew and Burke, 2004; Pinsky and Biggins, 2005 and Chapter 3). Recent evidence suggests that absence of tension does not activate the spindle checkpoint *per se* (Burke and Stukenberg, 2008). Rather, Mad2, Bub1 and Bub3 can associate with unattached kinetochores and can turn on a two-stage "switch" that the arrests cellular division by inhibiting the APC (Fig. 1-13). When kinetochores are occupied by microtubules, they cannot be occupied by the SAC factors and thus turning the "switch" off. The SAC checkpoint prevents the degradation of Pds1 (securin) in response to spindle damage. Pds1 degradation is required for the release of Esp1 (securin) which promotes anaphase onset (See chapter 3).

Checkpoints in meiosis

Checkpoints are also important in monitoring the events of meiosis (Carballo and Cha, 2007; Hochwagen and Amon, 2006). Many checkpoint functions are conserved between mitosis and meiosis, but some are unique to meiosis (Hochwagen and Amon,

Figure 1-11: The spindle checkpoint monitors microtubule binding at the kinetochore and tension created at the spindle pole body (SPB). Bub1, Bub3, Mad1, Mad2, and Mad3 are all required to detect unattached kinetochores. In the event of an unattached kinetochore, these five proteins inhibit the activity of Cdc20-APC (anaphase promoting complex) by binding to it. Inactive Cdc20-APC prevents the destruction of Pds1 (securin), thereby keeping Esp1 (separase) protected. Once proper microtubule connections have been made, APC becomes activated and targets Pds1 for degradation. This frees Esp1, which allows cleavage of the Scc1 cohesin, causing separation of sister chromatids. When there is a lack of tension at the SPB, Bub2 along with Bfa1 and Tem1 inhibits the activity of Cdh1-APC. Once proper tension is detected, Bub2 relieves its inhibition. Cdh1 then becomes inactivated through dephosphorylated, which causes activation of APC. The B-type cyclin, Clb2 is targeted for degradation, which causes exit from mitosis. This figure was adapted from (Gardner and Burke, 2000).

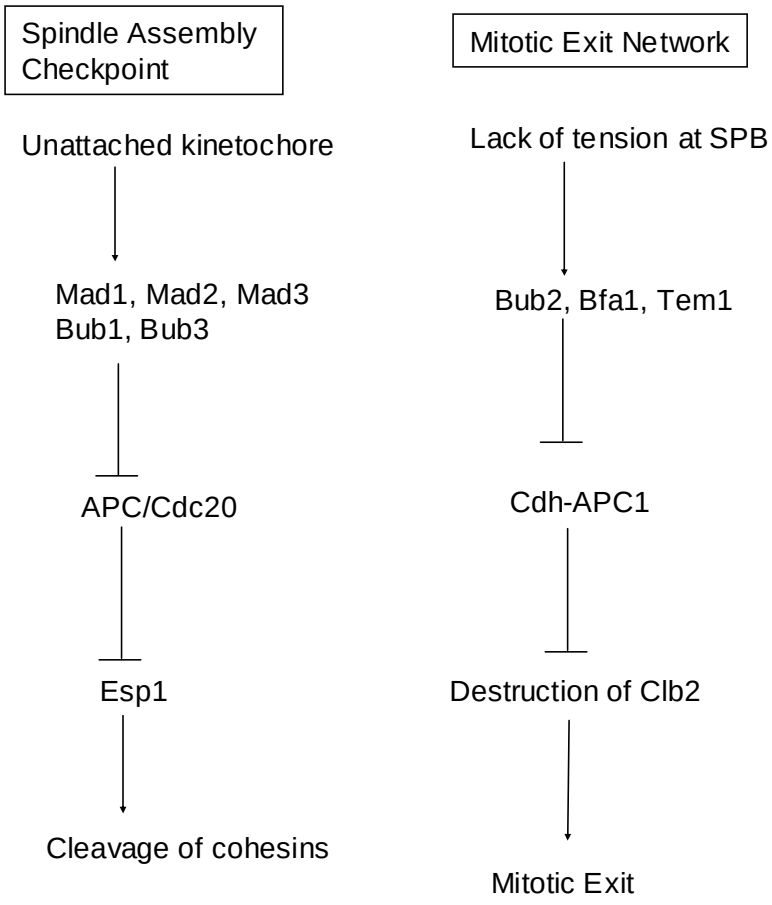
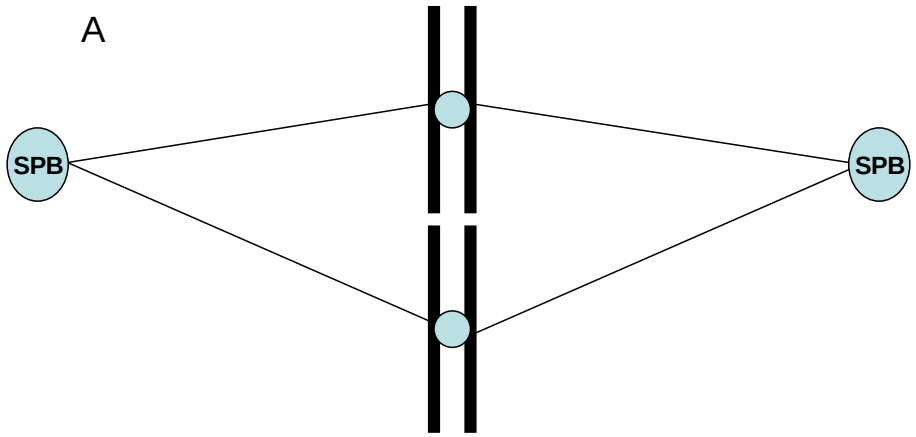
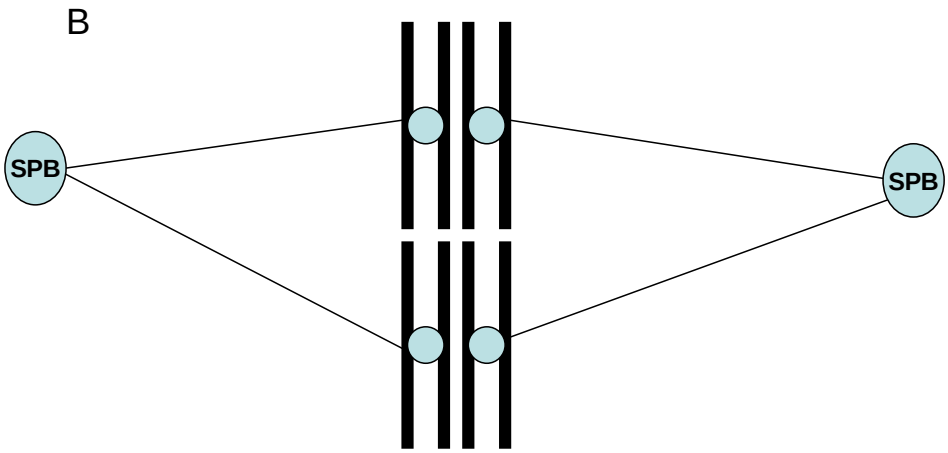

Spindle Assembly Checkpoint
Unattached kinetochore
Mad1, Mad2, Mad3
Bub1, Bub3
APC/Cdc20
Esp1
Cleavage of cohesins
Mitotic Exit Network
Lack of tension at SPB
Bub2, Bfa1, Tem1
Cdh-APC1
Destruction of Clb2
Mitotic Exit

Figure 1-12: Spindle attachment during meiosis and mitosis. [A] Spindle attachment is bipolar during meiosis II and mitosis. Spindle fibers from both faces of the kinetochore extend to spindle pole bodies (SPBs) on opposite sides. [B]. Spindle attachment is monopolar during meiosis I. Spindle fibers extend from one face of the kinetochore to an SPB on the same pole of the cell.

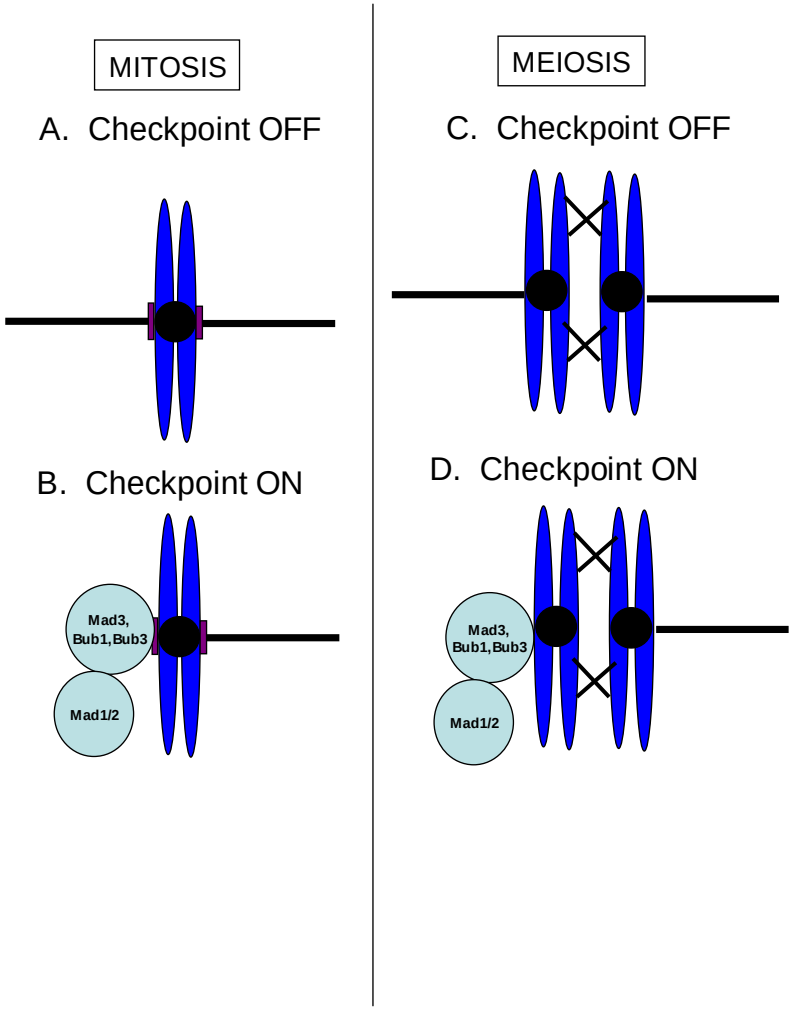


Mitosis and Meiosis II



Meiosis I

Figure 1-13: The 2-stage switch model for the SAC. Outer surface of kinetochores are shown in purple. [A] When microtubules are occupying the outer surface the kinetochore, the spindle checkpoint is off. [B] When the outer surface of the kinetochores is unoccupied by microtubules, members of the spindle assembly checkpoint can bind. Mad3, Bub1 and Bub3 bind directly to the kinetechore. Mad2 and Mad1 bind together. When Mad2 is bound to the kinetochores, it is in the "open" state. This causes a checkpoint-mediated arrest by inhibiting the APC/C. [C] and [D] show SAC in meiosis. Simplified from Burke, D.J., and Stukenberg, P.T. (2008). Linking kinetochore-microtubule binding to the spindle checkpoint. Dev Cell *14*, 474-479.


MITOSIS
A. Checkpoint OFF
B. Checkpoint ON
Mad3, Bub1,Bub3
Mad1/2
MEIOSIS
C. Checkpoint OFF
D. Checkpoint ON
Mad3, Bub1,Bub3
Mad1/2

2006). The S phase, DNA damage and spindle checkpoints are all present in meiosis with a few key differences. The Pachytene checkpoint (sometimes called the recombination checkpoint) is a checkpoint that causes mononucleate cell arrest before the reductional division in response to an excess of unprocessed ssDNA such as is found in *dmc1* mutants. This checkpoint can also respond to SC defects.

S phase checkpoint in meiosis

Meiosis is preceded by pre-meiotic S phase that is regulated by the S phase checkpoint. Treating cells with HU shortly after being placed in sporulation medium results in cell cycle arrest (Simchen et al., 1976). Furthermore, meiotic transcription ceases and recombination does not occur (Lamb and Mitchell, 2001). Replication and recombination have been shown to be coupled events. Recombination does not occur unless it is preceded by replication (Borde et al., 2000; Lamb and Mitchell, 2001). Borde et al. demonstrated this by deactivating all of the origins of replication along the left arm of chromosome 3 by mutating the entire ARS consensus sequences in that region. This delayed S phase by 60 minutes, as demonstrated by 2-D gel electrophoresis. In cells without ARS mutations, DSBs occur at the same time in both the left and right arms of chromosome 3. When replication is delayed in the left arm, however, Borde et al. (2000) observed that DSB formation was also delayed compared to the right arm.

One key difference between S phase in mitosis and pre-meiotic S phase is that pre-meiotic S phase takes much longer (25-30 minutes for mitotic S-phase vs. ~75 minutes for pre-meiotic S-phase) (Cha et al. 2000). Borde et al.'s (2000) observation leads to the intriguing notion that this extra time needed for pre-meiotic S phase might be because the chromatic must be altered to prepare for meiotic recombination only after origins of replication have fired. One such alteration might be the assembly of recombination initiation complexes.

Spindle checkpoint in meiosis

During meiosis there are two rounds of cellular division, thus the spindle must assemble and disassemble twice (Amon, 1999; Lew and Burke, 2003). During MI, meiotic spindles form monopolar attachments: each homolog is only connected to one SPB allowing sister chromatids to remain attached by cohesins present at the centromere during the reductional division. Spindle attachment during the second division of meiosis is similar to the bipolar attachment found in mitosis. The spindle checkpoint functions to delay anaphase in both MI and MII until proper spindle attachments are formed (Clarke and Bachant, 2008). In addition, anaphase onset depends on a meiosis-specific, Cdc20-related factor, Fzr1/Mfr1, which contributes to anaphase cyclin decline and anaphase onset and is partially inhibited by the spindle assembly checkpoint (SAC) (Yamamoto et al., 2008).

Murray and Dawson have proposed that the spindle checkpoint may coordinate the initiation of recombination and the start of the reductional division (Shonn et al., 2000; Shonn et al., 2003). Murray's lab hypothesizes that the normal delay in start of MI seen in wild type strains compared to the timing of MI in RIS mutants is due to a lack of interhomolog tension created by recombination intermediates that physically link homologous chromosomes prior to MI. Murray's lab has observed that eliminating recombination *(e.g*., in *spo11*), permits homologous chromosomes to segregate early. This is in agreement with our RIS model. However, Murray and colleagues have proposed that the early division in a *spo11* strain is due to a lack of inter-homolog tension. This tension is present in a wild type cell due to the presence of chiasmata. The Murray lab has proposed that members of the spindle checkpoint monitor this tension and delay the reductional division until tension is achieved. This hypothesis is in disagreement with the RIS model because we have observed that *mei4* mutants start the reductional division earlier than wild type strains. *mei4* mutants make no DSBs, and thus would have no inter-homolog tension.

In order to investigate the two different hypotheses for explaining the delay of the reductional division in a wild type cell, Rachel Gast examined MI kinetics in strains that had either or both branches of the spindle checkpoint genes eliminated. If a checkpoint is absent, the reductional division would no longer be delayed and the division would occur early. She reproducibly found that MI started at the same time as in wild type cells in *mad2*, *bub2*, or *mad2bub2* strains. These results suggest that spindle checkpoint proteins do not regulate the timing of the start of the reductional division. After Gast finished these experiments, a paper from the Dawson lab was published suggesting that Mad3, a member of the spindle assembly checkpoint might be responsible for monitoring the timing of the start of the reductional division (Cheslock et al., 2005). Dawson's lab examined nuclear, SPB and spindle kinetics in *mad3* mutants and found that these strains post-prophase I" or "post-anaphase I" earlier than wild type. Although they did not distinguish in their graphs whether SPB duplication, spindle formation or nuclear division (or a combination of all three) led them to the conclusion that *mad 3* mutants start the reductional division early, Gast found the result intriguing. She examined the MI timing of a *mad3* strain and reproducibly found that *mad3* strains start the reductional division at the same time as wild type cells. Gast also examined MI spindles and SPB duplication and found that these were also present in *mad3* cells at the same time as in wild type. After Gast graduated, I further investigated the role of Mad3 in meiosis using transcriptional analysis of a transcription factor, *NDT80*. These results are presented in Chapter 5.

The DNA damage checkpoint in meiosis

The DNA damage checkpoint found in mitosis, including Rad9 and Tel1 is fully active in meiosis prior to recombination (Cartagena-Lirola et al., 2008; Hochwagen and Amon, 2006). If cells are cells are treated with DSB-inducing agents shortly after introduction into sporulation medium, Rad53 becomes phosphorylated, indicating that the

DNA damage checkpoint was triggered (Cartagena-Lirola et al. 2008). Rad53 does not become phosphorylated after meiotic recombination normally initiates. The role of the DNA damage checkpoint prior to the initiation meiotic recombination has also been demonstrated by examining temperature-sensitive *cdc13*^{ts} mutants to a non-permissive temperature at the beginning of sporulation, prior to DSB formation and recombination. Cdc13 is a protein required for telomere capping; *cdc13*^{ts} mutants at the non-permissive temeperature form an abundance of ssDNA at telomeres (DNA damage) and subsequently arrest (Lydall, 2003). This arrest requires Rad17, Rad24, Rad9, Tel1 and Mec1, components of the DNA damage checkpoint.

The DNA damage checkpoint is also present in the absence of meiotic recombination. In fact, the spore viability of recombination initiation mutants is partially restored in strains that have been exposed to ionizing radiation because induced DSBs can stimulate proper chromosomal pairing and segregation during the first division (Thorne and Byers, 1993).

The Pachytene checkpoint

One of the distinguishing hallmarks of meiosis that differentiates it from mitosis is the intentional creation of ~200 DSBs at hotspots during prophase I. Creation of these DSBs is not completely random. They occur preferentially at hotspots in zygotene in meiosis. This intentional meiotic DNA damage requires that the role of the mitotic DNA damage checkpoint be altered. The components of the mitotic DNA damage checkpoint have been shown to play an additional, important role in monitoring the state of meiotic DNA, however. *dmc1* and some other late recombination mutants cannot properly process DSBs and therefore have hyperresected (exonuclease digested) DNA (Bishop et al., 1992). Dmc1 is a RecA homolog expressed only in meiosis and is important for DSB repair from homologs rather than sister chromatids (Bishop et al., 1992). The hyperresected DNA found in *dmc1* cells is perceived by the Pachytene checkpoint

proteins Rad17, Rad24, Mec1, Mec3 and Ddc1 which arrest the cell before the reductional division (Hochwagen and Amon, 2006; Lydall et al., 1996; Roeder and Bailis, 2000). Hed1, an inhibitor of Rad51 (a RecA homolog expressed in both mitosis and meiosis) has also been shown to be important for the Pachytene checkpoint (Busygina et al., 2008; Tsubouchi and Roeder, 2006). Unlike in the mitotic DNA damage checkpoint, Rad9, Tel1 and Rad53 are not necessary for this pachytene arrest (Hochwagen and Amon, 2006). The Pachytene checkpoint appears to be conserved; mice lacking Dmc1 or other homology search factors have arrested gametogenesis, although it is followed by apoptosis (Ashley et al., 2004; Pittman et al., 1998).

Mek1 is a meiosis specific Rad53 paralog important in the function of the Pachytene checkpoint (Bailis and Roeder, 2000; de los Santos and Hollingsworth, 1999; Niu et al., 2005; Wan et al., 2004) . It has been proposed that Mek1 is a meiosis-specific substitute for the effector kinase Rad53 (Carballo and Cha, 2007). Mek1 is conserved in *S. pombe*, but is not found in *C. elegans*, *Drosophila* or mice (Hochwagen and Amon, 2006). Mek1 is expressed early in meiosis and has been shown to interact with the SC components Hop1 and Red1 (de los Santos and Hollingsworth, 1999; Niu et al., 2005; Rockmill and Roeder, 1991; Wan et al., 2004; Woltering et al., 2000). Hop1 and Red1 have been proposed to be required for the Pachytene checkpoint (Bailis et al., 2000), though it is seems unlikely because these proteins are required for ~90% of DSB formation, hence little hyperresected DNA could form in *hop1*, or *red1* strains.

The synaptonemal complex is also monitored by the Pachytene checkpoint (Roeder and Bailis, 2000). *zip1*, *zip2* and *zip3* mutants all arrest in certain strain backgrounds (see Chapter 3 for details). Rad17, Rad24, Mec1, Mec3, Ddc1 Hop1, Red1 and Mek1 are all required for this arrest. In addition to these proteins, the nucleolar ATPase Pch2 and the histone methyltransferase Dot1are also required (San-Segundo and Roeder, 1999, 2000). Dot1 and Pch2 are also required for the checkpoint arrest in *dmc1* cells (San-Segundo and Roeder, 1999, 2000).

Because the Pachytene checkpoint has defined in mutant cells, it provokes the question of what role Pachytene checkpoint proteins play in a normal meiosis. Some of these proteins (Mek1, Mec1, Rad24, Rad17 and perhaps others) serve in aspects of normal meiotic progression (described below).

Other roles of checkpoint proteins during meiosis

In addition to their roles in the Pachytene checkpoint, Rad24, Rad17 and Mec1 have been implicated in other aspects of normal meiotic progression. These genes are necessary for suppressing ectopic recombination and for promoting recombination between homologs (partner choice) (Carballo and Cha, 2007; Grushcow et al., 1999; Thompson and Stahl, 1999). Ectopic recombination is recombination between non-homologous chromosomes. During meiotic recombination, repair of DSBs preferentially occurs using homologous chromosomes rather than sister chromatids (Szostak et al., 1983). Rad24, Rad17 and Mec1 may also play a role in proper SC assembly. *rad17*, *rad24* and *mec1* mutants all display polycomplexes (abnormal large aggregates of Zip1-4 proteins) (Grushcow et al., 1999).

Mek1 has been implicated in promoting recombination from homologous chromosomes. To ensure interhomolog recombination occurs, a barrier to sister chromatid repair (BSCR) exists, which prevents meiotic DSB repair from a sister chromatid. The BSCR is at least partly dependent upon the kinase activity of Mek1 in association with the SC protein Hop1 and Red1 (Niu et al., 2007; Niu et al., 2005; Wan et al., 2004). Mek1-analog sensitive mutant cells grown in the presence of an inhibitor that specifically inactivates Mek1's kinase activity (1-NA-PP1) are able to overcome *dmc1*-induced pachytene arrest by repairing DSBs through an intersister pathway (Niu et al., 2005; Wan et al., 2004). This suggests that Mek1 kinase activity is one of the components of the BSCR. However, an alternative explanation is that Mek1 is required for assembly of the SC which could promote homologous recombination.

The function of Mek1 in influencing partner choice requires a specific residue in the C –terminal domain (referred to as the C domain by Hollingsworth) of Hop1 (Lysine 593), which appears to promote dimerization of Mek1. *hop1-K593 dmc1 MEK1* diploids bypass the Pachytene checkpoint and are unable to prevent intersister repair, resulting in inviable spores due to non-disjunction. In cells where Mek1 is able to dimerize via another route (*hop1-K593A dmc1 GST-MEK1*, where Mek1 dimerizes because of the GST moiety), the *dmc1*-induced arrest is maintained (Niu et al., 2005). These data indicate that part of the BSCR is due to Hop1-promoted dimerization of Mek1. Hop1 is phosphorylated by Mec1 and Tel1 during prophase I, and this phosphorylation is also required for the BSCR (Carballo et al., 2008). Whether this Hop1 phosphorylation is also linked to Mek1-dimerization is unknown.

Target of the RIS

The RIS creates a delay in the start of the first division of meiosis. *NDT80* seemed like a potential target for the RIS because it is the transcription factor that upregulates genes necessary for the first division of meiosis. *ndt80* null mutants arrest in pachytene as mononucleate cells (Hepworth et al., 1998; Xu et al., 1995). Furthermore, Ndt80 is capable of responding to signaling from the Pachytene checkpoint. *dmc1* mutant cells arrest in pachytene and do not accumulate phosphorylated Ndt80 in their nuclei (Tung et al., 2000). This arrest can be bypassed by disabling members of the Pachytene checkpoint such as *RAD24*. Furthermore, overexpressing *NDT80* in a *dmc1* cell can restore transcription of middle meiotic genes (Pak and Segall, 2002b). While the Pachytene checkpoint is not activated during a normal meiosis (unlike the RIS) and it involves different signaling transducers than the RIS, these findings do illustrate that *NDT80* expression is malleable in response to checkpoint stimuli and is therefore an excellent candidate for a target of the RIS.

Description of thesis

I present work in this PhD thesis which furthers the understanding of the coordination of the initiation of meiotic recombination and the reductional division. The presence of a subset of recombination initiation proteins sends a signal that delays the start of MI in wild type cells. I present experiments demonstrating the first division kinetics of mutants of the two remaining recombination initiation genes that our lab had not yet studied. Rec107 is part of the RIS, while Ski8 is not. Because Ski8 is required for the initiation of recombination, this lends further support to our hypothesis that DSBs are not the signal that delays MI in a wild type cell.

Because the initiation of recombination and the reductional division are two major events in meiosis, it is reasonable to propose that the coordination of these events is conserved in other organisms. To begin the study of this question, we examined whether the RIS is conserved between the strain background that we use (S288C) and a very divergent strain background (SK1). The history and evolutionary relationships of these two strains are discussed further in the introduction of Chapter 2. We found that the RIS is conserved in SK1 strains. *rec102* and *rec104* SK1 strains both start the first division earlier that wild type SK1 strains.

As discussed above, the degradation of cohesins is necessary for the division of homologous chromosomes during a normal meiosis. Securin (Pds1 in *S. cerevisiae*) must be degraded in order for cohesins to degrade. In Chapter 3, I show that *rec104* mutants can perform the reductional division without first degrading Pds1 suggesting that the RIS acts independently of the pathway that controls securin (PDS1) degradation.

.The work in this thesis expands our knowledge of the mechanism by which the RIS delays the reductional division. Specifically, my goal was to determine how the RIS was transduced and what the target of the RIS is (Fig. 1-8). In Chapter 4 I present data showing that the RIS delays transcription of the regulator of middle meiotic gene expression, *NDT80*. *NDT80* transcription is earlier in the RIS mutants *rec102* and

rec104. I observed that ski8 mutants do not transcribe *NDT80* early, supporting my conclusion that DSBs are not the signal that delays the reductional division in wild type cells. Lastly, I demonstrate that expression of *NDT80* is both necessary and sufficient to determine the timing of the reductional division.

To study the transduction of the RIS, we used a candidate gene approach to evaluate members of the spindle, DNA damage and S phase checkpoints as candidates for transducing the RIS (Chapter 5). Our experiments excluded members from all three of these checkpoints in transducing the RIS. I establish the meiosis-specific kinase Mek1 as a candidate for relaying the RIS.

CHAPTER 2

THE RECOMBINATION INITIATION SIGNAL

Abstract

The initiation of recombination and the first division are two major events in meiosis. Ten genes are essential for the initiation of meiotic recombination in *S.cerevisiae*. Our lab has previously demonstrated that a subset of recombination initiation mutants start the reductional division early. These observations have led us to hypothesize that a Recombination Initiation Signal (RIS) consisting of a subset of these proteins delays the start of MI in wild type cells. In this chapter I present experiments demonstrating the MI timing of remaining two mutants that our lab had not yet tested. The results show that *rec107* strains start MI early, indicating that *REC107* is a part of the RIS. *ski8* strains start the reductional division at the same time as wild type, however, indicating that *SKI8*, while essential for recombination, is not a part of the RIS. Lastly, I show experiments demonstrating that the RIS is conserved in the divergent *S. cerevisiae* strain background SK1.

Introduction

Meiotic recombination requires many genes

Double stranded breaks (DSBs) initiate meiotic recombination in *Saccharomyces cerevisiae* (Borde, 2007; Keeney, 2000). Two general classes of genes are required for meiotic recombination: early recombination genes (early exchange or EE) act to make the DSBs that initiate homologous recombination, while late recombination genes (late exchange or LE) act after the breaks are formed to process and repair the breaks (Engebrecht and Roeder, 1989; Malone and Esposito, 1981). A third class of genes, the early synapsis (ES), is required for full levels of DSB formation.

Early exchange genes

The EE genes in *S. cerevisiae* include ten genes that are required to make meiotic DSBs; four of these genes also play a role in mitotic processes in the cell. The remaining six recombination initiation genes are meiosis specific for function (an exception is *REC107* which is transcribed in mitotic cells, but only translated during sporulation (Li et al., 2006b)). Mutations in any one of the ten recombination initiation genes confer a similar phenotype: elimination of meiotic recombination, genetic epistasis to mutations in genes required later in the meiotic recombination process, incomplete synaptonemal complex formation, a reduction in sporulation and inviable spore production (Malone et al., 1991). In other words, all ten of these genes are required for the initiation of meiotic recombination. There is significant evidence that these ten proteins form a complex (Chapter 1). *SPO11* encodes a homolog of the A subunit of archaebacterial type II topoisomerase (topoisomerase VI) and has been proposed to catalyze DSB formation (Bergerat et al., 1997; Keeney et al., 1997). Each of these ten genes will be discussed below. *MRE11, XRS2* and *RAD50* are genes that encode the proteins that make up the MRX complex (Borde, 2007). This complex has a dual role in both the initiation of recombination and in later recombination events (see below). In addition to its role in meiotic recombination, the MRX complex is required for DNA repair, telomere maintenance and non-homologous end joining (NHEJ). The EE gene *SKI8* is also important during vegetative growth and in degradation of non-polyadenylated mRNAs (Searfoss and Wickner, 2000). *REC102, REC114, MEI4* and *REC107* are expressed only during meiosis. These five genes have no clear homologs outside of fungi (R. Malone, personal comm.). A detailed description of the ten EE genes that initiate meiotic recombination was presented in Chapter 1.

Late exchange genes

The late exchange (LE) genes are necessary for processing DSBs through a number of steps to form recombinants. After removal of the Spo11-oligonucluotide complexes by the MRX complex, 5' ends are partially digested by MRX and Sae2 (Moreau et al., 2001). Exo1 nuclease and/or Sgs1 helicase digest the DNA further, to produce the long stretches of single-stranded DNA required for strand invasion (Mimitou and Symington, 2008; Moreau et al., 2001; Tsubouchi and Ogawa, 2000). The single stranded 3' ends that result become coated with replication protein A (RPA is a homolog of the bacterial single-stranded DNA binding protein (SSB); (Wold, 1997)). RPA subsequently becomes replaced with strand transfer proteins. These include homologs of the bacterial RecA strand-exchange protein, Rad51 and Dmc1 (Sheridan and Bishop, 2006). Dmc1 is a meiosis-specific protein, while Rad51 functions in repair of both mitotic and meiotic DSBs (Bishop et al., 1992; Shinohara et al., 1997a; Shinohara et al., 1992). In mitosis, Rad51 acts through a mechanism that uses the sister chromatid as a template for repair (Kadyk and Hartwell, 1992). While both Rad51 and Dmc1 strand-transfer proteins are required for normal recombination of meiotic DSBs, they associate with different cofactors and have some non-overlapping functions (Dresser et al., 1997; Shinohara et al., 1997a). It has been proposed that the repair of meiotic DSBs can follow two different pathways: a Rad51-only repair pathway or a Dmc1-dependent pathway (Dresser et al., 1997; Tsubouchi and Roeder, 2003). Rad51 associates with Rad52, Rad55, Rad57, and Rad54 (Sung et al., 2000), while Dmc1 associates with Mei5, Sae3, Hop2, Mnd1, and Rdh54 (Chen et al., 2004; Ferrari et al., 2009; Henry et al., 2006; Shinohara et al., 1997b; Zierhut et al., 2004). When any of the above mentioned proteins are absent, the efficiency of recombination is moderately to severely reduced (Bishop et al., 1992; Paques and Haber, 1999; Shinohara et al., 1997a; Soustelle et al., 2002).

Early synapsis genes

An additional class of genes, called early synapsis (ES) genes, is necessary for axial element and early synaptonemal complex formation as well as contributing to recombination initiation (Mao-Draayer et al., 1996). Hop1 and Red1 are necessary for wild type levels of homologous recombination (Hollingsworth and Byers, 1989; Mao-Draayer et al., 1996; Rockmill and Roeder, 1990; Zickler and Kleckner, 1999). Therefore, Hop1 and Red1 are not absolutely required for DSB formation, although they do play some sort of role since levels of DSB formation are reduced 2-100-fold, depending on the locus or if recombination genes are absent. Our lab has shown a 20-fold reduction in DSB formation at the *HIS2* hotspot in *hop1* and *red1* mutants (Mao-Draayer et al., 1996), while Kleckner's lab has shown a 4-fold reduction in DSB formation at the *his4::LEU2* hotspot in *red1* mutants (Mao-Draayer et al., 1996). This demonstrates that there is likely some variability in the necessity of the SC to promote recombination in different loci.

The Recombination Initiation Signal

Members of the Malone lab have shown that several recombination initiation mutants start the reductional division earlier than wild type strains. *rec102, rec104, rec114, red50, xrs2, mre11 and spo11* deletion mutants start the reductional division earlier than wild type strains [see Chapter 1 and (Galbraith et al. 1997, Jiao et al., 1999, Malone et al., 2003). However, *mei4* mutants, which, like the aforementioned mutants, do not initiate DSBs, do not start the reductional division earlier than wild type. These results have led us to hypothesize there is a Recombination Initiation Signal (RIS) which delays the start of the reductional division.

Two synaptonemal complex genes have also been shown to be important in the timing of the reductional division. Lindsay Carpp showed that *hop1* and *red1* mutants start the first division of meiosis earlier than wild type, similar to a *rec104* mutant

(Malone et al., 2004). It is unclear whether Hop1 and Red1 are a part of the RIS or whether they are downstream sensors of the RIS. This idea will be explored in the final chapter.

One alternate explanation for the earlier first division observed in some recombination initiation mutants is that pre-meiotic DNA synthesis is shortened in these mutants. Cha *et al*. (2000) proposed that initiation mutants with an earlier MI are the result of cells having a shorter pre-meiotic S phase. They reported that *spo11* mutants in the SK1 strain background have a shorter duration of pre-meiotic replication and proposed that this was the reason that *spo11* mutants started the first division of meiosis early. Curiously, in the same paper, they report that pre-meiotic S phase in *rec102* cells is indistinguishable from wild type. This is perplexing because Spo11 and Rec102 are thought to be present in the same sub-complex of the putative recombination initiation complex (see Chapter 1). Stuart Haring, a graduate student from our lab, used the same approach as Cha et al. (2000) to test this hypothesis in our S288C-derived strain background and found that the duration, time of entry and time of 50% of cells completing S phase in *rec102*, *rec104*, *rec114*, *spo11 rad50* or *ski8* mutants was not significantly different from wild type (Malone et al., 2004). Furthermore, Haring calculated that S phase was 51-59 minutes for all strains tested, including wild type; these values are similar to the 59 minutes reported for *spo11* SK1 strains by Cha *et al.* (2000). This supports the conclusion that the length of S phase is not affected by recombination initiation mutants and does not support the hypothesis of Cha *et al.* (2000).

In this chapter, I present data showing the timing of the reductional division in two recombination initiation mutants, *rec107* and *ski8*. I show that *REC107* strains start MI early, indicating that Rec107 is a part of the RIS. The reductional division starts at the same time as in wild type in *ski8* strains, thus Ski8 is not a part of the RIS.

Is the RIS conserved in other strain backgrounds?

In contrast to research done with other model systems, (e.g., *Caenorhabditis elegans*), there are several consensus wild type strain backgrounds in *Saccharomyces cerevisiae*. The majority of *Saccharomyces* genetics labs use S288C as a strain background and it was the strain chosen for the *Saccharomyces* Genome Database (http://wiki.yeastgenome.org/index.php/Commonly_used_strains). Some fields of study in yeast genetics require an alternative strain background that has additional characteristics desirable for that field. For example, the sigma 1278Bstrain has been used to study filamentous growth in *Saccharomyces*. In contrast to other areas of investigation, a significant number of yeast meiosis researchers use the strain background SK1 as an alternative to S288C, though S288C derivatives are used by many labs, including the Malone lab. S288C was used predominantly prior to 1992 (R. Malone, personal comm.).

S288C was developed by R. Mortimer to study auxotrophies (Mortimer and Johnston, 1986). It is 88% congenic with the strain EM93 isolated by Emil Mrak in 1938 at UC Davis. The rest of its genetic background is comprised of the strains EM126 (also isolated by E. Mrak), NRLL YB-210 (isolated from Costa Rican bananas) and the three commercial baking strains Yeast Foam, FLD and LK. Many of these strains were first used by the pioneering yeast researcher Carl Lindegren in the 1940's (Lindegren and Lindegren, 1943a, b). S288C is a non-flocculent (non-clumpy) strain that disperses well in liquid culture and requires only a nitrogen source, glucose, a few salts and trace elements and biotin to grow (Beam et al., 1954; Mortimer, 1955; Mortimer and Johnston, 1986; Mortimer and Tobias, 1953). Additionally, S288C contains no amino acid or base auxotrophies.

In my research in the Malone lab, I have used the homothallic strain background K65-3D exclusively for my work. K65-3D is primarily derived from S288C and was created by crosses to have markers useful in heteroallelic recombination studies, is

homothallic and has a high sporulation rate and spore viability (Malone et al., 1991). Sporulation levels are typically 60-80% in K65-3D. In labs where meiosis is not the focus, S288C derivatives can accumulate mutations that reduce sporulation rates to <10%.

The origins of SK1 are somewhat unclear. Nancy Kleckner popularized the use of this strain for meiotic recombination studies in the late 1980's. She does not give a description of the strain background that she uses; rather she writes that the yeast strain NKY274 (*Mat α, ho::LYS2, ura3, lys2*) was "derived from crosses made from the strain SK1" from Kane and Roth, 1974 (Alani et al., 1987). Kane and Roth did indeed use a strain called SK-1 in their studies of sporulation rates of different strains in different types of media. The SK-1 strain background can be traced back to the strain Z113 which Roth used in experiments in 1969 and 1970 (Roth and Halvorsen 1969; Roth, 1970). The origins of strain Z113 are unclear. Several apparent modifications have been made from the original SK-1 strain. For example, the original strain had no auxotrophies, but the modern SK1 strains in use have several available markers.

Kane and Roth (1974) report that the SK-1 strain has a rapid synchronous sporulation. This rapid and synchronous sporulation is the primary benefit to using SK1 in studying meiosis (Alani et al., 1990, Cao et al., 1990, Padmore et al., 1991) The sporulation levels of SK1 are typically ~90% and it has high spore viability (>95%) (Argueso et al., 2004; Shinohara et al., 1997). The main disadvantage of using SK1 as a strain background is that it is flocculent (clumpy) making it difficult to sample and count under the microscope from liquid culture without vigorous sonication first. Additionally, some have reported that SK1 strains can spontaneously sporulate in rich media suggesting that glucose repression may not be complete (Keeney and Kleckner, 1995, Roth, 1970; Roth and Halvorsen, 1969). Consistent with this, Doug Pittman and Kai Jiao, two graduate students from our lab, have reported seeing spores in YPD-grown SK1 strains (R. Malone, personal comm.).

Recently, a phylogenetic analysis constructed using single-nucleotide polymorphism analysis of 147 loci in 32 different strains of the genus *Saccharomyces* strains ,including several common laboratory strains, was performed (Ben-Ari et al., 2005). Ben-Ari et al. (2005) found that SK1 and S288C strains are quite divergent; S288C differed from all of the other laboratory strains at only 3-17% of the loci studied while SK1 differed from all the other laboratory strains at 83-97% of the loci studied. An earlier phylogenetic analysis done by the Hartl lab using high-density oligonucleotide arrays revealed 11,115 single feature polymorphisms in 14 different *S. cerevisiae* strain backgrounds (Winzeler et al., 2003). These experiments examined single base changes between two sequences 25 base pairs in length. If there were base changes in the 25 bp sequence there would be a decrease in hybridization when it is used as a probe, allowing the genomic DNA hybridization patterns of two different strain backgrounds to be compared. Their analysis allowed them to conclude that SK1 is only distantly related to S288C, even though it is generally considered to be *S. cerevisiae* (Winzeler et al., 2003).

The experiments presented in this thesis test the hypothesis that the RIS is not unique to the S288C background of *S. cerevisiae*. Differences have been reported in meiotic phenotypes among different strain backgrounds. For example, Shirleen Roeder's laboratory analyzed the kinetics of meiosis in mutants overexpressing *NDT80* in wild type and in *zip1* backgrounds in BR and SK1 strains (Tung et al., 2000). Zip1 proteins form the central elements of the synaptonemal complex (Zickler and Kleckner, 1998). In the BR2459 strains, sporulation was abolished and cells arrested as mononucleates. This arrest is dependent on members of the Pachytene checkpoint including *MEK1*, implying that the Pachytene checkpoint is important for monitoring the state of the SC in BR strains (Bailis and Roeder, 2000). In contrast to BR strains, in SK1 *zip1* strains, sporulation was only delayed and reduced to 60%. Our laboratory recently investigated the timing of a *zip1* mutant in our S288C (K65-3D) background. Sarah Nord Zanders, an Honors undergraduate, found that the timing of the first division in our strain background

is intermediate between the kinetics displayed by SK1 and BR (S. Nord Zanders, Honors thesis). Zanders found that the timing of the first division in cells that actually do divide is delayed by two hours or more compared to wild type K65-3D. At 30 hours only 5% of *zip1* cells completed both divisions compared to 85-95% for wild type.

The *zip1* arrest in BR strains prevents accumulation and phosphorylation of Ndt80 in the nucleus. To see if this arrest could be overcome by overexpression of *NDT80*, Tung *et al*. (2000) used a high copy vector with *NDT80* expressed from its native promoter in both BR and SK1 strain backgrounds. The overproduction of Ndt80 improves sporulation in a *zip1* BR mutant although it is not as high as wild type. In the SK1 strain background the overexpression of *NDT80* causes nuclear division and spore formation to occur earlier than in the *zip1* mutant without *NDT80* overexpression (Tung et al., 2000). In the case of the Ndt80 overexpression in the *zip1* mutant, both the SK1 and BR strain backgrounds have an earlier reductional division.

If the recombination initiation signal that we have identified in our strain background were an important feature of meiosis, it should be present in all *S. cerevisiae* strain backgrounds. In fact, it should be present in all *Saccharomyces* species. We hypothesize that although SK1 and S288C are distantly *S. cerevisiae* strains, they will both show an early first division in a *rec102* and *rec104* mutant, thus confirming that the RIS is present in both strain backgrounds.

In this chapter I present results showing the timing of the reductional division in SK1 *rec102* and *rec104* strains. The reductional division starts earlier in *rec102* and *rec104* strains compared to wild type SK1 strains. This implies that the RIS is conserved in a strain that is evolutionarily divergent from S288C leading us to speculate the RIS may even be conserved in other species.

[Morgan Pansegrau, an Honor's undergraduate performed the timepoint analysis of *rec107*. I made the strains for this experiment. I performed the entire *ski8* experiment.

The SK1 experiments were a collaborative effort between Rachel Gast, a Masters student, and I. Gast and I each performed one trial of the timepoint experiments.]

Materials and methods

Yeast strains used

The S288C yeast strains used for the experiments described in this chapter are derived from the homothallic diploid K65-3D (Malone and Esposito, 1981). K65-3D is homozygous for the following markers: *HO, lys2-1, tyr1-1, his7-2, can1*^{r}*, ura3-13, ade5, met13-d, trp5-2, leu1-12, ade2-1*. Complete descriptions of all strains are detailed in the Appendix.The *rec104* deletion strain used in this chapter is *rec104-Δ1* (Galbraith and Malone, 1992). The *rec104-Δ1* is a deletion of the entire coding region of *REC104.* The *rec102* and *ski8* mutant strains are deletion strains containing an insertion of the G418^{r} gene. These strains are precise deletions of the entire coding region of these genes and were obtained from the Research Genetics deletion collection (Brachmann et al., 1998). The proper designation of G418^{r}-deleted *ski8* mutant strains is *ski8Δ::G418*^{r}, but I will refer to this strain and others like it as *ski8* in this chapter. The deletions are described by the *Saccharomyces* Genome Deletion Project (http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.htmL). All deletion and deletion/insertion mutations were tested by both genetic and Southern analysis using at least two restriction enzymes that would give different sizes and number of bands in the deletion and wild type copy of the gene (Southern, 1975). *G418*^{r} -deleted strains were selected using YPD medium (2% dextrose, 1% yeast extract, 2% bacto-peptone, 1.8 % agar) containing 200mg/L of G418, an antibiotic used in section of *G418*^{r} mutants.

All of the K65-3D-derived strains used in this chapter are homozygous diploids created by sporulating and dissecting a heterozygous strain and then selecting segregants containing the desired mutations. Strains containing a *G418*^{r} insertion could be selected

by growth on rich medium containing 200mg/L of G418. The *rec104-Δ1* mutation was detected in strains by using PCR with the primers "*rec104* colony F (5'GAGCTGTTCGGGTATTGCGT3') and "*rec104* colony R" (5'GAAAGTATCAGTTCTATGGACAGTTC3').

SK1 haploid isogenic strains (containing either *rec102Δ::URA3* or *rec104Δ-1* mutations) were provided by Scott Keeney and contained the following mutations: *ho::LYS2, lys2, ura3, leu2::hisG*. To create Rec- homozygous strains, the appropriate *MAT a* and *MAT A* haploids were crossed. To create a wild type SK1 strain *rec102 MAT α* and *rec104 MAT A* strains were mated creating heterozygous diploids that were dissected to obtain Rec^+ haploid segregants. *MAT α* and *MAT A* Rec^+ haploids were crossed to obtain the Rec^+ diploid.

Media and growth conditions

YPD is a rich medium containing glucose as a carbon source (2% dextrose, 1% yeast extract, 2% bacto-peptone, 1.8% agar). Auxotrophies were tested on synthetic complete (SC) drop-out media containing all amino acid and bases necessary for growth *except* for the one being tested (e.g. SC-ura is synthetic complete without uracil). Strains were sporulated on SpoIII-21 medium containing 2% potassium acetate, 0.1 % glucose, 0.25% yeast extract, 1.8% agar and 0.34 g/L of SS21, pH 6.8. SS21 is a complete amino acid and base mixture, which was developed in the Malone lab in 1980 (15 g each of the following: adenine sulfate, histidine, leucine, lysine, methionine, threonine, tryptophan, tyrosine and uracil and 2 g of p-aminobenzoic acid. The minimal amount of glucose in SpoIII-21 plates allows for cells not in G1 to finish the cell cycle and reach G1 for entry into sporulation, however, is not high enough to inhibit sporulation. Strains were sporulated on plates at 30° C for 4-6 days.

Liquid sporulation and timepoints

To obtain sporulated cells, a 10 mL pre-culture of cells grown in liquid YPD medium was inoculated using a fresh colony and grown for 10-12 hours at 30°C in a water bath shaker set at 175rpm. These cells were used to inoculate 250 or 500 mL cultures of YPA pre-sporulation medium (1% potassium acetate, 2% bacto-peptone, 1% yeast extract) 1L or 2.8L Fernbach flasks, respectively. Cultures were grown for 7-12 hours to a concentration of 2 x 10^7 cells/mL, as determined by counting on a hemicytometer. The doubling time of 135 minutes was used for *ski8* strains while a doubling time of 120 minutes was used for all other strains used in this chapter. Cells from the YPA cultures were harvested by centrifuging for 5 minutes at 5000 rpm in a Sorvall RC-5B centrifuge then washed 1x with water and centrifuged again. These cells were then resuspended in half the original volume of Spo II-21 sporulation medium, a completely defined sporulation medium where little vegetative growth occurs (2% potassium acetate, 0.34 g/L supplement 21 [SS21] pH 6.8). Either 1 liter or 2.8 L Fernbach flasks were used, depending on the amount of culture being sporulated. Cultures were grown in a 30º C airshaker at 195 rpm and cells were removed at appropriate timepoints. Timepoints were typically started at 3-4 hours and at sixteen hours. A late (approximately 30h timepoint was taken for counting final sporulation values for the experiment.

It has been reported that SK1 strains have a tendency to sporulate in rich medium and in the pre-sporulation medium, YPA (Keeney and Kleckner, 1995, Roth, 1970; Roth and Halvorsen, 1969). In order to limit the amount of sporulation occurring prior to transfer to the sporulation medium, the SK1 cells were only allowed to grow for 2 generations in YPA. The protocol was performed as described for K65-3D-derivatives, except that cells were inoculated into the YPA pre-sporulation medium at a concentration of 0.5 x 10^7 cells/mL and grown to a concentration of 2 x 10^7 cells/mL. Aliquots taken from SK1 strains had to be sonicated before concentrations could be determined by

hemacytometer. Aliquots of the YPD or YPA cultures were first diluted 10X in PO_4 buffer then they were sonicated three times for a 10 second pulse each time. Between each sonication period there was a 5 second break; a 10 µL aliquot could then be pipetted onto a hemacytometer to determine concentration. Formaldehyde treated samples were sonicated as described before slides were prepared for DAPI analysis.

Microscopy

The method by which we monitor the timing of the first meiotic division is by direct fluorescence microscopic examination of the DNA in nuclei using 4', 6-diamidino-2-phenylindole (DAPI). Even in Rec^- mutants, the reductional division is followed in almost all cells by a second division. We only count a cell as binucleate when there are two distinguishable masses of DNA, indicating that MI has occurred. The presence of four DNA masses indicates that MII has occurred. The only way this division of DAPI-staining can happen is if the chromosomes have segregated from each other. To perform DAPI-staining, 1 mL of formaldehyde-fixed cells were centrifuged for 30s in at top speed in a microfuge at room temperature and resuspended in 1 mL of 50% ethanol for at least 5 minutes. The remainder of the steps was performed in dim light using only a red safety light or by just barely opening the door to the microscope room. The samples were centrifuged again for 30s and resuspended in 0.1 mL of a 1mg/L DAPI solution for 10 minutes, washed once with water and resuspended in 10 µL of mounting medium (55% glycerol, 0.001g/ mL 1,4 phenyline diamine in 8.4 % sodium bicarbonate buffer, pH 9.0. The DAPI solution was diluted from a 1mg/mL stock solution that was stable, if stored at 4˚C in the dark indefinitely, but mounting medium is only stable for 4 weeks when stored in a similar manner. 3 µL of cell suspension was placed on a slide, an 18 x 18 mm^2 #1 coverslip was placed over it and the slide was pressed very firmLy on the bench using my thumbs and considerable force to ensure that the stained cells were a monolayer on the slide. The edges of the coverslip were sealed with Sally Hanson's Ultimate shield or

Double Duty clear nail polish. The slides were examined using a Leitz Laborlux 12 microscope equipped with a fluorescence filter for DAPI (excitation = 344 nm; emission = 466 nm). Cells were counted visually and were categorized according to whether they contained one nucleus (mononucleate cells which have not undergone the first division), two nuclei (binucleate cells that had completed the reductional division), or four nuclei (tetranucleate cells that had completed both the reductional and equational division). The final sporulation values for each experiment were counted from a late (~30 hour) timepoint.

Results

Rec107 is a component of the RIS

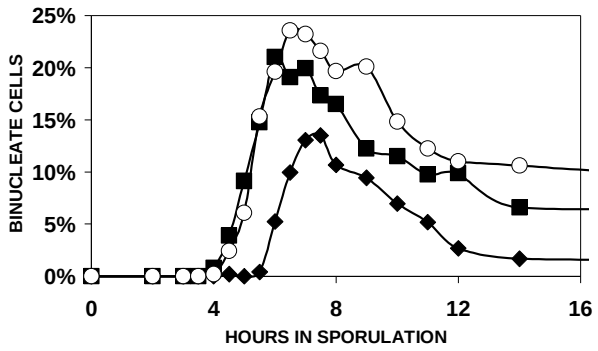
Cells which have segregated their chromosomes into two separated and distinguishable nuclei are defined as having undergone the first meiotic division. Recombination initiation genes previously shown to be required for the normal delay of the first division include *REC102, REC104, REC114*, and *RAD50, SPO11, MRE11 and XRS.* (See introduction to this chapter and Chapter 1.) The synaptonemal complex genes *HOP1* and *RED1* have also been shown to be required for this normal delay. The data in Fig. 2-1A indicate that Rec107 is also required for the delay; a mutation in this gene confers an earlier reductional division similar to *a rec104* mutant. *rec107* start the reductional division ~1.5 hours earlier than wild type. The second division in *rec107* cells starts at nearly the same time as wild type, or at most 30 minutes earlier than wild type (Fig. 2-1B). This is consistent with the equational division phenotype observed in other RIS mutants.

Ski8 is not a component of the RIS

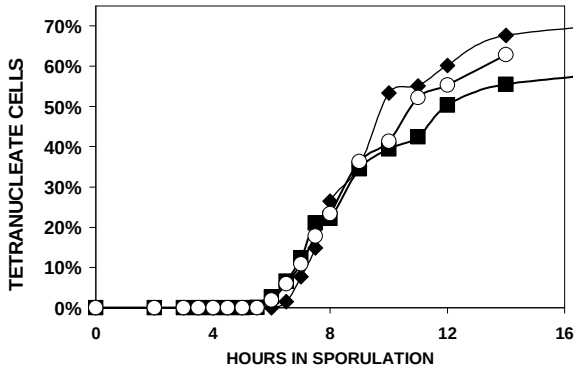
In contrast to our observations of *rec107* strains, I did not observe an early first division in *Ski8* mutants. A strain lacking *SKI8* began the MI division at the a time

Figure 2-1: Timing of the meiotic divisions in a *rec107* mutant. In all experiments WT cells are indicated by solid diamonds (◆) and *rec104* strains are shown as solid squares (■). In panels A-D the *rec107* mutant is shown as open circles (○). The data for *rec107* is the average of two cultures. Wild type and *rec104* contain data from one culture. The average sporulation values in the *rec107* experiments were: WT, 78%; *rec104*, 26%; *rec107*, 28%. Timing of the first division is indicated by the appearance of binucleate cells. MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells. [A] Timing of the reductional division in a *rec107* mutant. [B] Timing of the equational division in a *rec107* mutant. One of two representative experiments is shown. A second experiment was performed showing similar results, but is not shown.

A. First Division in *rec107*



B. Second Division in *rec107*



initiation signal (Fig. 2-2A and C). It should be noted the height of the first division curve representing the maximal percentage of binucleate cells is still elevated in a *ski8* mutant, similar to the height of the MI curve seen in a *rec104* mutant even though a *rec104* mutant starts MI earlier than *ski8* (Fig. 2-2 A and C). Sporulation levels in *ski8* mutants were reduced compared to wild type (20% vs.75%), typical of other recombination initiation mutants (Galbraith et al., 1997; Jiao et al., 1999; Malone et al., 2004).

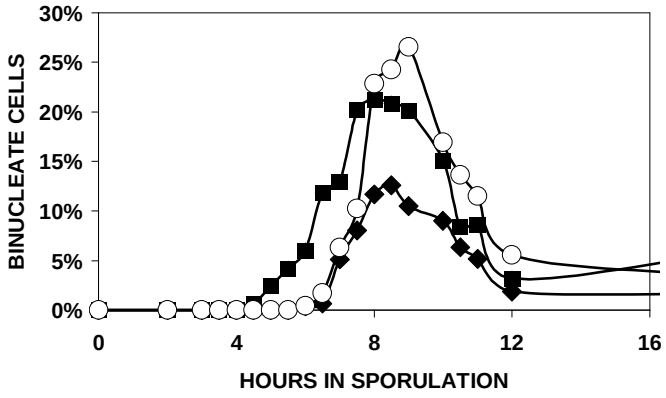
SK1 *rec102* and *rec104* strains start MI earlier than wild type

Thus far, all of the experiments conducted in our laboratory examining the effects of a recombination initiation mutation on the timing of the start of the first division have been performed in the S288C-derived K65-3D strain background. Strains of the SK1 background are also frequently used in meiotic experiments because they are purported to go through meiosis more rapidly and synchronously than standard yeast strain backgrounds (Bailis et al., 2000; Kane and Roth, 1974; Storlazzi et al., 1996; Tung et al., 2000). If the recombination initiation signal were an important feature of meiosis, it should be present in all *S. cerevisiae* strain backgrounds, even one as divergent as SK1. Cha, *et al*., (2000) and Kee and Keeney (2002) reported that *spo11* mutants start the first division in an SK1 background earlier than SK1 wild type strains, though it was unknown whether this would be true for other mutants of the RIS in the SK1 background.

To test whether other initiation mutants also conferred an earlier start to the first division in SK1 strains, Rachel Gast and I examined the first meiotic division in SK1 wild type, *rec102* and *rec104* mutants. We included a Rec^{+} wild type from our K65-3D strain background as a control in these experiments. The data in Fig. 2-3A illustrate that the reductional division begins in the wild type SK1 and our wild type strain at about the

Figure 2-2: Timing of the meiotic divisions in a *ski8* mutant. In all experiments WT cells are indicated by solid diamonds (◆) and *rec104* strains are shown as solid squares (■). The *ski8* mutant is shown as open circles (○). . Two independent experiments were performed. [C] and [D] are same as [A] and [B], but an independent experiment. The wild type and *rec104* are data from one culture. The data for *ski8* is the average of two cultures The average sporulation values in these experiments were: WT, 75%; *rec104*, 28%; *ski8*, 20%. Timing of the first division is indicated by the appearance of binucleate cells. MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells. [A]Timing of the reductional division in a *ski8* mutant. [B] Timing of the equational division in a *ski8* mutant. Sporulation for this experiment was WT, 77%; *rec104*, 29%; *ski8*, 21%.

A. First Division in *ski8* (exp. 1)



B. Second Division in *ski8* (exp.1)

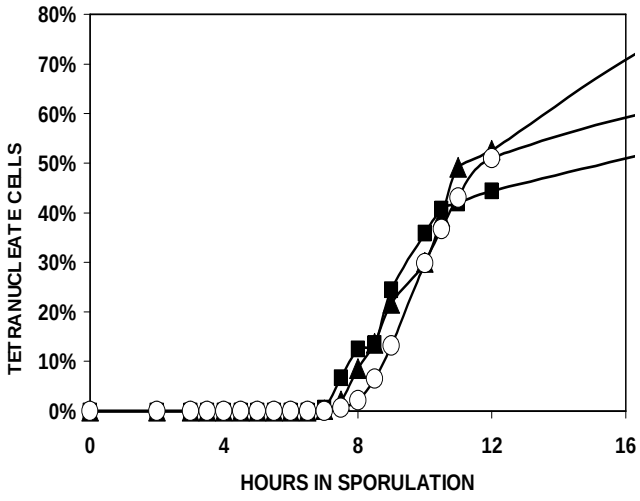
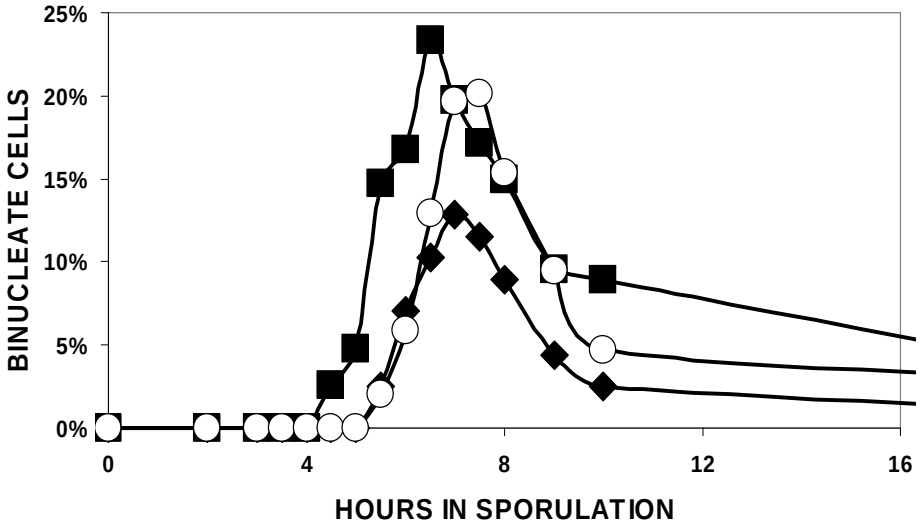
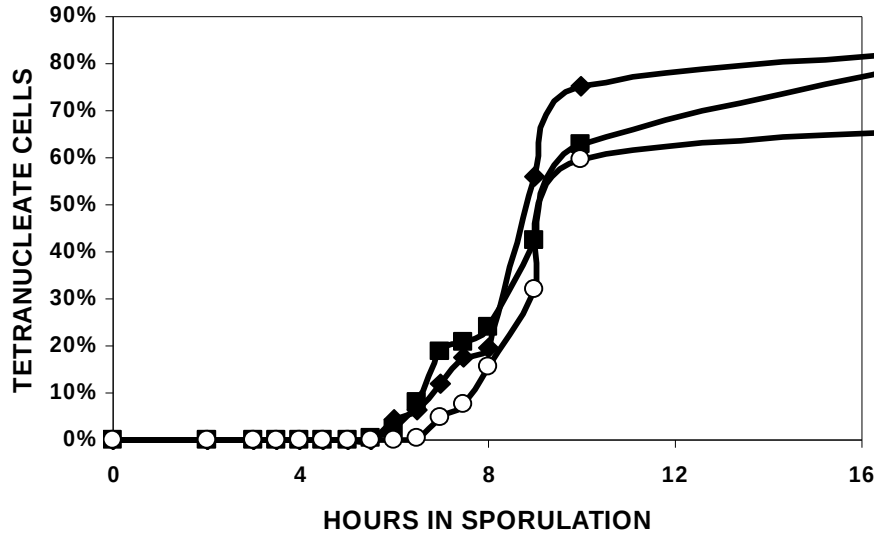


Figure 2-2, continued

C. First Division in *ski8* (exp. 2)



D. Second Division in *ski8* (exp. 2)



same time (5 h) (See discussion). Therefore, most of the rapid progression through meiosis displayed by SK1 strains relative to our strains appears to occur after the first division; this is apparent when the kinetics of the second division are examined (Fig. 2-3B). The data in Figure 2-4 also demonstrate that a *rec102* mutation in the SK1 background starts the reductional division earlier than the SK1 wild type (at 4h). The average sporulation value for the *rec102* mutation in the SK1 background was 56%. This is higher than the values seen in the *rec102* mutation our K65-3D background which average about 25%; however SK1 Rec^{+} wild type sporulation is also higher, and thus the increase in *rec102* sporulation is not unexpected (S. Keeney, personal comm. to R. Malone).

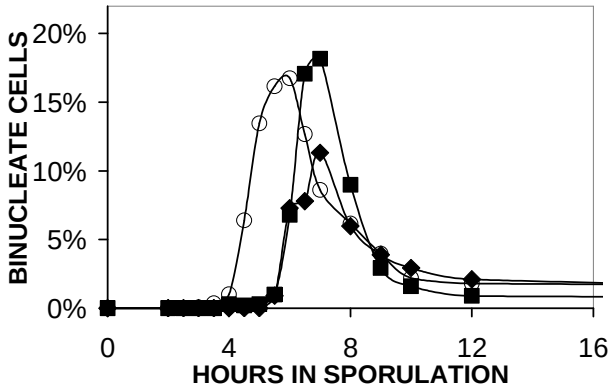
Fig. 2-4A also clearly shows that the reductional division begins at the same time (4 h) in both wild type *S. cerevisiae* strain backgrounds in *rec104* mutants. The data in Figure 2-5 demonstrate that a *rec104* mutation in the SK1 background starts the reductional division at an earlier time than either the S288C or SK1 wild type.

The result of the experiments testing *rec102* and *rec104* recombination initiation mutations in a SK1 strain background is that both begin the first division earlier than SK1 wild type. The differences between the timing of the mutants and that of wild type is comparable to that seen in S288C experiments (Malone et al., 2004).

The sporulation kinetics of an SK1 Rec+ strain and K65-3D are shown in Fig. 2-5. Mature asci start to form ~3-4 hours earlier in SK1 than in K65-3D, though final sporulation levels are similar from both strains. (77% for the K65-3D and 78% for the SK1 strain)

Figure 2-3: Timing of the divisions in a *rec102* mutant in the SK1 background. In all graphs, the divisions in the K65-3D WT are shown as solid diamonds (◆), the SK1 WT are shown as solid squares (■), the SK1 *rec102* are shown as open circles (○). For each mutant, at least two independent cultures were examined for each experiment and the data is the mean. The average sporulation values for these experiments were: K65-3D WT, 70%; SK1 WT, 92%; SK1 *rec102*, 56%. [A] Timing of the first division indicated by the appearance of binucleate cells. [B] MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells. Two other independent experiments were done for each mutant with indistinguishable results. I have not shown these data.

A. SK1 *rec102* BINUCLEATES



B. SK1 *rec102* TETRANUCLEATES

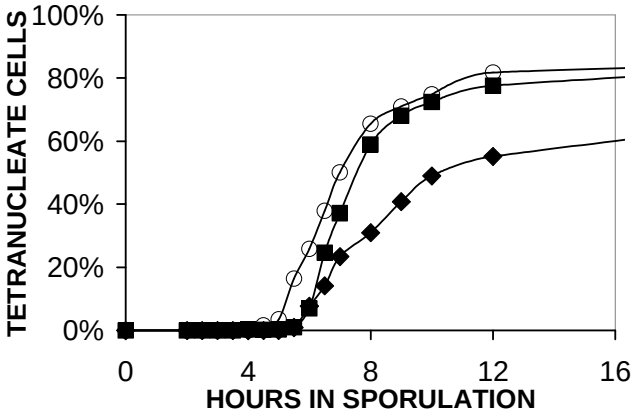
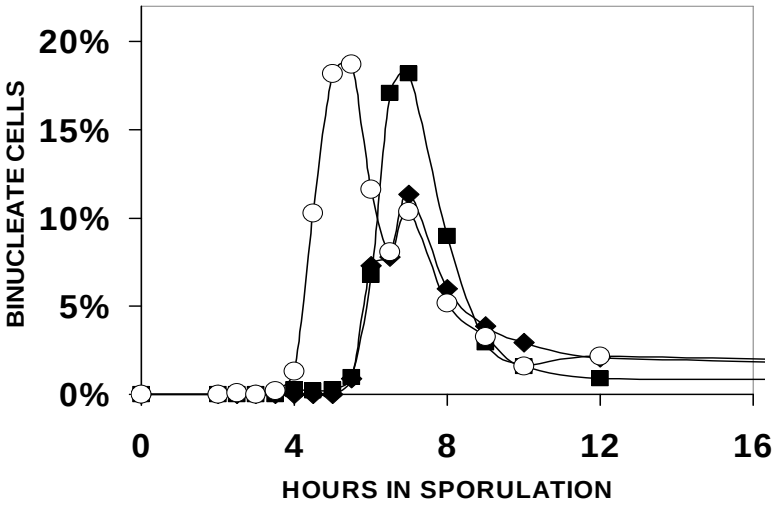


Figure 2-4: **Timing of** t**he divisions in a *rec104* mutant in the SK1 background.** In all graphs, the divisions in K65-3D WT are shown as solid diamonds (◆), the SK1 WT are shown as solid squares (■), and the SK1 *rec104* are shown as open circles (○). For each mutant, at least two independent cultures were examined for each experiment and the data is the mean. Two other independent experiments were done for each mutant with identical results. I am showin the data from one experiment. The average sporulation values for these experiments were: K65-3D WT, 70%; SK1 WT, 91.5%; SK1 *rec104*, 57%. [A] Timing of the first division indicated by the appearance of binucleate cells. [B] MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells.

A. First Division in SK1 *rec104*



B. Second Division in SK1 *rec104*

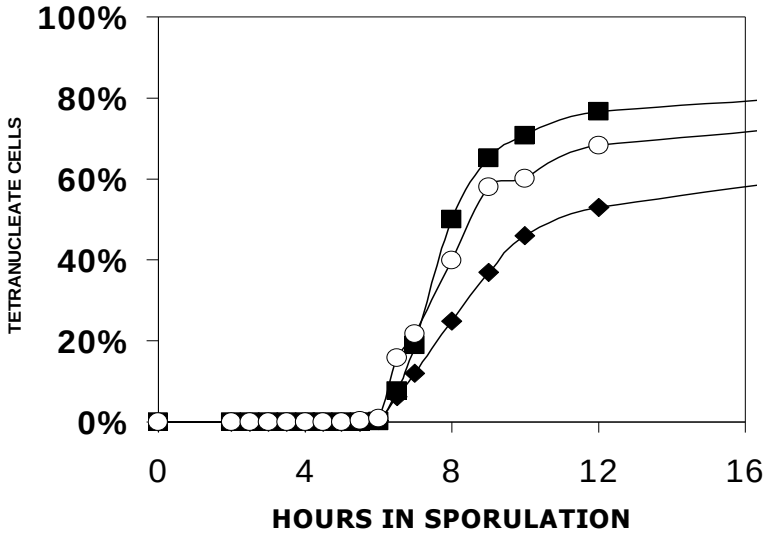
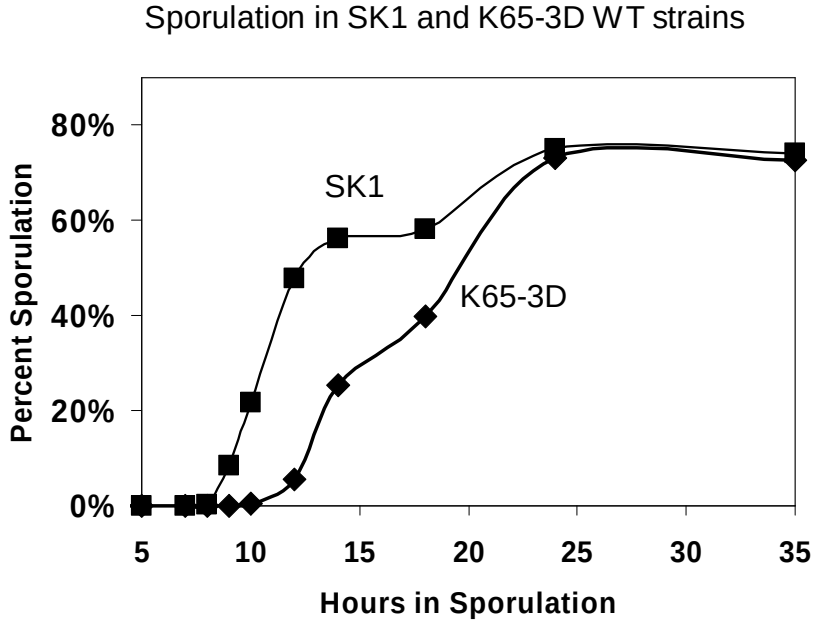


Figure 2-5: Comparing sporulation in wild type SK1 and K65-3D. K65-3D (Rec^+) shown in diamonds. SK1 wild type (Rec^+) shown in squares. Data is from one culture for each. Data from one of two experiments is shown. A second experiment was done and showed similar results. Final sporulation for this experiment were 77% for the K65-3D and 78% for the SK1 strain.


Sporulation in SK1 and K65-3D WT strains
Percent Sporulation
80%
60%
40%
20%
0%
5
10
15
20
25
30
35
Hours in Sporulation
SK1
K65-3D

Discussion

Rec107 is a member of the RIS, while Ski8 is not

There are still many questions in the field of meiotic recombination regarding how the recombination initiation proteins interact with each other and with chromatin to carry out the formation of DSBs. Given the complexity of a putative protein complex made up as many as ten proteins and interacting with chromatin in a transient manner during meiosis, this is not entirely surprising. After the process of recombination is complete, the reductional division separates homologous chromosomes. There must be communication between recombination and the first division to ensure that homologous chromosomes are not separated too early. If nuclear division were to start before recombination was complete, this would result in broken chromosomes and inviable products.

Our laboratory had previously demonstrated that seven of the eight recombination initiation proteins and two synaptonemal complex proteins that we had examined are involved in signaling to delay the reductional division (Galbraith et al., 1997; Jiao et al., 1999; Malone et al., 2004) (Malone lab, unpublished results). If any one of these seven proteins is absent, the first division starts at an earlier time. In this chapter, I present data examining the remaining two recombination initiation genes: *SKI8* and *REC107*. We found that *rec107* mutants started the first division of meiosis earlier than wild type (Fig. 2-1) (Malone et al., 2004). However, *ski8* mutants start the reductional division at the same time as wild type (Malone et al., 2004 and Fig. 2-2). We have proposed that eight of the ten recombination initiation proteins (not including Mei4 or Ski8) form a signal that indicated that initiation will occur (Galbraith et al., 1997; Jiao et al., 1999; Malone et al., 2004). We have shown that *hop1* and *red1* strains start the reductional division early (Malone et al., 2004), however, it is unknown whether Hop1 and Red1 are a part of the RIS or are downstream sensors of the RIS. This will be discussed in Chapter 6.

It should be noted the peak height of the first division curve is still elevated in a *ski8* mutant, similar to a *rec104* mutant even though a *rec104* mutant starts MI earlier than *ski8* (Fig. 2-2 A and C). One explanation for this is that *ski8* strains are perhaps impaired in their ability to exit the first division even though Ski8 is not required for the RIS. Our lab has also observed a similar increase in the maximal percentage of binucleate cells in a *mei4* strain (Galbraith et al., 1997; Jiao et al., 1999). An elevated MI peak would also be seen if *ski8* cells started the reductional division early but exited the division either later than, or at the same time as wild type cells. However this was not the case; we observed that *ski8* cells started the reductional division at the same time as wild type. Rachel Gast has shown that the spindle checkpoint is important for determining when cells exit the reductional division. She found that *mad2 rec104* mutants have an early reductional division, but did not have an elevated MI peak as seen in a *rec104* strain.

Neither Mei4 nor Ski8 are required for the recombination initiation signal, yet both of these proteins are required for the formation of DSBs. The observation that *ski8* and *mei4* mutants do not start the reductional division earlier than wild type strains indicated that DSBs are not the signal that delays the start of MI in wild type strains. Instead, we believe that the presence of a subset of recombination initiation proteins sends a signal to delay the start of MI. Furthermore, the *ski8* and *mei4* observations suggest that you do not need an entire recombination initiation complex to send this signal. One possible explanation for this could be that Ski8 and Mei4 serve structural roles in the complex that are required to position Spo11 on the DNA, but do not send the recombination initiation signal (see Chapter 1). Ski8 is a WD-repeat containing protein that is required for the formation of the Ski complex which is involved in RNA degradation during mitosis. Ski8 is required for formation of the Ski complex; however, Ski8 does not actually have any RNA metabolic domains. If Ski8 is plays a similar structural in assembling the recombination initiation proteins in meiosis, this could

explain why Ski8 is required for break formation, but not for the RIS. Keeney and colleagues have proposed that Ski8 acts as a scaffold for the assembly of Spo11-Rec102-Rec104 based on immunofluorescence experiments show that Spo11may be required to recruit Ski8 to meiotic chromatin (Arora et al., 2004; Kee et al., 2004) (Also Chapter 1). Furthermore, cell fractionation experiments show that Ski8 is required to form stable complexes of Spo11-Rec102-Rec104 on meiotic chromatin. In addition, Rec102 and Rec104 are proposed to require Ski8 for their recruitment to meiotic chromosomes (Kee et al., 2004). If Spo11 were dependent on Ski8 for proper localization and entry into the nucleus, and if Rec102 and Rec104 were dependent on Ski8 for proper chromatin association, then a *ski8* mutant should show the same early MI timing as a null *spo11, rec102,* or *rec104* mutants. Because *ski8* mutants do not start MI early like *spo11, rec102,* and *rec104* mutants, it appears that intact recombination initiation complexes are not required for the recombination initiation signal. Full protein-protein interactions as mediated by the WD motif of Ski8 are required, however, for DSB formation.

The second recombination initiation protein with normal MI kinetics is Mei4. The observation that *mei4* mutants start the first division at the same time as wild type strains also creates a paradox because Mei4 is reportedly needed to remove Spo11 after the DSBs are made (Prieler et al., 2005). If the only role of Mei4 in the initiation complex is to remove Spo11, then this would be consistent with Mei4 not being a part of the RIS. However, Mei4 is clearly required for DSB formation, implying that Mei4 is not just needed when DSB formation is over. Prieler *et al's* (2005) observation is inconsistent with the normal timing of *mei4* because *spo11* strains start the reductional division early. A further indication that Mei4 is required for more than just Spo11 removal is that Rec114 and Mei4 proteins can associate with the hotspot *YCR048w* in a *spo11* mutant suggesting that the Rec107/Rec114/Mei4 subcomplex precedes the binding of Spo11 to chromatin (Li et al., 2006b; Sasanuma et al., 2008). It is also likely that Ski8 is recruited after Mei4 because all evidence suggests that Ski8 forms a subcomplex with Spo11, as

well and is almost certainly present in a separate subcomplex from Mei4 (Arora et al., 2004; Li et al., 2006b; Sasanuma et al., 2008). If Mei4 is needed at a later time in the assembly of the recombination initiation complex and if it is required for the normal removal of Spo11 from the DNA, then what function is it playing to initiate DSBs and why does the first division begin with wild type kinetics in the absence of Mei4? The paradoxes raised by Ski8 and Mei4 will continue to problematic until more is known about the stoichiometry, timing of assembly and disassembly and specific function of each of the components the recombination initiation proteins and the recombination initiation signal. I will address this conundrum further in the final chapter of the thesis.

Demelza Koehn has observed has shown that strains with DB fusions of *SPO11, REC104, REC114, REC102, REC107, MEI4* and *SKI8* can all make DSBs at *GAL2*, suggesting that each these proteins are capable of attracting and forming a functional recombination initiation complex, despite presumably altering the wild type order of assembly (Koehn et al., 2009). Of the DB fusions that she tested, only a *DB-MRE11* strain could not make DSBs at *GAL2*. This suggests that the MRX subcomplex may be the last subcomplex to associate with hotspot DNA, yet Mre11, Rad50 and Xrs2 are all clearly required for the RIS. It is not possible to determine the MI timing of any of the DB-fusion strains that Koehn used in her studies due to the presence of a *rad50S* mutation in the strain background. *rad50S* strains are used to evaluate DSBs by Southern analysis because DSBs are formed in *rad50S* strains, but not resected, making DSBs visible on a blot as a clear band as opposed to a smear. While the presence of *rad50S* is necessary for the quantification of DSBs using Southern analysis, it decreases sporulation to less than 5% and delays the reductional division by several hours (Alani et al., 1990, Stuart Haring, personal comm.).

The RIS is conserved in SK1 strains

Because there are several consensus wild type strains in use in *S. cerevisiae* research, several different strain backgrounds are used (*e.g.*, SK1, S288C, BR2495), different results can sometimes be observed, especially in meiosis. Generally the conclusions from experiments in one strain background can be applied to all *S. cerevisiae* backgrounds but exceptions have been documented. The Roeder lab has reported that there are differences in the kinetics of the meiotic divisions in *zip1* mutants in BR2495 and SK1 strain backgrounds (Tung et al., 2000). In the BR2495 strain background, *zip1* mutant cells arrest at pachytene, but in the SK1 background, *zip1* mutant cells still sporulate (Tung et al., 2000). Although the *zip1* SK1 cells do sporulate, the levels are reduced compared to wild type (40% in zip1 SK1 cells compared to 70% for SK1 wild type cells). These results show that different strain backgrounds can influence the phenotype caused by a particular mutation. Although Tung *et al*, (2000) found differences in the *zip1* phenotype in BR2495 and SK1, they demonstrated that an overexpression of *NDT80* had the same effect on the *zip1* mutant in both strain backgrounds. This suggests that overexpression of genes regulated by *NDT80* is sufficient for the first division, even when synapsis is defective, regardless of strain background.

The difference in phenotype in *zip1* mutants between BR2495 strains and SK1 strains led us to ask the question whether RIS mutants would have the same MI timing phenotypes in other strain backgrounds. The SK1strain background is used by many labs who work on meiosis in *S. cerevisiae* and is quite divergent from the S288C-derived strain background that we use (Ben-Ari et al., 2005).

Although previous work demonstrated that *spo11* mutants start the MI division at an earlier time in a SK1 background (Cha et al., 2000), we wanted to determine if the general properties of the RIS signal were conserved by examining two other recombination initiation mutants, *rec102* and *rec104*. The results in this chapter

demonstrated that the reductional division actually begins in the SK1 strain with timing very similar to that in our wild type K65-3D background. Our results demonstrate that the differences in timing of meiosis do occur after the first division. Both *rec102* and *rec104* result in cells starting the first division of meiosis about 1.5 earlier in our strain background. Because *spo11*, *rec102*, and *rec104* mutations all confer an earlier MI in SK1, I conclude that the RIS is not unique to the S288C strain background. Because the SK1 strain background and our strain background are so divergent, it is likely that the RIS is conserved in all *S. cerevisiae* backgrounds. In the future it will be important to examine the MI kinetics of *ski8* and *mei4* mutants in the SK1 background. In an S288C-derived background these two mutants start the first meiotic division at a time that is indistinguishable from wild type (Galbraith et al., 1997; Malone et al., 2004). If the earlier start of MI is due to a recombination initiation signal and is not related to recombination or DSBs, then the *ski8* and *mei4* mutants should start the reductional division at the same time as wild type in the SK1 background.

Later events, including the second meiotic division and sporulation, do occur more rapidly in SK1 (Fig. 2-5). This could be due to allelic differences between SK1 and K65-3D. These differences do not affect the timing of the first division, however. If SK1 and S288C have S phase times, which are indistinguishable (which is consistent with available data), and if these two strains have similar MI kinetics, then the differences in sporulation timing must occur after the first division. Figures 2-3 and 2-4 demonstrate that SK1 wild type strain background is proceeding through the second meiotic division faster than the S288C strain background. Because MI occurs at the same time in K65-3D and SK1 strains, it is reasonable to assume that all events prior to the reductional division (S phase, recombination, SC formation, etc.) occur at the same time in the two strain backgrounds, despite the published statements that SK1 strains have more rapid events (Cha et al., 2000).

One study has shown that found *SPO11* is conserved in the ascomycete fungus *Sordaria macrospora* and that *spo11* mutants display a phenotype analogous to the early MI timing seen in *S. cerevisiae* (Storlazzi et al., 2003). In this work, *spo11* bivalents segregated earlier than wild type. Given the phenotypes of recombination initiation mutants in *Sordaria*, S288C, and SK1 *S. cerevisiae* strains, the RIS may be more widely conserved in fungi. In this view, *Sordaria* cells can detect the presence of recombination initiation proteins and respond by adjusting the time at which the reductional division starts. This mechanism coordinates the initiation of recombination and the first meiotic division perhaps to insure that the formation of chiasmata is completed before the separation of homologous chromosomes or to ensure that chromosomes do not segregate before the DNA is repaired.

Summary and final remarks

The experiments presented this chapter represent the completion of our study of all ten recombination initiation genes in their role in determining timing of the reductional division. We have found that Xrs2, Mre11, Rad50, Rec102, Rec104, Rec107, Spo11 and Rec114 are all required for the RIS. Of the ten initition proteins, only Mei4 and Ski8 are not required for the RIS. The structural nature of the RIS has yet to be determined, including any required phosphorylation of the RIS proteins. Xrs2, Rec107 and Rec104 are all phosphoproteins (See Chapter 1), and it would be beneficial to know whether phosphorylation of any of these proteins is required for the RIS. This could most easily be studied by examining the well-characterized phosphorylation of Rec107. Rec107 is phosphorylated in a Cdc7/Dbf4-dependent manner on residues Ser11, 15, 19, 22 and 29 of the N-terminal region of the Rec107 protein (Sasanuma et al., 2008). Phosphorylation of all of these residues contributes to DSB formation, but phosphorylation of Ser 29 is absolutely required for DSBs. Cdc28/Clb5 also directly phosphorylates Rec107 on Ser30 and Ser271 and is important for DSB formation but not

for chromatin association (Henderson et al., 2006; Wan et al., 2008). It would be interesting to know whether the phosphorylation that is required for full or partial DSB formation is also required for the RIS. For example, the MI timing of *rec107-ser15* could be examined. These mutants make reduced DSBs. If *rec107-ser15* started the reductional division early as observed in *rec107* deletion mutants, this would suggest that Ser15 is important in determining the RIS, because Rec107 is clearly present in at least some initiation complexes if at least some DSBs are made.

By its nature, the RIS must be a transient signal that must be terminated in order for the reductional division to finally occur. I present my speculations on the nature of this termination in the final chapter of the thesis.

The experiments presented in this chapter demonstrate that the RIS is conserved among two distant *S. cerevisiae* strains. This conclusion is important because it means the RIS is not unique to the S288C strain background and it allows one to hypothesize that the RIS may be conserved among other ascomycetes as well as other eukaryotes.

CHAPTER 3

SECURIN DESTRUCTION IS NOT REQUIRED FOR MI IN *rec104* CELLS

Abstract

In order for chromosomes to segregate during mitosis, the cohesins that hold sister chromatids together must first be degraded. Degradation of cohesins requires the degradation of Securin (Pds1 in yeast). Degradation of Pds1 is also necessary in wild type meiosis. It has been previously demonstrated, however, that nuclear division can occur without first degrading epitope-tagged Pds1 in a *spo11* mutant (Shonn et al., 2000). The experiments in this chapter support this observation by demonstrating that the first invasion can also occur in a *rec104* strain without first degrading HA-tagged Pds1. I conclude that Pds1 degradation is controlled by an alternate route than the RIS and that the RIS occurs prior to the signal responsible for degrading Pds1.

Introduction

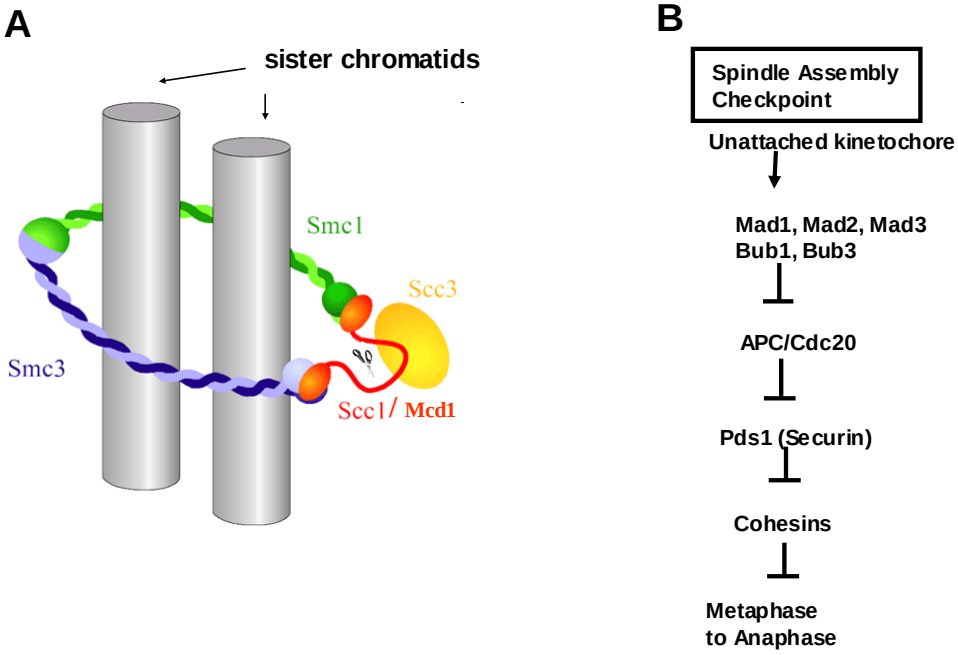
Cohesins in mitosis

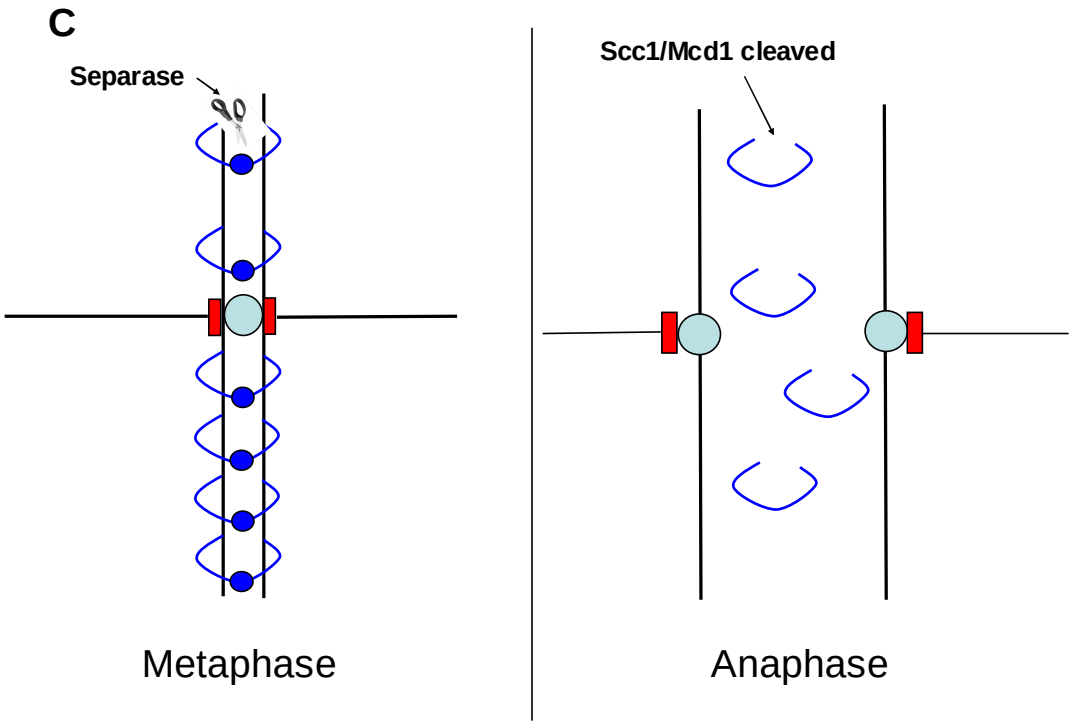
The correct segregation of genetic material during cell division is crucial for the propagation of organisms. Improper segregation of chromosomes leads to aneuploidy. To facilitate proper chromosome segregation, eukaryotes have evolved a system that holds recently- replicated sister chromatids together and that can be disassembled in a careful and controlled manner. Though the mechanism of chromosome segregation has features common to most eukaryotes, this introduction will focus exclusively on *S. cerevisiae*. Cohesins form a large tripartite ringed structure of proteins comprised of the subunits Smc1, Smc3, the kleisin subunit Scc1/Mcd1 and Scc3 that encircle sister chromatids and hold them together until spindle fibers have attached to kinetichores (Haering and Nasmyth, 2003; Nasmyth, 2002; Nasmyth and Haering, 2005) (Fig. 3-1 A).

The complex is deposited during or shortly after S phase. The Smc1 and Smc3 subunits of cohesin both form rod-shaped molecules that heterodimerize by means of 'hinge' domains situated at the ends of 30-nm-long intramolecular antiparallel coiled-coil ATPase 'heads' at the other ends are connected by the Scc1/Mcd3 subunit of cohesin, thereby forming a tripartite ring with a 35 nm diameter (Fig. 3-1A). Recent evidence suggests that the cohesin complex holds sister chromatids together by entrapping sister chromatids within a single ring complex (Haering et al., 2008). Protein-DNA crosslinking studies using yeast mini-chromosomes have shown that there are connections at the interfaces between the three subunits of the cohesin ring, thereby creating a covalently closed cohesin structure. Haering *et. al.* (2008) concluded that a single ring, as shown in Fig. 3-1 A, rather than a double ring is the most likely structure formed by cohesins.

Once spindle attachments have formed, the kleisin subunit (Scc1/Mcd1) of the cohesin ring is cleaved by the cysteine protease separase (*ESP1* in *S. cerevisiae*), severing the cohesin ring and allowing sister chromatids to segregate due to the force created by the pull of the spindle fibers (Nasmyth and Haering, 2005) (Fig. 3-1B and C). The timing of separase function is controlled by the anaphase promoting complex (APC) including Cdc20, an E1 ubiquitin ligase. APC/C is only activated when each chromosome is correctly attached to spindle fibers radiating from opposite poles (amphitelic attachment) as opposed to the same pole (syntelic attachment). When attachment is achieved, the APC ubiquitinates the inhibitory chaperone protein securin (encoded by *PDS1* in *S. cerevisiae*) targeting it for destruction by the 26S proteosome (Cooper et al., 2009; Nasmyth and Haering, 2005) (Fig. 3-1B). Pds1 binds and sequester securin (encoded by *ESP1* in *S. cerevisiae*). Deagradation of Pds1 allows Esp1 to become active and the kleisin subunit of cohesins to be cleaved. Once the cohesin ring has severed, the sister chromatids are pulled to opposite poles by the force of the mitotic spindle.

Figure 3-1: Role of cohesins in mitosis. [A]Sister chromatids are represented by grey cylinders. Smc1, Smc3 and a subcomplex of Scc1/Mcd3 and Scc3 form the three subunits of the cohesin subcomplex in *S. cerevisiae*. Scc1/Mcd3 (the kleisin subunit) is cleaved by separase (represented by scissors) to allow segregation of sister chromatids. Modified from Haering, C.H., Farcas, A.M., Arumugam, P., Metson, J., and Nasmyth, K. (2008). The cohesin ring concatenates sister DNA molecules. Nature *454*, 297-301. [B] Pathway showing the mechanism for the cleavage of cohesins. [C] Cohesins, shown in dark blue, hold sister chromatids together during metaphase in mitosis. Spindle fibers are shown extending from kinetochores, shown in red. In Anaphase, cohesins are degraded allowing sister chromatids to segregate.


A
sister chromatids
Smc1
Scc3
Smc3
Scc1/ Mcd1
B
Spindle Assembly Checkpoint
Unattached kinetochore
Mad1, Mad2, Mad3
Bub1, Bub3
APC/Cdc20
Pds1 (Securin)
Cohesins
Metaphase to Anaphase


C
Separase
Scc1/Mcd1 cleaved
Metaphase
Anaphase

Cohesins in meiosis

Cohesin assembly and disassembly is somewhat different in meiosis than in mitosis. Cohesins are deposited during or shortly after pre-meiotic S phase. Spindle attachment is monopolar in Meiosis I, unlike the bipolar attachment found in Meiosis I. This means that spindle fibers radiate from one face of the kinetochore on a homolog to a single SPB. Another key difference between mitosis and meiosis is that homologs are held together by chiasmata prior to anaphase I. The kleisin subunit Scc1/Mcd1 is replaced with the meiosis-specific homolog Rec8 in meiosis and is removed in a step-wise manner (Brar et al., 2006; Molnar et al., 1995). Rec8 is first cleaved along the length of chromosome arms during anaphase I. This allows the segregation of homologs. Rec8 is then cleaved from regions near centromeres in Meiosis II allowing sister chromatids to segregate (Lee et al., 2002; Shonn et al., 2002). In *S. cerevisiae* this step-wise loss of chromosome cohesion is controlled, in part, by the meiosis-specific protein Spo13, which protects Rec8 from being cleaved by Esp1 (separase) (Brar et al., 2006).

Recently it has been shown that Rec8 affects the localization of Spo11 (Kugou et al., 2009). Using ChIP with high-density tiling arrays, they found that Spo11 initially accumulated around centromeres before being localized to chromosome arms as pre-meiotic S phase progresses. They showed that deletion of *REC8* influenced the localization of Spo11 to centromeric regions and in some of the intervals of the chromosomal arms. In *rec8* strains Spo11 distribution was random. This observation suggests that Rec8 can influence the distribution of Spo11 along chromosomes.

Pds1 destruction precedes chromosome segregation in meiosis

At the onset of sporulation in *Saccharomyces*, Pds1 is diffusely expressed in the cytosol and then becomes concentrated in the nucleus before the MI spindle forms in anaphase I (Shonn et al., 2000). In wild type cells, nuclear Pds1must be completely

degraded before the onset of anaphase I, thus some investigators consider that Pds1 destruction is the essential marker of anaphase I rather than division of nuclear material (Shonn et al., 2000) (See Discussion). *pds1* null mutants are inviable during vegetative growth . During mitosis Pds1 must be fully degraded to allow sister chromosomes to segregate equationally. A temperature-sensitive *(ts)* mutant of *PDS1* shifted to the non-permissive temperature at the start of sporulation causes an arrest in prophase I suggesting that Pds1 is essential for meiotic division (Cooper et al., 2009). This arrest can be partially suppressed by a *spo11* mutation. Cooper *et al.* (2009) also suggest that Pds1 has a role in promoting break formation. This is underscored by the absence of DSB formation and heteroallelic recombination in $pds1^{ts}$ mutants that haave been sporulated at the non-permissive temperature (Cooper et al., 2009). An alternative interpretation is that in $pds1^{ts}$ mutants, cohesin cannot properly be assembled because separase is always active, thus setting off a checkpoint response prior to recombination. Cohesins are normally assembled at the end of S phase in mitosis and the same is presumably true during pre-meiotic S phase, implying that cohesins are present during the time when recombination initiates in late leptotene and early zygotene. Cooper *et al.* (2009) observed that the $pds1^{ts}$ arrest partially requires the Pachytene checkpoint proteins Mek1 and Red1, though not the DNA damage checkpoint proteins Rad17 and Rad24. This suggests that the state of meiotic chromatin, including cohesins is monitored by the cell. Cooper *et al.* (2009) also observed that Pds1 is required for proper SC formation suggesting that proper chromatin context is also necessary for formation of the SC.

Pds1 destruction is not required for the reductional division in a recombination mutant

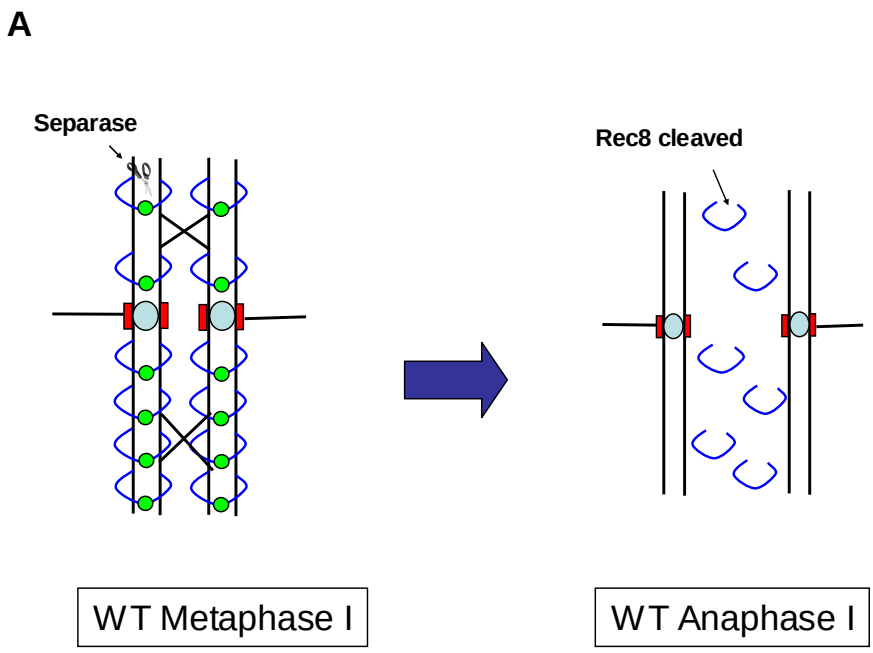
Andrew Murray's lab examined Myc-tagged Pds1 in sporulating wild type and *spo11* cells (Shonn et al., 2000). They observed that nuclear Pds1 was always degraded in WT cells that were undergoing Anaphase I. In contrast, 77% of *spo11* cells that had an

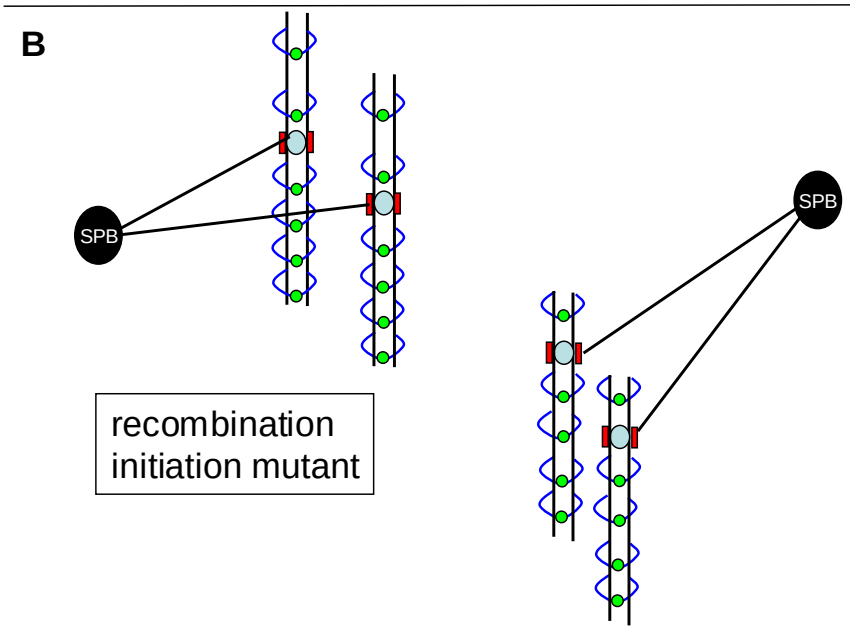
elongated MI spindle and divided DNA masses had Pds1-Myc present. They interpreted this observation to mean that the absence of tension created by chiasmata was responsible for this premature chromosome segregation. They proposed that this tension present in wild type cells is monitored by the spindle checkpoint (Fig. 3-2) and discussed in Chapter 1 and Chapter 5). Their view was that spindles formed and attached at the same time in wild type cells and *spo11* mutants. However, homologous chromosomes are not held together by chiasmata in *spo11* cells. Thus, as soon as the spindles attached, they were pulled apart. Mechanistically, Cohesins do not need to degrade for nuclear division in *spo11* strains (Fig, 3-2A).

Because of Murray's lab's important observations in *spo11* strains, and because the Shonn *et al*. (2000) model does not support the idea of a recombination initiation signal, we examined epitope-tagged Pds1 in a *rec104* background. *REC104* is a recombination initiation gene that is essential for DSB formation, similar to *SPO11*, meaning that no chiasmata are formed in *rec104* cells (Malone et al., 1991). Both *SPO11* and *REC104* are members of the RIS. Our lab has shown that *spo11* and *rec104* mutants both start the reductional division early (Galbraith et al., 1997, Malone et al., 2004). I used an integrated HA- tagged strain created using a construct obtained from Orna Cohen-Fix (Cohen-Fix and Koshland, 1997) transformed into *rec104* and WT strain backgrounds. This allowed us to visualize Pds1 presence to see whether Anaphase I could occur in a *rec104* strain without degradation of Pds1.

[Sonja Smith, an R.A. II from the Malone lab, created the epitope-tagged heterozygous *Pds1* strain that I dissected to create the strains used in this study. The immunostaining, imaging and analysis were performed by me.]

Figure 3-2: Cohesin degradation during MI. [A]. In a normal meiosis chiasmata hold homologs together. This allows pairing of homologous chromosomes. Cohesins, shown in dark blue with the Rec8 subunit represented by a green dot, must degrade in order for Anaphase I to occur. [B]. In a recombination initation mutant, nuclear division does not require cleavage of cohesins.


A
Separase
Rec8 cleaved
WT Metaphase I
WT Anaphase I


B
SPB
SPB
recombination
initiation mutant

Materials and Methods

Yeast strains used in Pds1-HA experiments

The yeast strains used for the experiments described in this chapter are derived from the homothallic diploid K65-3D (Galbraith et al., 1997) ultimately derived from the S288C background. K65-3D is homozygous for the following markers: *HO, lys2-1, tyr1-1, his7-2, can1r, ura3-13, ade5, met13-d, trp5-2, leu1-12, ade2-1*. Complete descriptions of all strains are detailed in the Appendix. To create the HA-tagged Pds1 strains used in this study, Sonja Smith (R.A. II from the Malone lab) transformed a fragment from the plasmid pOC52 into the strain K65-3D-104Δh, a strain that is heterozygous for the *rec104Δ* mutation creating the strain SMS1-1. The plasmid pOC52 was a gift from Orna Cohen-Fix (Cohen-Fix et al., 1996). pOC52 is pUC19-based plasmid carrying *PDSI::HA* and *URA3,* which was integrated at an *XbaI* site located 3' of the *PDS1* open reading frame. *PDSI::HA* is an in frame fusion of the triple HA repeat at the *BglII* site of *PDS1* (position 520 relative to the first open reading frame base). This heterozygous strain SMS1-1was sporulated and dissected to obtain the homozygous Pds1-HA *rec104* and Pds1-HA wild type strains, SMS4-1-1C (stock # M3738) and SMS4-1-2B (stock # M3736), respectively. The *PDS1::HA* fusion was detected by selecting segregants that grew on SC-Ura media. The *rec104*-Δ*1* mutation was detected in strains by using PCR with the primers "*rec104* colonyF (5'GAGCTGTTCGGGTATTGCGT3') and "*rec104* colony R" (5'GAAAGTATCAGTTCTATGGACAGTTC3').

Media and growth conditions

All media not described below has been described in Chapter 2. Sporulation and growth conditions are also described in Chapter 2.

Liquid sporulation and timepoints

Liquid sporulation media is described in Chapter 2. 20 mL of cells were harvested at either 7 or 8 hours later in a 50 mL conical tube. The tubes were centrifuged for 2 minutes in a clinical centrifuge at room temperature and immediately fixed for later use (see below).

A late (approximately 30h) 1 mL timepoint was taken and added to an equal volume of 8% formaldehyde for counting final sporulation values for the experiment.

Pds1-HA immuno-staining and microscopy

Cells were fixed and stained using a protocol derived from methods used in the Koshland laboratory (Cohen-Fix et al., 1996) (http://www.ciwemb.edu/labs/koshland/Protocols/MICROSCOPY/pds1if.htmL). The cell pellet from 20 mL of sporulating cells (see above) was resuspended in 10 mL of 1x KPO_4 buffer with 4% formaldehyde (2x KPO_4 buffer contains 120 mL 1M $K2HPO_4$, 280 mL 1M $KH2PO_4$ per liter, pH 6.5) in 50 mL conical tubes. Tubes were placed on a rotating shaker at room temperature and allowed to fix for 12 minutes then spun down again in the clinical centrifuge at top speed for 2 minutes. The fixed pellet was washed twice with 40 mL 1x KPO_4 buffer and twice with 1x KPO_4 buffer, 1.2 M Sorbitol. The pellet was resuspended in 5 mL of 1x KPO_4 buffer, 1.2 M Sorbitol and stored in a 4º C refrigerator for up to three weeks.

To spheroplast the cells for immuno-staining, a 1 mL aliquot was removed from the stored 5 mL of cells, placed in a 1.5 mL eppindorf tube, and centrifuged at 10,000 rpm at 4º C for 1 minute and resuspended in 250 µL of 1x KPO_4 buffer. 30 µL of a freshly prepared stock containing 1 mg/mL Zymolyase 100T and 1 µL / mL of β-mercaptoethanol were added to each tube and incubated at 37º C in an incubator block for 35 minutes, inverting to mix once or twice.

While the cells were incubating, poly-lysine coated slides were prepared. A 1:10 dilution of a 1mg/mL thawed frozen stock of poly-lysine in ddH_2O (Sigma) was prepared and 1 mL of the 1:10 dilution was spotted onto 10-well Teflon-coated slides (Polysciences, Inc.) by dropping ~100 µL onto each well using a P-1000. After 5 minutes the solution was aspirated off the wells, the slides were briefly held (~2 seconds) under the distilled water tap in the lab sink to rinse them and then aspirated dry using the vacuum aspirator attached to the water supply. I devised the method of rinsing poly-lysine slides under the tap, because it is easy. I never paid any attention to the angle at which I held the slides or water pressure that I used.

After incubating for 35 minutes, the spheroplasts were gently centrifuged at 3000 rpm in a microfuge for 5 minutes at 4º C. The spheroplasts were washed twice with 1 mL of 1x KPO4, 1.2M sorbitol and gently resuspended in 250 µL of 1x KPO4, 1.2 M sorbitol. 20 µL of the spheroplast suspension was spotted onto the wells of a polylysine-coated slide and allowed to settle in a humid chamber for 20 minutes. The humid chamber was made by inverting an opaque Tupperware box over the slides with 4-5 folded, damp paper towels placed next to the slides. The slides were then submerged in a Coplin jar of -20º C methanol for 6 minutes in the -20º C freezer and then immediately removed and plunged into a coplin jar with -20º C Acetone for 30 seconds. I kept bottles of acetone and methanol in the -20º C freezer, so I would always have cold reagents available. The slides were then air dried for a few minutes on the bench.

To immunostain the slides, 1 mL of blocking buffer (1 % BSA in PBS (0.04 M K_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl)) was first spotted (~ 0.1 mL/well) onto each slide. This spotting technique was also used for the washing steps described below. The slides were placed in a humid chamber for 5 minutes. The blocking buffer was aspirated and 20 µL of a 1: 100 dilution in blocking buffer of both primary antibodies was applied to the wells and the slides were placed in a humid chamber. The rat monoclonal antibody 3F10 (Roche) was used as a primary antibody to detect Pds1-HA and the mouse

monoclonal anti-α-tubulin antibody 12G10 (Developmental Studies Hybridoma Bank, University of Iowa) was used as a primary antibody to visualize meiotic spindles (α-tubulin is encoded by the *TUB1* gene in *S. cerevisiae*). I stained for spindles and Pds1-HA simultaneously (*i.e.,* both of these antibodies were applied at the same time.

After 1-3 hours the primary antibody was aspirated and the slides were washed 6 times with 1 mL of blocking buffer. A 1:500 dilution containing both secondary antibodies in blocking buffer was then applied to each well. From this point, the slides were only exposed to dim room light. To dim the lights, I shut the doors, and closed the blinds, and turned off the main lights, but my lab mates could keep their desk lights on. The goat anti-rat antibody Alexa 488 (Invitrogen) was used as a secondary antibody to detect Pds1-HA and the goat anti-mouse Alexa 546 (Invitrogen) was used as a secondary antibody to detect spindles. After 1 hour the secondary antibody was aspirated and the slides were washed 6 times with 1 mL (~0.1 mL/well) of blocking buffer. One mL of a 1 mg/L solution of DAPI in plain PBS was freshly-prepared and applied to each slide (~0.1 mL/well) and allowed to incubate for five minutes in a dark, humid chamber. This solution was then aspirated and the slides were then washed 6 times with plain PBS. The slides were very thoroughly aspirated and approximately 20 µL of mounting medium (55% glycerol, 0.001g/ mL 1,4 phenyline diamine in 8.4 % sodium bicarbonate buffer, pH 9.0) was applied to each well. A 24 x 50 mm coverslip was carefully placed on each slide without pressing. The edges of each slide were sealed with clear nail polish (Sally Hanson's Double Duty) and the polish was allowed to dry. Slides were stored at 4º C in the dark for up to 5 days in a foil-wrapped box before being examined under the microscope.

Slides were viewed using a Leica MMAF microscope at 1000x magnification and photographed using a Leica CCD camera located in the Biology Department's Carver Center for Imaging. All images were captured with MetaMorph software and analyzed using Adobe Photoshop. Cells were scored by zooming in to photographs on the

computer and marking each cell with a dot in Photoshop after it was counted. Two independent experiments were performed: one from a seven hour timepoint and one from an eight hour timepoint and the results were averaged. One culture of Pds1-HA WT and one culture of Pds1-HA *rec104* were scored for each experiment. Only binucleate cells were scored (cells with two separate masses of DAPI staining). Cells were classified as either having one, two or no masses of Pds1-HA staining. Spindle morphology was classified as 1-long, 2-short, or 2-long.

Results

Andrew Murray's lab has previously shown that a *spo11* mutant can divide its nuclear material without first degrading nuclear Pds1. This observation led Murray and colleagues to propose the hypothesis that initiation mutants segregate DNA earlier because they have no inter-homolog tension (See Introduction). Because this observation contrasted with our own work and to extend the generality of the "no recombination-no need to destroy Pds1 observation", I performed a similar experiment comparing HA-tagged *rec104* and wild type strains. I sporulated and harvested cells from two different experiments. One trial was performed with cells taken at 7 hours and the second trial was performed with cells collected at 8 hours. Though Pds1 is expressed before MI and before MII, I only scored binucleate cells because the RIS signals the delay of the first meiotic division; the timing of the equational division is not detectably different in *rec104* strains. Pds1-HA is shown in green and meiotic spindles (Tub1) are shown in orange. Nuclear material labeled by DAPI is shown in blue (Fig.3-3, 4 and 5). In wild type, Pds1-HA staining is expressed diffusely in the cytosol at the start of meiosis then becomes concentrated in the nucleus (Fig. 3-2 and 3-3). Pds1 is then localized to the nucleus again prior to MII.

When anaphase I occurs, Pds1-HA staining is always absent from the nucleus of WT cells (76/76 cells scored) (Table 3-1). This can be seen in figure 3- 2, as indicated by

the white arrows. In a *rec104* background, however, nuclear Pds1-HA staining was frequently present in one or two masses even though Anaphase I is occurring and the nuclear material has divided (115/130 cells scored) (Fig. 3-3). Nuclear segregation without degradation of Pds1 was never observed in wild type cells (Table 3-1 and Fig. 3-4B). Representative Anaphase I cells are shown in Fig. 3-4A.

Discussion

I observed that Pds1 degradation is not required for the first division in a *rec104* mutant (Fig. 3-4). In contrast, I never observed the first division in a wild type cell before Pds1-HA degraded. This observation is consistent with the Murray lab's observation in a *spo11* mutant, but our interpretations of the results differ. Murray and colleagues attribute the division of *spo11* nuclear material before the destruction of Pds1 as a consequence of a lack of interhomolog tension created by recombination intermediates (Shonn et al, 2000). They propose that this interhomolog tension is monitored by the spindle checkpoint and that the spindle checkpoint is responsible for the early reductional division seen in a *spo11* mutant. I agree that there is no interhomolog tension in mutants that do not make DSBs (*e.g.*, *spo11* or *rec104)*; however, it cannot be an absence of tension alone that causes an early first division. The Malone lab has shown that *ski8* and *mei4* mutants are also required for DSB formation, yet do not start the reductional division of meiosis earlier than wild type (Galbraith et al., 1997; Jiao et al., 2002; Malone et al., 2004) and (Chapter 2). *ski8* and *mei4* cells therefore do not have any interhomolog tension. If Murray's hypothesis were correct, we would observe an earlier MI in *ski8* and *mei4* strains.

Furthermore, I have shown that *ski8* mutants do not express the transcription factor *NDT80* earlier than wild type (Chapter 4). *NDT80* is the transcription factor necessary for middle meiosis and MI spindle formation (Hepworth et al., 1998; Xu et al., 1995). Logan Vidal, an Honor's undergraduate, and I have shown that *NDT80* is targeted

Table 3-1: Pds1-HA staining in WT binucleate cells

	No Staining			1 Mass	2 Masses		
Spindles	**1 long**	**2 short**	**2 long**	**1 long**	**1 long**	**2 short**	**total**
Exp. 1: 7h in spo.	20	5	10	0	0	30	65
Exp. 2: 8h in spo.	56	33	15	0	0	52	156
total	76	38	25	0	0	82	221
% total	34	17	11	0	0	37	
st. dev.	± 4.0	± 1.0	± 4.1	0	0	± 9.1	

Note: Combined quantification of Pds1-HA WT data for two different experiments**.** One experiment was performed using cells harvested at 7 hours into sporulation. The other experiment was performed using cells harvested at 8 hours. Only binucleate cells with two distinguishable masses of DAPI staining were scored. Pds1-HA staining was scored as either absent (No staining), one mass or two masses. Spindles were either classified as being MI anaphase (1 long), post-MI anaphase (2 short), or MII anaphase (2 long). % total refers to the percentage of cells in the particular data class.

Table 3-2: **Pds1-HA staining in *rec104* binucleate cells**

	No Staining			1 Mass	2 Masses		
Spindles	**1 long**	**2 short**	**2 long**	**1 long**	**1 long**	**2 short**	**total**
Exp. 1: 7h in spo.	6	17	9	58	5	65	160
Exp. 2: 8h in spo.	15	16	3	44	2	24	104
total	21	33	12	102	7	89	264
% total	8.0	12.5	4.5	39	2.7	34	
st. dev.	± 7.5	± 3.4	± 1.9	± 4.3	± 0.85	± 12.4	

Note: Combined quantification of Pds1-HA *rec104* data for two different experiments**.** One experiment was performed using cells harvested at 7 hours into sporulation. The other experiment was performed using cells harvested at 8 hours. Only binucleate cells with two distinguishable masses of DAPI staining were scored. Pds1-HA staining was scored as either absent (No staining), one mass or two masses. Spindles were either classified as being MI anaphase (1 long), post-MI anaphase (2 short), or MII anaphase (2 long). % total refers to the percentage of cells in the particular data class.

Figure 3-3: Pds1-HA expression in WT cells. Representative images from two different experiments. Pds1-HA is shown in green, meiotic spindles are shown in orange and nuclear staining (DAPI) is shown in blue. The white arrows indicate binucleate cells with an elongated MI spindle.

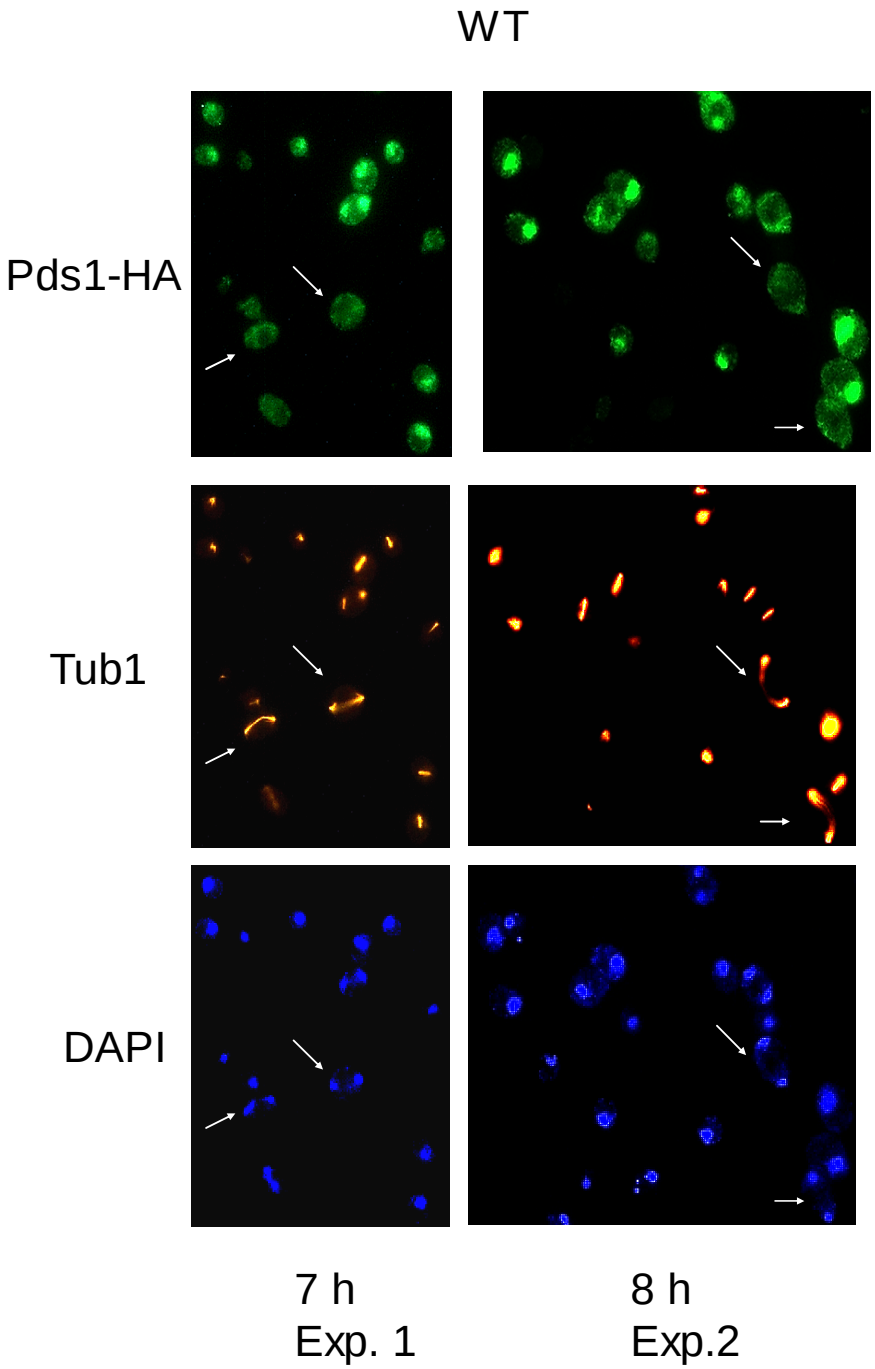

WT
Pds1-HA
Tub1
DAPI
7 h
Exp. 1
8 h
Exp.2

Figure 3-4: Pds1-HA expression in *rec104* cells. Representative images from two different experiments. Pds1-HA is shown in green, meiotic spindles are shown in orange and nuclear staining (DAPI) is shown in blue. The white arrows indicate binucleate cells with an elongated MI spindle.

rec104

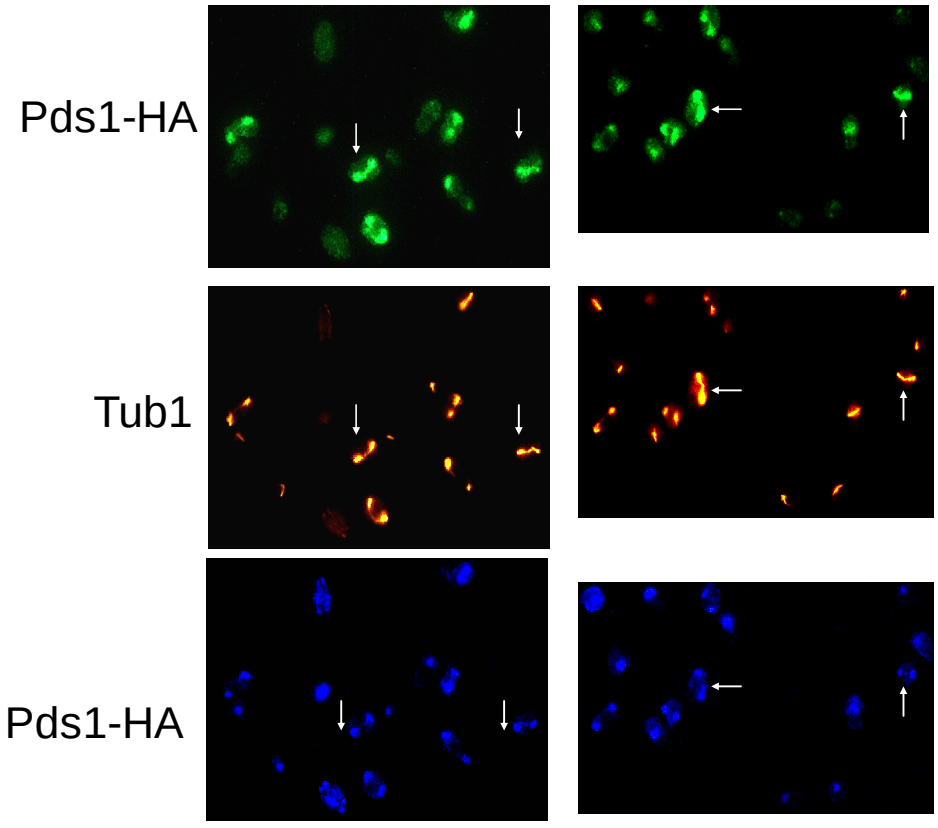
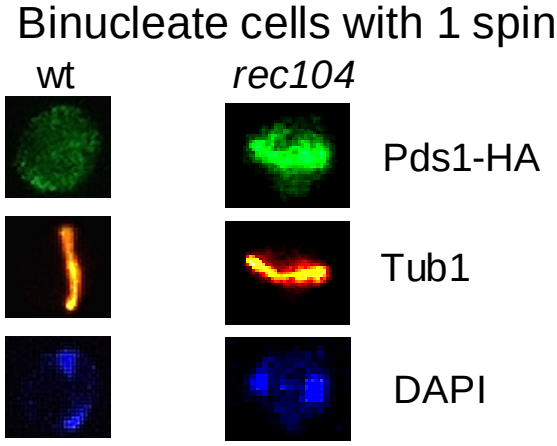
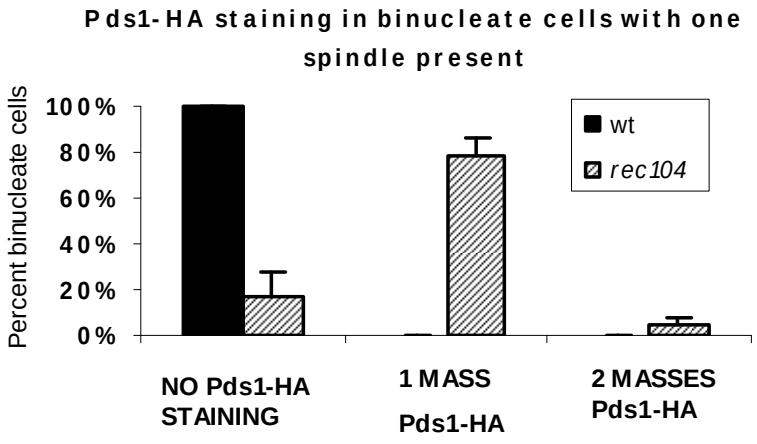


Figure 3-5: Pds1-HA staining in binucleate cells with one spindle present. [A] Representative binucleate cells from 7-8 hours in sporulation. Pds1-HA is labeled in green, Spindles (Tub1) are labeled in orange and DNA is shown in blue. [B] Quantification of nuclear Pds1-HA staining in WT (solid bars) and *rec104* (shaded bars) in binucleate cells with fully elongated MI spindles. Data is shown from a combination of two independent experiments. Of the 221 WT binucleate cells scored, 76 were in anaphase I (had a fully elongated MI spindle). Of the 264 binucleate *rec104* cells that were scored, 130 had a fully elongated MI spindle.

A.


Binucleate cells with 1 spindle
wt
rec104
Pds1-HA
Tub1
DAPI

B.


Pds1-HA staining in binucleate cells with one spindle present
Percent binucleate cells
100%
80%
60%
40%
20%
0%
wt
rec104
NO Pds1-HA STAINING
1 MASS Pds1-HA
2 MASSES Pds1-HA

by the RIS and that early transcription of *NDT80* is sufficient for an early reductional division. We propose that it is the absence of the members of the RIS, including Rec104 and Spo11, but not Ski8, that delays the start of MI.

We also disagree with Andrew Murray and colleagues about the role of the spindle checkpoint in monitoring a lack of recombination-induced tension in MI. Rachel Gast, a Master's student who graduated from our lab, extensively studied the role of the spindle checkpoint in monitoring the first division of meiosis. Gast examined the reductional division timing of *mad2, bub2, mad3* and various double mutants. She found that each of these strains started MI at the same time as wild type strains. She concluded that no aspect of the spindle checkpoint is involved in transducing the signal to delay the first division in wild type. Furthermore, we have shown that that the RIS impinges on a target (*NDT80*) that is required for spindle formation (Hepworth et al., 1998; Xu et al., 1995). Members of the spindle checkpoint cannot be required to delay the start of the reductional division because the spindle checkpoint is not active prior to spindle formation.

A further experiment that could be done would be to look at HA-tagged Pds1 in a *ski8* background along with meiotic spindles and nuclei. I predict that results would be similar to wild type. The reductional division would not occur in *ski8* strains until nuclear Pds1 had degraded. This would provide further evidence that it is the proteins of the RIS and not DSB formation that signals the delay of the reductional division.

Summary and final remarks

Cohesins hold sister chromatids together during mitosis and meiosis. Nuclear division can only occur when the inhibitory chaperone protein Pds1 (Securin) is degraded allowing Esp1 (Separin) to cleave the cohesin ring structure, this freeing the chromosomes. During MI in wild type cells, chiasmata hold chromosomes together. These chiasmata must be degraded in order for Anaphase I to occur (Fig. 3-2). It has

been previously shown by the Murray lab that a recombinationless *(spo11)* meiosis does not require the degradation of Pds1 for nuclear division (Shonn et al., 2005). They have also proposed that the lack of chiasmatic tension in a *spo11* cell explains why *spo11* strains divide early. This hypothesis cannot be correct, however, because *rec103* and *mei4* mutants do not make DSBs, yet start the reductional division at the same time as wild type.

These results do not exclude members spindle checkpoint from having a role in a normal meiosis, however. Cohesins must be degraded in order for chromosomes to to segregate in wild type meiosis because homologs are paired due to chiasmata.

CHAPTER 4

NDT80 IS THE TARGET OF THE RECOMBINATION INITIATION SIGNAL

Abstract

The reductional division is a major meiotic event which requires the transcription of many genes. The primary transcriptional regulator of middle meiosis, including the reductional division, is the transcription factor Ndt80. Because the RIS delays the start of the reductional division, I hypothesized that the RIS targets the transcription of *NDT80*. To test this, I examined the transcriptions of *NDT80* in *rec102*, *rec104* and wild type strains. I showed that *NDT80* is transcribed earlier in these strains. Ndt80 is also active earlier in these strains because *SPS4*, a gene whose transcriptional regulation is entirely dependent on Ndt80, is also transcribed earlier in these strains. These data are consistent with *NDT80* being the transcriptional target of the RIS. To test whether it is the RIS signaling activity or RIS protein-mediated DSB formation which delays *NDT80* transcription, I examined the transcription of NDT80 and SPS4 in a ski8 mutant. *ski8* is required for DSBs, but not for the RIS. I found that transcription of *NDT80* and *SPS4* was not early in a *ski8* mutant, thus DSBs (and hence meiotic recombination) are not the signal that delays the reductional division in a wild type cell. Lastly, I present data demonstrating that altering the timing of *NDT80* transcription is both necessary and sufficient for controlling the timing of MI. To test this, I showed that *NDT80* expressed from an early meiotic promoter is sufficient to cause an early reductional division.

Introduction

During sporulation in *Saccharomyces cerevisiae*, cells exit the mitotic cell cycle and commit to a determined sequence of events that includes meiosis and results in the formation of four haploid spores. The decision to sporulate is largely a transcriptionally controlled process (Honigberg and Purnapatre, 2003; Mitchell, 1994; Vershon and Pierce,

2000) (Chapter 1). There are two primary transcriptional regulators of sporulation: Ime1 controls the initiation of sporulation and early sporulation gene transcription, while Ndt80 is the primary regulator of middle sporulation gene transcription. Null mutants of *IME1* fail to initiate sporulation (Kassir et al., 1988), while null mutants of *NDT80* arrest in pachytene as mononucleate cells (Hepworth et al., 1998; Xu et al., 1995).

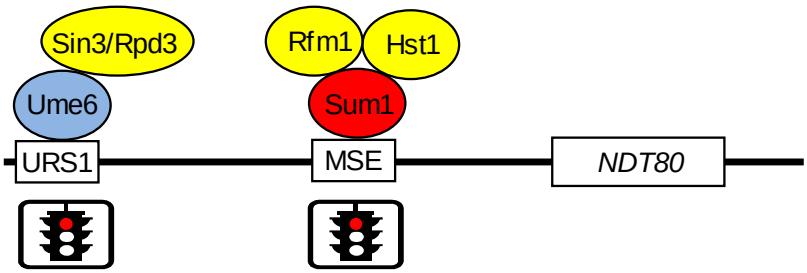
We have shown that the Recombination Initiation Signal, comprised of eight of the ten recombination initiation proteins and possibly the two SC proteins Hop1 and Red1, causes a transient delay of the reductional division. Because *NDT80* is the transcription factor essential for the reductional division, I hypothesized that *NDT80* is the target of the RIS.

Ndt80: a transcriptional activator required for the reductional division

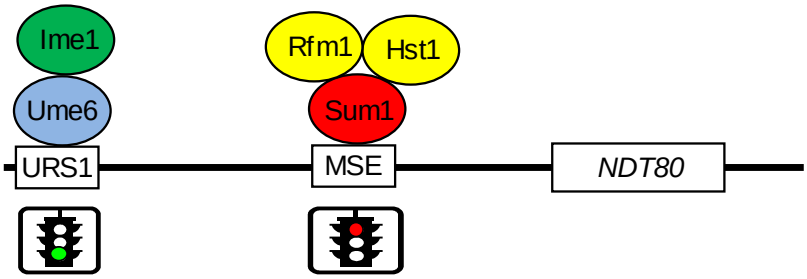
Initiation of the meiotic program requires the transcription factor Ime1 (Honigberg and Purnapatre, 2003; Vershon and Pierce, 2000). The control of transcription of *IME1* is determined by the presence of mating type proteins, and starvation for nitrogen and glucose. Additionally, transcription of *IME1* is promoted by the presence of acetate in the sporulation medium. The regulation of *IME1* transcription is discussed in Chapter 1. *IME1* is required for the transcription of early meiotic genes including the recombination initiation genes *(e.g., spo11, rec104, ski8,* and *rec102)* and synaptonemal complex (SC) genes *(i.e., hop1* and *red1).* Ime1 binds to URS1 sites along with Ume6, a constitutively bound transcription factor, to activate transcription. Early meiotic genes are repressed by Sin3/Rpd3/Ume6 binding to URS1 sites during vegetative growth. Early gene transcriptional regulation is also discussed in Chapter 1. *NDT80* transcription requires Ime (Fig. 4-1).

Figure 4-1: The regulation of *NDT80* transcription. [A] The promoter of *NDT80* is repressed at two different sites during vegetative growth. A complex of Sin3 and Rpd3 bind to Ume6 which is bound to URS1 to repress *NDT80* transcription. Ume6 is required for both positive and negative regulation of *NDT80* transcription. Sum1 represses *NDT80* transcription by binding to an MSE along with Hst1 and Rfm1. [B] At early-middle meiosis, the transcriptional activator Ime1 can bind to Ume6 located at URS1 to activate low levels of transcription of the *NDT80* promoter. [C] During pre-middle meiosis, the Ime2 kinase can phosphorylate Sum1 causing it to release from the MSE. [D] This allows *NDT80* to bind to its own promoter to activate high levels of transcription. Blue arrows below the *NDT80* gene indicate level of transcription. Red traffic light icons indicate that a promoter is repressed while green traffic light icons indicate that indicate that a promoter activated. Modeled after Pak J & Segall J (2002) *Mol Cell Biol* **22,** 6417-6429.

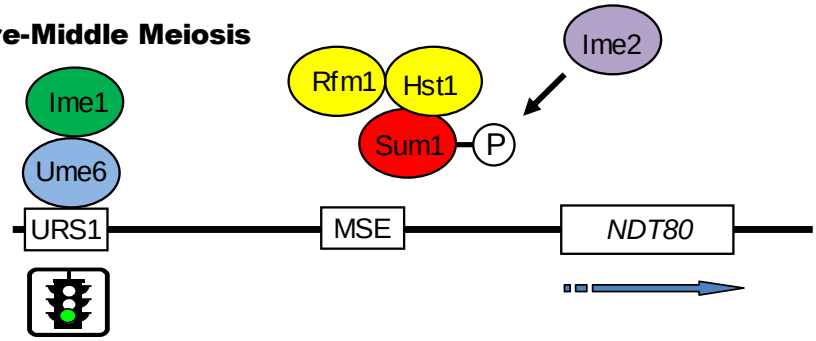
A. Vegetative Growth



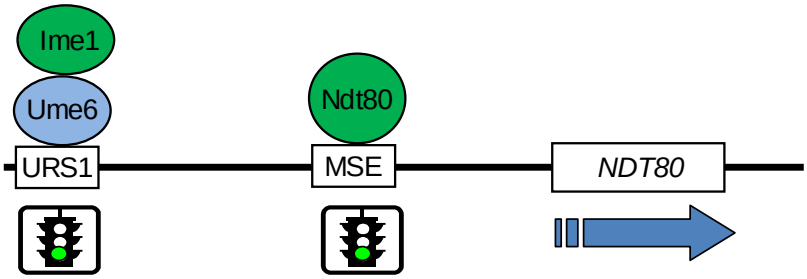
B. Early Meiosis



C. Pre-Middle Meiosis



D. Middle Meiosis



Ndt80 is a transcriptional activator of Middle Meiotic Genes (MMGs) (Chu et al., 1998). *ndt80* mutants arrest as mononucleate cells lacking MI spindles (Hepworth et al., 1998; Xu et al., 1995). Initial transcription of *NDT80* is mediated by the transcriptional activator Ime1, but subsequent transcription is mediated by Ndt80 itself [(Hepworth et al., 1998) and Chapter 1 and Fig. 4-1. Early transcription of *NDT80* is followed by Ndt80 binding to MSE sites to promote high levels of transcription of *NDT80* (Pak and Segall, 2002a; Pierce et al., 2003). *NDT80* transcription is negatively regulated by the repressor Sum1 during vegetative growth and early meiosis (Xie et al., 1999). Sum1 must be removed in order for high levels of *NDT80* transcription to occur.

During vegetative growth and early meiosis MSEs are repressed by a complex of the Sum1 repressor, the Hst1 histone deacetylase and the tethering factor Rfm1 (McCord et al., 2003) . In order for high levels of *NDT80* transcription to occur, Ndt80 must displace the Sum1 complex (Pierce et al., 2003) . This process occurs because the kinase Ime2 phosphorylates Sum1 and causes it to lose affinity for the MSE (Chu and Herskowitz, 1998; Hepworth et al., 1998) (Fig. 4-1).

Not all Ndt80-controlled promoters are repressed in a Sum1-dependent manner. For example, *SPS4* is a middle meiotic gene whose transcriptional regulation is entirely dependent on Ndt80 (Xie et al., 1999). This property makes *SPS4* an ideal reporter in *NDT80* transcriptional studies.

NDT80: a possible target for the RIS

NDT80 seemed like a potential target for the RIS because it is the transcription factor that upregulates genes necessary for the first division of meiosis. These include genes for the MI spindle, spindle checkpoint and the B-type cyclins Clb 5 and 6 (Chu et al., 1998). Furthermore, Ndt80 is capable of responding to signaling from the Pachytene checkpoint, implying that altering the expression of Ndt80 is sufficient to delay the reductional division. Cells arrested at the Pachytene checkpoint do not accumulate

phosphorylated Ndt80 in their nuclei (Tung et al., 2000). This arrest requires the DNA damage checkpoint gene *RAD24* and *rad24 dmc1* cells still have phosphorylated Ndt80 present in their nuclei. *NDT80* transcription is reduced in *dmc1* mutants. *rad24 dmc1* strains show wild type levels of *NDT80* transcription, as well as full levels of transcription of *NDT80*-controlled genes (Pak and Segall, 2002b). *rad24 dmc1* strains still show reduced sporulation, and make few mature spores because meiotic recombination cannot be finished in the absence of Dmc1 and segregation of chromosomes will occur randomLy in the absence of crossing over (Pak and Segall, 2002b). Furthermore, overexpressing *NDT80* in a *dmc1* cell can restore transcription of middle meiotic genes, though only ~20% of *dmc1 NDT80* O/E cells complete MI and MII compared ~90% for wild type (Pak and Segall, 2002b). While the Pachytene checkpoint is not activated in wild type cells (Carballo and Cha, 2007) (unlike the RIS) and may involve different signaling transducers than the RIS, these findings do illustrate that *NDT80* transcription is malleable in response to checkpoint stimuli and is therefore an excellent candidate for a target of the RIS.

To test my hypothesis that the transcription of the transcription factor *NDT80* is the target of the RIS, I examined transcription of *NDT80* in *rec102* and *rec104* strains compared to wild type. *REC102* and *REC104* are both required for the RIS. I also examined the transcription of *SPS4*. To test whether the proteins of the RIS or the formation of double-stranded breaks targets *NDT80* transcription, I examined *NDT80* and *SPS4* transcription in a *ski8* background. *ski8* mutants do not form DSBs, yet start the first division of meiosis at the same time as wild type (Gardiner et al., 1997; Malone et al., 2004). Lastly, data presented in this chapter show that early *NDT80* transcription is sufficient to create an early first division. This is consistent with the hypothesis that *NDT80* is the only target of the RIS.

[Logan Vidal, an Honors undergraduate performed the *NDT80* overexpression timepoint analysis. All the remaining experiments, including all of the northern analyses were performed by me.]

Materials and methods

Yeast strains used in experiments

The yeast strains used for the experiments described in this chapter are derived from the homothallic diploid K65-3D ultimately derived from S288C. K65-3D is homozygous for the following markers: *HO, lys2-1, tyr1-1, his7-2, can1r, ura3-13, ade5, met13-d, trp5-2, leu1-12, ade2-1*. Complete descriptions of all strains are detailed in in the Appendix. The *rec104* deletion strain used in this chapter is *rec104-Δ1* (Galbraith and Malone, 1992). The *rec102* and *ski8* mutant strains are deletion strains containing an insertion of the $G418^r$ gene. Creation and growth of these strains was as described in Chapter 2.

The *pHOP1-NDT80* (see below) containing-strains used in this project were created by transforming appropriate strains using lithium acetate-based transformation and selecting on SC-URA medium. SC medium (Synthetic Complete) medium contains 2% glucose, 0.5% Ammonium sulfate and complete supplementation of amino acids. SC-URA medium lacks the amino acid uracil.

Plasmids used in experiments

The pHOP1-*NDT80* (Malone stock number: B1493) plasmid used in this chapter was a generous gift from Jackie Segall (Pak and Segall, 2002a, b). It contains 990 bp of the *HOP1* promoter fused to the 1884 bp *NDT80* ORF plus 350 bp downstream cloned into the NotI-ClaI sites of the MCS of pRS316.

Media and growth conditions

All media not described below was described in Chapter 2. Sporulation and growth conditions are also described in Chapter 2.

Liquid sporulation and timepoints

Liquid sporulation experiments were as described in Chapter 2. 1 mL of cells were harvested for DAPI-staining as previously described. 15-20 mL of cells were harvested in a 50 mL conical tube with approximately 10-15 mL of crushed ice obtained from our ice machine and immediately centrifuged in a cold clinical centrifuge at top speed for 2 minutes. The supernatant was poured off and RNA was immediately extracted from the resulting cell pellet without freezing it first. For liquid sporulation experiments where a *URA3*-containing plasmid was present in a *ura3* strain background, cells were first grown 10-12 hours in 250 mL SC-URA medium in 1 L flasks before being harvested and resuspended in YPA medium to a concentration of 5×10^6 cells/mL. These cultures were grown for only two generations to a concentration of 2×10^7 cells/mL (typically about 7 hours to allow for any growth lag the cultures were experiencing). Sporulation and timepoint sampling was completed as described above. Plasmid retention was confirmed by plating appropriate dilutions of cells collected at zero hours on both synthetic complete and SC-URA medium and calculating the percentage of colonies which grew on the SC-URA medium divided by the number of colonies growing on SC-complete medium after at least 48 hours of growth.

Microscopy

DAPI staining and nuclei analysis was performed as described in Chapter 2.

RNA isolation and northern analysis

RNA was immediately extracted from 10-15 mL of sporulated cells (see above) using a protocol adapted from the Cross laboratory (McKinney et al., 1993). It takes ~40

minutes for me to extract the RNA from 6 timepoint samples, so timepoints could not be taken more frequently than every hour. All RNA solutions used were treated with 0.04 % Diethyl Pyrocarbonate (DEPC) overnight and then autoclaved. The entire isolation procedure was performed in the 4° C cold room on ice. The cell pellet was washed one time with dH_20, transferred to a 2mL round-bottomed microcentrifuge tube and centrifuged 30s in a microfuge. To the washed cell pellet, 400 µL of NETS buffer (0.3M NaCl, 1mM EDTA, 10mM Tris-HCl pH 7.5 and 0.2% SDS), 400µ l of Phenol-Chloroform-Isoamyl Alcohol solution (25:24:1) and approximately 500 µL of 500 micron glass beads (Biospec Products) were added. Samples were then vortexed at top speed on a Fisher Mini Vortexer using a hand-held multi-vortex holder to hold the tubes for 6 minutes. The samples were then centrifuged for 5 min. in a microfuge and the aqueous layer was carefully transferred to a new tube containing 1 mL of cold 95% ethanol. These were placed in the -80° C freezer until all of the timepoint samples were extracted then centrifuged for 5 minutes in a microfuge. The pellet was aspirated and dried for 10 minutes in a spin-vac at room temperature before being resuspended in 50 µL of ETS buffer (10 mM Tris-HCl pH 7.9, 1 mM EDTA, 0.2%, 0.2 % SDS). Samples were placed in a 65°C water bath for 10-15 min with occasional vortexing to aid resuspension. All samples were quantified using the Nanodrop spectrophotometer (Thermoscientific) in the Center for Comparative Genomics (CCG). Typical yields from 10 mL of cells were 100-200 µg of RNA.

A formaldehyde-based denaturing gel (1 % agarose, 6.3% formaldehyde, 0.02 M MOPS buffer pH 7.0) was used to run all RNA samples. Thirty micrograms of RNA was loaded into each gel lane using 2X RNA gel loading buffer. 2 x gel loading buffer contains 50% Formamide, 0.02 M MOPS buffer pH 7.0, 5.9% Formaldehyde, 6.66% Glycerol, 13.33 % Saturated Bromophenol Blue stock solution (to make this , bromophenol blue is dissolved in dH2O until the point of saturation) and 0.006% Ethidium Bromide. The loading dye was freshly prepared each time. The formamide

used to make the 2X loading buffer was freshly-deionized by adding 5-7 mL formamide with approximately 1 mL of AG 501-X8 (D) resin (Bio-Rad) in a 15mL conical tube, then shaking the tube for 1-2 minutes (by hand) and decanting the formamide into a fresh tube. Samples with loading dye added were heated at 65° C for 10 min, and then placed on ice while the gel was loaded. Large Gels (22 cm length) were run overnight (12-15 h) at ~ 70 V until the bromophenol blue in the dye had migrated to the bottom of the gel. The gel was photographed then soaked for 20 minutes in DEPC-treated water and blotted using standard capillary-transfer methods to Hybond N nylon membrane (Amersham, GE Health Products). Because the hybridization bottles that I used to probe my membranes can only accommodate 10 cm-long membranes, it was necessary to cut the membranes to size before proceeding. A 10 cm region of the membrane was excised from the center of each blot encompassing the region around the ribosomal bands with a razor blade using the gel photograph as a rough guide. All three of the transcripts of interest were present between the ribosomal bands. I then cross-linked the membrane for 5 min in the Malone lab homemade UV trans-illuminator box. Blots were placed in large hybridization bottles with 25 mL of Perfect Hyb Plus hybridization buffer (Sigma) and put into a 67° C Bellco Autoblot microhybridization rotating oven to pre-hybridize for at least 1 hour. Approximately 200 ng of ^{32}P-labelled probe were added to the hybridization bottle and returned to the rotating oven for 12-20 hours. Probes used were generated by Random-prime labeling PCR products made from the full-length coding region of the desired gene using the Invitrogen Random prime labeling kit and 20-50 μCi of alpha ^{32}P dATP (NEN, GE Healthcare). All probes used incorporated 90% or more of the ^{32}P, as estimated by the Geiger counter.

Blots were washed once with 100 mL 2X SSPE (20X SSPE is 3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA) at 67° C for 10 minutes in the hybridization oven and three times with 100 mL 2X SSPE, 0.1 % SDS at 67° C for 10 minutes. Blots were

exposed at least 24 hours on phosphorimager screens (Molecular Dynamics) and scanned using a Bio-Rad Phosphorimager imaging system at the CCG.

Blots were stripped before being probed with subsequent probes by washing 2 X 5 minutes with 200 mL boiling 1 % SDS in the hybridization bottle placed in the 67° C hybridization oven. After stripping, the blots were rinsed with 1 L of 2 X SSPE. All blots were probed first with *NDT80*, second with *SPS4* and last with the loading control *ENO1*.

All phosphorimager data were analyzed using Quantity One software. *SPS4* and *NDT80* levels are presented as both raw data and normalized to the loading control *ENO1*. Normalization was done by dividing the quantity of transcription of either *SPS4* or *ENO1* in a lane by the quanitity of transcription of *ENO1* in the same lane.

Results

A target of the signal for recombination initiation

A candidate for a target of the recombination initiation signal that delays the first division is the *NDT80* gene. This gene encodes a positive regulator of the middle meiotic genes, and *ndt80* mutants arrest before the first division (Hepworth et al., 1998; Xu et al., 1995). I therefore examined the transcription of *NDT80* in WT, *rec104*, and *rec102* mutants (Figure 4-2). Rec102 and Rec104 are both members of the RIS. *rec104* mutants start MI ~1.5 hours earlier than wild type, while *rec102* mutants start MI ~ 2 hours earlier. If *NDT80* transcription were a target of the RIS, I would expect to see *NDT80* transcription start earlier in *rec102* and *rec104* strains. Evidence indicates that Ndt80 must also be post-translatioanlly modified for full activity, therefore *SPS4*, a gene whose transcription is entirely dependent on *NDT80*, was used as a reporter of Ndt80 activity (Pak and Segall, 2002b). *NDT80* transcription begins about one hour earlier in both *rec102 and rec104* mutants compared to WT cells (Fig. 4-2 A, B and D). (Figure 4-2 B). Transcription of *NDT80* is 1.9 and 4.3-fold higher in *rec104* and *rec102* mutants,

respectively than in wild type at 5 hours (4-2 D). Interestingly, transcription of *NDT80* decreases in both *rec102* and *rec104* mutants by 8 hours. Presumably, I would notice a similar turnover in wild type at ~10 hours.

SPS4 transcription begins earlier and reaches higher levels in *rec102* mutants than in *rec104* mutants (e.g., compare *SPS4* transcription at 5 and 6 hours of sporulation in Figure 4-2 B and C) consistent with the earlier start of the MI division. Minimal transcription of *SPS4* is detected at 4 hours in wild type strains in *rec102* strains; no transcription wasdtected in either *rec104* cells or WT cells at this time. Transcription of *SPS4* is 4.4 and 5.4-fold higher in *rec102* and *rec104* mutants, respectively, than in wild type at 6 hours (Fig. 4-2 E). These data suggest that *NDT80* is a target of the recombination initiation signal.

Absence of DSBs does not cause an early transcription of *NDT80* in RIS mutants

We have hypothesized that the presence of eight RIS proteins sends a signal that delays the start of MI in wild type cells, rather than the DSBs made by the initiation complex. The previous data suggests that the target of the RIS is *NDT80*. To further test this hypothesis, we examined transcription of *NDT80* in a recombination initiation mutant that is not a member of the RIS. *ski8* mutants cannot make DSBs (Malone et al., 1991), yet start the reductional division at the same time as wild type (Malone et al., 2004) (Chapter 2). Our model predicts that *NDT80* and *SPS4* will not be expressed early in *ski8* strains as in *rec102* mutants. Neither *NDT80* nor *SPS4* are expressed earlier than in wild type; the transcription of *NDT80* and *SPS4* may actually be somewhat later in *ski8* mutants than in wild type cells (Figure 4-3).

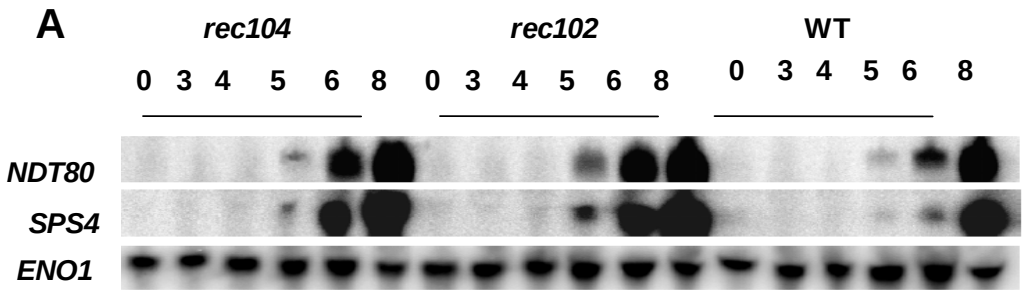
The *SPS4* transcription differences between *rec102, ski8* and WT are even more striking (Fig. 4-3 C and E). At eight hours, transcription of *SPS4* is 19-fold higher than in wild type, while *SPS4* transcription in *ski8* is only 26% of wild type levels (Fig. 4-3 E).

Table 4-1: Spore viability in mutant strains examined in this chapter

Strain name	Malone Lab Stock number	Relevant Genotype	% Viability	Total Spores
K65-3D [p*HOP1-NDT80*]	M3675	*NDT80* O/E	41	80
EG 1-1-2B	M3371	*rec104*	0*	400
SEN 2-1-7D	M3577	*rec102*	0*	400
KF 2-11-1B	M3497	*ski8*	0*	80
K65-3D	M156	WT	97	1220

Note: Spore viability in wild type (WT) and mutant strain tetrads. Viability of strains was assessed by dissecting tetrads and examining individual spores. **rec104, rec102* viability was assessed by setting out whole asci on sporulation plates rather than dissecting individual spores. Viability of *rec104, rec102* and WT was determined by Kai Jiao, a former graduate student.

Figure 4-2: Transcription/activity of *NDT80* in Rec$^-$ mutants undergoing an early first division. [A] The genotype of the strain examined is shown at the top. Numbers below the genotypes indicate the time in sporulation that RNA was isolated. The probes used for the Northerns are shown to the left. [B] The amount of *NDT80* transcription (corrected for loading by *ENO1*) is shown vs. time. The black bar represents transcription in WT cells, the red bar transcription in *rec104* cells, and the blue bar transcription in *rec102* cells. [C] Same as in [B], but showing the transcription of *SPS4*. [D] Quantification of *NDT80* transcription. All values are shown divided by the counts per mm^2 of *ENO1* (See methods). Transcription: wild type refers to the *ENO1*-normalized *NDT80* transcription of a strain/ the ENO1-normalized transcription of WT. [E] Same as [D], but showing the transcription of *SPS4*. All data are shown from one experiment. A second experiment was performed, giving similar results, that is not shown. All Northerns were done by first probing for *NDT80*, followed by *SPS4*, then *ENO1*. The blots were stripped in between. See methods for details.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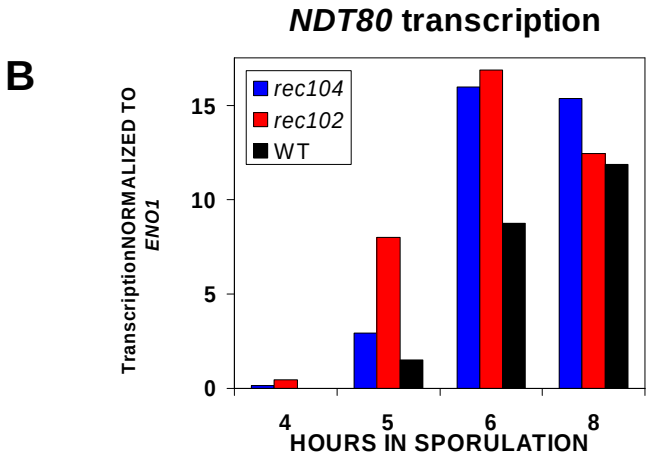
rec102
WT
0 3 4 5 6 8 0 3 4 5 6 8 0 3 4 5 6 8
NDT80
SPS4
ENO1


B
NDT80 transcription
rec104
rec102
WT
TranscriptionNORMALIZED TO ENO1
0
5
10
15
4
5
6
8
HOURS IN SPORULATION

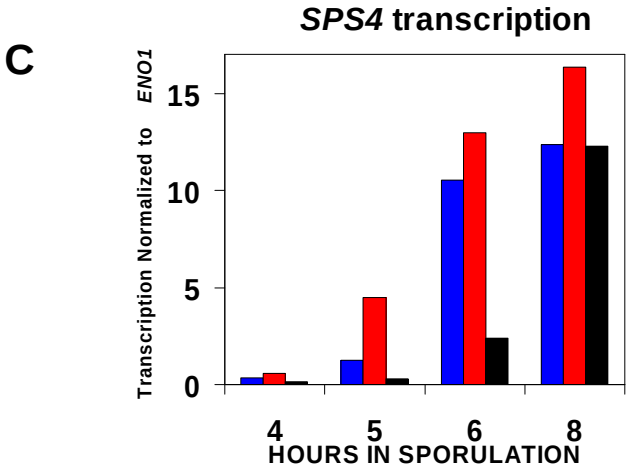

C
SPS4 transcription
Transcription Normalized to ENO1
0
5
10
15
4
5
6
8
HOURS IN SPORULATION

Figure 4-2, continued:

D. Quantification of *NDT80* transcription in Rec$^-$ mutants

Strain Name	Relevant Genotype	Time (h)	transcription normalized to *ENO1*	transcription / wild type
EG 1-1-2B	*rec104*	4	n.d. *	-
		5	2.9	1.5
		6	16	1.8
		8	15	1.3
SEN 2-1-7D	*rec102*	4	n.d. *	-
		5	8.0	5.3
		6	6.9	1.9
		8	12	1.0
K65-3D	WT	4	n.d. *	-
		5	1.5	1.0
		6	8.8	1.0
		8	12	1.0

*No transcription detected.

Figure 4-2, continued:

E. Quantification of *SPS4* transcription in Rec$^-$ mutants

Strain Name	Relevant Genotype	Time (h)	transcription normalized to *ENO1*	transcription : wild type
EG1-1-2B	*rec104*	4	n.d. *	-
		5	1.3	-
		6	11	4.4
		8	12	1.0
SEN 2-1-7D	*rec102*	4	n.d. *	-
		5	4.5	-
		6	13	5.4
		8	16	1.3
K65-3D	WT	4	n.d. *	-
		5	n.d. *	-
		6	2.4	1.0
		8	12	1.0

*No transcription detected.

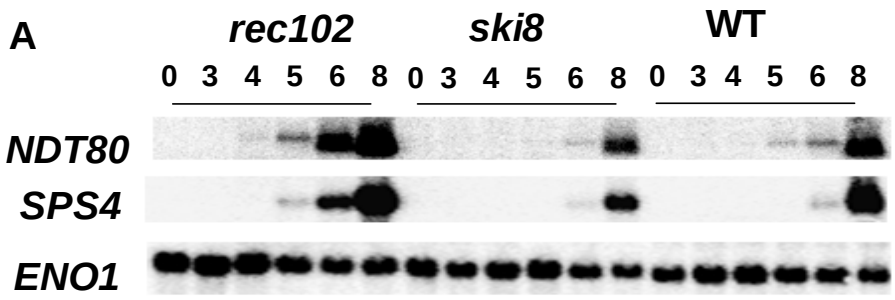
Because *NDT80* is clearly not expressed or active at a time earlier than wild type, I conclude that it is the proteins of the RIS rather than DSBs that delay the transcription of *NDT80* in wild type cells.

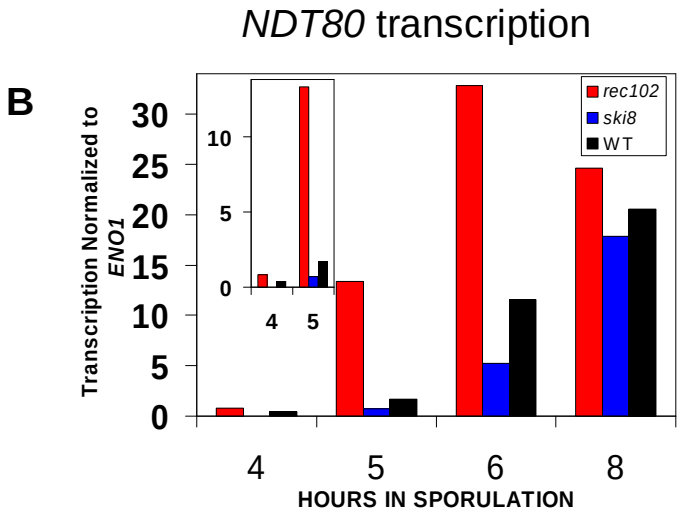
Earlier transcription of *NDT80* results in an earlier first division

The experiments described above support the hypothesis that *NDT80* is a downstream target of the RIS. I have shown that *NDT80* is expressed earlier in both *rec102* and *rec104* mutants (also in Malone, et al., 2004). Logan Vidal, an Honors undergraduate student, and I analyzed the meiotic divisions in a strain expressing *NDT80* at an earlier time from a *HOP1* promoter. The transcription of *HOP1* is controlled by the transcriptional activator Ime1; thus, *HOP1* is an early meiotic gene. According to a micoaaray study of the genes transcribed during sporulation, abundance of the *HOP1* transcript increases 11.1 fold over the amount detected in vegetative cells after 1 hour in sporulation medium (Chu et al., 1998). The pHOP1-*NDT80* plasmid is a CEN vector meaning that only one copy is present per cell. The two chromosomal copies of *NDT80* were also present in these strains, suggesting that *NDT80* should also be overexpressed at normal times. *NDT80* is induced 33.4-fold at 5 hours in sporulation (Chu et al., 1998). Our model predicts that wild type cells containing a high copy plasmid with pHOP1-*NDT80* should start the reductional division earlier than wild type.

Early expression of *NDT80* in a wild type strain caused a decrease in spore viability (41% for pHOP1-*NDT80* strains vs. 97% for wild type), but the reduction in viability was not as severe as eliminating DSB formation (0% viability for *rec102, ski8* and *rec104*) (Table 4-1). The reductional division may even begin earlier in overexpression strains than in *rec102* strains (Fig. 4-4 A) (see Discussion). MI appears to start at approximately 4 hours in the early expression strain compared to at 5 hours for *rec102* and 7 hours for wild type. In any case, the fact that the early expression strain started MI earlier than in WT cells shows that transcription of *NDT80* is sufficient for an

Figure 4-3: Transcription/activity of *NDT80* in *ski8* mutants [A] The genotype of the strain examined is shown at the top. Numbers below the genotypes indicate the time in sporulation that RNA was isolated. The probes used for the Northerns are shown to the left. [B] The amount of *NDT80* transcription [corrected for loading by *ENO1 (*see methods)] is shown vs. time. The black bar represents transcription in WT cells, the red bar transcription in *rec102* cells, and the blue transcription in *ski8* cells. [C] Same as in [B], but showing the transcription of *SPS4*. [D] Quantification of *NDT80* transcription. All values are shown divided by the counts per mm^2 of *ENO1* (See methods). Transcription: wild type refers to the *ENO1*- normalized *NDT80* transcription of a strain/ the ENO1-normalized transcription of WT. [E] Same as [D], but showing the transcription of *SPS4*. All data are shown from one experiment. A second experiment was, performed giving similar results, that is not shown. All Northerns were done by first probing for *NDT80*, followed by *SPS4*, then *ENO1*. The blots were stripped in between. See methods for details.


A
rec102
ski8
WT
0 3 4 5 6 8 0 3 4 5 6 8 0 3 4 5 6 8
NDT80
SPS4
ENO1


NDT80 transcription
B
Transcription Normalized to ENO1
rec102
ski8
WT
HOURS IN SPORULATION

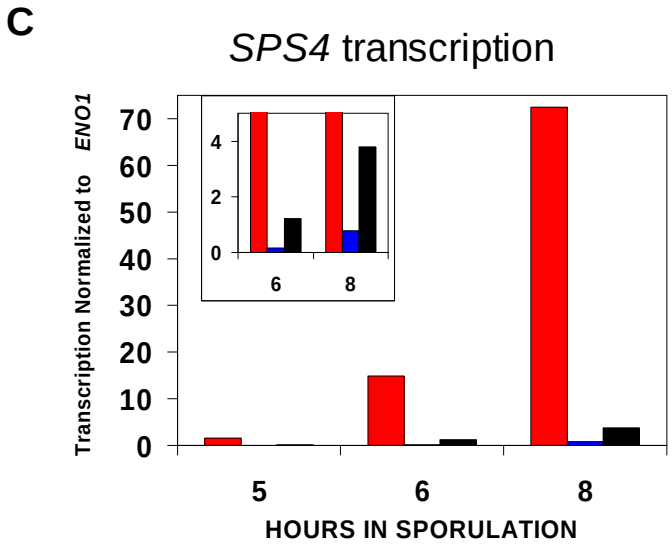

C
SPS4 transcription
Transcription Normalized to ENO1
HOURS IN SPORULATION

Figure 4-3, continued

D. Quantification of *NDT80* transcription in *ski8*

Strain Name	Relevant Genotype	Time (h)	transcription normalized to *ENO1*	transcription / wild type
SEN 2-1-7D	*rec102*	4	n.d. *	-
		5	13	7.9
		6	33	2.8
		8	25	1.2
KF 2-11-1B	*ski8*	4	n.d.*	-
		5	n.d.*	0.42
		6	5.3	0.46
		8	18	0.87
K65-3D	WT	4	n.d.*	-
		5	1.7	1.0
		6	12	1.0
		8	21	1.0

*No transcription detected.

Figure 4-3, continued:

E. Quantification of *SPS4* in *ski8*

Strain Name	Relevant Genotype	Time (h)	transcription normalized to *ENO1*	transcription/ wild type
SEN 2-1-7D	*rec102*	5	2.0	-
		6	15	12
		8	73	19
KF 2-11-1B	*ski8*	5	n.d.*	-
		6	n.d.*	-
		8	1.0	0.26
K65-3D	WT	5	n.d.*	-
		6	1.2	1.0
		8	3.8	1.0

*No transcription detected

early reductional division. This experiment was performed twice; each trial showed similar results.

Transcription of the pHOP1-*NDT80* overexpression plasmid was confirmed by Northern analysis of *NDT80* and the reporter gene *SPS4* (Fig. 4-3 C). The strain containing the pHOP1-*NDT80* plasmid showed both earlier and higher levels of transcription of *NDT80* and *SPS4* than either wild type or *rec102* strains (Fig. 4-3 D and F). While at 3 hours there is no significant transcription of *NDT80* in wild type cells, the pHOP1-*NDT80* strain has already begun. At the same time, the *rec102* strain has no significant amount of *NDT80* mRNA. At 4 hours, the wild type amount of *NDT80* transcription is still undetectable, though transcription of *NDT80* has clearly started in both the *rec102* control strain and the overexpression strain. I conclude that the transcription of *NDT80* occurs even earlier in the presence of the early expression plasmid than it does in *rec102* mutants.

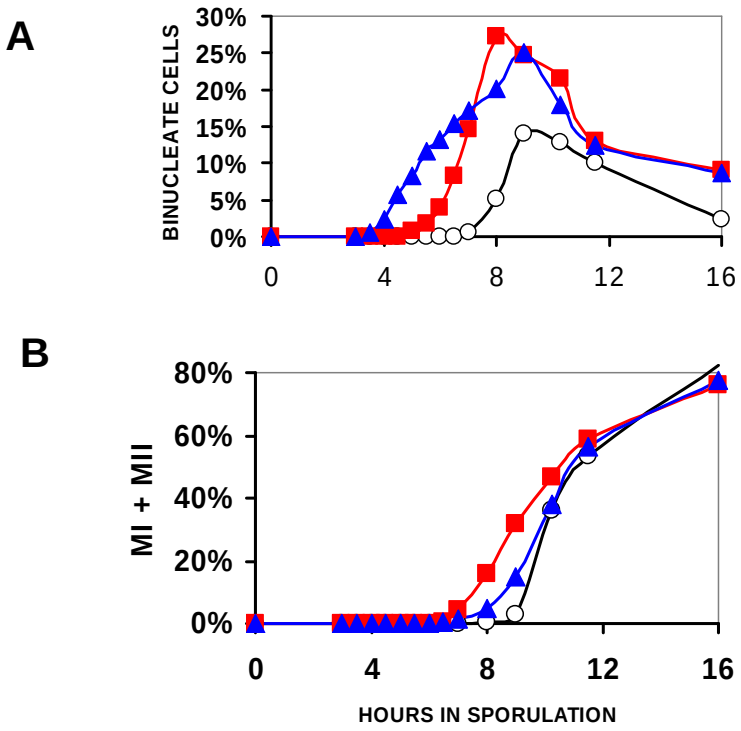
Taken together these results support the hypothesis that *NDT80* is the only target of the RIS. *NDT80* is both necessary and sufficient to mediate an early reductional division.

Discussion

NDT80 is a target of the RIS.

We have shown that the presence of eight of the ten recombination initiation proteins plus Hop1 and Red1 are required to delay the start of MI. Strains with mutations in any one of these ten genes start the reductional division earlier than wild type strains Our lab has shown that the RIS acts prior to MI spindle formation (Malone et al., 2004), suggesting that a target of the RIS should be required for MI spindle formation , hence the first division. The *NDT80* gene is just such a candidate. Null mutations in *NDT80* arrest in pachytene and although the spindle pole body duplicates, it does not separate and MI spindles do not form (Hepworth et al., 1998; Xu et al., 1995)

Figure 4-4: Meiotic divisions in a strain expressing *NDT80* early. In all graphs, the divisions in WT cells are shown as black open circles, the *rec102* early control is red squares, and the WT strain containing pPHOP1-*NDT80* is blue triangles and is the average of two cultures. A second independent experiment was performed for this strain with identical results. The average sporulation values for this experiment were: WT, 82%, *rec102*, 31%; *NDT80* early expression strains, 70.5%. [A] Timing of the first division indicated by the appearance of binucleate cells. [B] MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells. [C] Northern analysis of *NDT80* in strains with and without the plasmid pHOP1-*NDT80*. Transcription of *SPS4* indicates the presence of active *NDT80* product. *ENO1* served as a loading control. [D]Graph *NDT80* in *rec102*, shown in red, a wild type strain containing the pHOP1-*NDT80* plasmid, shown in blue, and WT shown in black. Transcription is normalized to the loading control, *ENO* (see Methods). [E] Same as [D], but showing transcription of *SPS4*. [F] [D] Quantification of *NDT80* transcription. All values are shown divided by the counts per mm^2 of *ENO1* (See methods). Transcription: wild type refers to the *ENO1*- normalized *NDT80* transcription of a strain/ the ENO1-normalized transcription of WT. [E] Same as [D], but showing the transcription of *SPS4*. All data are shown from one experiment. A second experiment was performed, with similar results, that is not shown. All Northerns were done by first probing for *NDT80*, followed by *SPS4*, then *ENO1*. The blots were stripped in between. See methods for details.


A
BINUCLEATE CELLS
30%
25%
20%
15%
10%
5%
0%
0
4
8
12
16
B
MI + MII
80%
60%
40%
20%
0%
0
4
8
12
16
HOURS IN SPORULATION

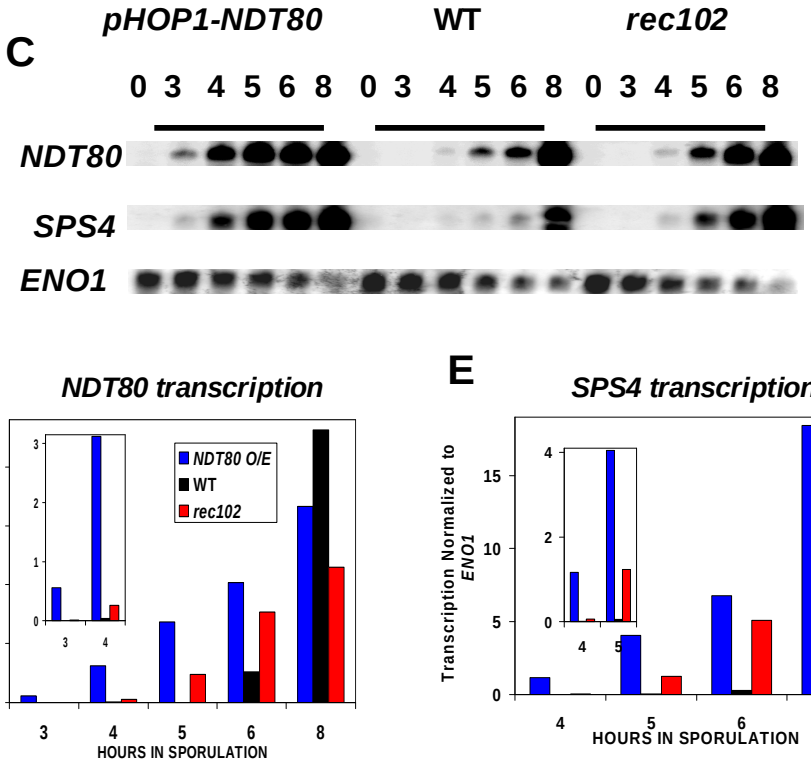

C
pHOP1-NDT80
WT
rec102
0 3 4 5 6 8 0 3 4 5 6 8 0 3 4 5 6 8
NDT80
SPS4
ENO1
D
NDT80 transcription
Ctranscription normalized to ENO1
NDT80 O/E
WT
rec102
HOURS IN SPORULATION
E
SPS4 transcription
Transcription Normalized to ENO1
HOURS IN SPORULATION

Figure 4-4, continued:

F. Quantification of *NDT80* Transcription in *NDT80* O/E strain

Strain Name	Relevant Genotype	Time (h)	transcription normalized to *ENO1*	transcription: wild type
K65-3D [p*HOP1-NDT80*]	*NDT80* O/E	3	5.6	-
		4	31	-
		5	69	67
		6	102	3.9
		8	170	0.74
SEN 2-1 7D	*rec102*	3	n.d*	-
		4	2.6	-
		5	24.1	24
		6	77	2.9
		8	120	0.52
K65-3D	WT	3	n.d*	-
		4	n.d*	-
		5	1.0	1.0
		6	26.0	1.0
		8	230	1.0

*No transcription detected.

Figure 4-4, continued:

G. Quantification of *SPS4* transcription in *NDT80* O/E strain

Strain Name	Relevant Genotype	Time (h)	transcription normalized to *ENO1*	transcription / wild type
K65-3D [p*HOP1-NDT80*]	*NDT80* O/E	4	12	-
		5	41	-
		6	68	25
		8	180	4.3
SEN 2-1-7D	*rec102*	4	n.d. *	-
		5	12	-
		6	51	19
		8	190	4.3
K65-3D	WT	4	n.d. *	-
		5	n.d. *	-
		6	2.7	1.0
		8	43	1.0

*No transcription detected.

This indicates that *NDT80* is involved in the communication between recombination initiation functions and the reductional division. The transcription of *NDT80 i*s increased at earlier times in both *rec102* and *rec104* mutants (Fig. 4-2). *SPS4* is a middle meiotic gene regulated only by *NDT80* and not by other meiotic transcriptional regulators

[Jacqueline Segall, personal communication to R. Malone, and (Pak and Segall, 2002a, b)]; *SPS4* transcription therefore is a reporter for active Ndt80. *SPS4* is clearly transcribed at earlier times in *rec102* than in *rec104*, and earlier in *rec104* than in WT cells (Fig. 4-2).

Transcription of *NDT80* ioccurs somewhat earlier in *rec102* stains than in *rec104* strains. This suggests that the RIS is composed of two parts. This is not likely due to a difference in order of assembly of the recombination initiation complex between Rec102 and Rec104 because all evidence indicates that Rec104 and Rec102 are present in the same subcomplex and are likely recruited to the DNA together (Arora et al., 2004; Jiao et al., 2003; Kee and Keeney, 2002). Phosphorylation or other post-translational modifications could determine the order of activation for the RIS. Both Rec102 and Rec104 are phospho-proteins, though it is unknown which kinase phosphorylates either of these proteins.

The early MI in RIS mutants is not due to the absence of DSBs

ski8 strains do not form DSBs. *NDT80* is not expressed earlier in a *ski8* mutant than in wild type; transcription of *NDT80* may actually be delayed by an hour in *ski8* strains compared to wild type (Fig. 4-3). This supports our model that *ski8* is not part of the RIS (discussed in chapter 2). If Ski8 were a part of the RIS, then *ski8* strains would show transcription of *NDT80* earlier than in wild type. It is somewhat perplexing that *NDT80* transcription is later than wild type in *ski8* strains because I have shown that *ski8*

strains start the reductional division at the same time as wild type (chapter 2). This is could be due to small variation in growth conditions between the two experiments. An alternative explanation is that perhaps only minimal Ndt80 is required for the reductional division. This could be tested by by modulating expression of *NDT80* from a controllable promoter.

Ndt80 activity is essential for MI spindle formation (Hepworth et al., 1998; Xu et al., 1995). Homologous chromosomes are held together by recombination intermediates. This causes tension of the MI spindle. Andrew Murray has proposed that this interhomolog tension is monitored by the spindle checkpoint and that inter-homolog tension regulates the timing of MI (Shonn et al., 2000) (discussed in detail in chapter 3). Because *NDT80* transcription is delayed by the RIS, this indicates that the MI spindle checkpoint is not responsible for regulating the start of MI. If *NDT80* is not yet expressed, there can be no MI spindle, hence no spindle checkpoint. The RIS acts before spindle formation occurs.

Delaying transcription of *NDT80* should be sufficient to delay the start of MI

All of our data support that the hypothesis that *NDT80* is a downstream target of the RIS (Malone et al. 2004) (this chapter). An *ndt80* mutant arrests prior to the first division with one nucleus and no meiotic spindles (Hepworth et al., 1998; Xu et al., 1995). If *NDT80* were the only downstream target of the RIS that delays the start of MI in WT cells, then overexpressing *NDT80 at* an earlier time should result in an earlier MI, thus mimicking the initiation mutants. We observed that overexpressing *NDT80* results in an earlier MI similar to the early MI seen in a *rec102* mutant (Fig. 4-4). We conclude that *NDT80* transcription is both necessary and sufficient for the MI division. Our data are consistent with the hypothesis that *NDT80* is a target of the RIS. Given its known essential role in meiosis, it may be the only target.

It is interesting to note that overexpression of transcription of *NDT80* from an early meiotic promoter decreases the viability of spores compared to wild type (Table 4-1). One would predict that expressing *NDT80* before recombination is complete would lead to aneuploidy and broken chromosomes. Therefore, it is somewhat surprising that any of the *NDT80* overexpression spores are viable at all. The viable spores must have had sufficient recombination to allow homolog pairing, and have finished recombination in order to avoid broken chromosomes. This is different from a *rec104* mutant, which has no recombination. Thus, homologous chromosomes in *rec104* cells cannot correctly pair, resulting in the formation of inviable spores.

How does the RIS target *NDT80*?

These experiments do not provide a mechanism for how the RIS affects the transcription of *NDT80*. It is very unlikely that the RIS directly interacts with the transcriptional machinery responsible for upregulating *NDT80*. Rather, it is more likely that a signaling cascade relays the RIS and ultimately delays the transcription of *NDT80*. In chapter 5, I present data implicating the meiosis specific kinase Mek1 as a transducer of the RIS.

The control of *NDT80* in meiosis is complex and occurs both at transcriptional and post-transcriptional level of events (Pak and Segall, 2002b; Tung et al., 2000) . Furthermore, regulation of *NDT80* transcription involves several protein factors that themselves have complex regulation (Fig. 4-1). The Sum1 repressor of *NDT80* would seem one likely candidate for a target of the RIS. Sum1 represses the transcription of middle sporulation genes including *NDT80* by binding to MSEs. During vegetative growth, Sum1 is bound to MSEs along with the histone deacetylase Hst1. Hst1 and Sum1 are tethered to MSEs by Rfm1 (McCord et al., 2003). It is unclear whether Rfm1 remains bound to MSEs, but complexes of all three of these proteins can be co-purified.

To fully derepress the MSE occupied by Sum1 complexes, in meiosis, phosphorylation by Ime2 is required (Moore et al., 2007).

The Stewart lab has published that a complex of the Set3 histone methyltransferase and Hos2 histone deacetylase repress MMGs by binding to MSEs rather than Sum1/Hst1 complexes (Pijnappel et al., 2001). They reported that *hos2 and set3* mutants started the first division of meiosis earlier than a wild type strain and thus Hos2 seemed like an excellent candidate for the RIS pathway. Their strain background and/or the method by which they sporulated their strains are much less efficient than ours, because they needed to take timepoints over a course of 75 hours to visualize both MI and MII. In contrast MI and MII are mostly complete by 16 hours in out strain background. They took timepoints only every five hours and counted only 300 cells/timepoint and showed *hos2* strains started MI maybe 5 hours earlier than wild type. To test their observation, Jaime Williams, an Honors undergraduate in our lab, examined the timing of the first division in a *hos2* mutant with our strains and techniques, to determine if Hos2 had a role in the RIS pathway. She found that a *hos2* strain started the first division at a time indistinguishable from wild type indicating that Hos2 is not involved in RIS pathway (Jaime Williams, Honors thesis). One explanation for this contradictory result is that perhaps in our strain background both Hos2 and Set3 must be absent in order to observe an earlier MI. This seems unlikely, as Hos2 has been shown to always require Set3 to silence promoters, so a *hos2* strain should have the same phenotype as a *set3* strain. An alternative explanation is that Hos2 is not actually a negative regulator of Sum1. Vershon's lab disagrees that Hos2 and Set3 are negative regulators of *NDT80* transcription (McCord et al., 2003). They found no significant decrease in the transcription of several MMGs in *hos2* and *set3* mutants. In contrast, in a *sum1* mutant they reported a 33-fold increase in transcription of the MMG loci *SMK1, YLR343w, YFL012w* and *YAL018c*. They found a significant, though smaller, increase in MMG transcription in *hst1* and *rfm1* mutant strains (4.2 and 5.5-fold increase,

respectively). From these observations, I conclude that Hos2 is not required for the RIS pathway in controlling *NDT80* transcription. Sum1, acting with Rfm1 and Hst1, is a more likely candidate, but further experiments are necessary.

CHAPTER 5

TRANSDUCTION OF THE RIS

Abstract

Checkpoints are systems of proteins which monitor the major events of the cell cycle. Checkpoints can regulate both the response to cellular damage (*e.g.*, a cell cycle arrest due to UV damage) and can also regulate normal cellular events (e.g., the timing of cohesin degradation). We have shown that the RIS delays the start of the reductional division by delaying the transcription of the middle meiotic regulator *NDT80.* In this chapter, I present experiments elucidating the transduction of the RIS. We used a candidate gene approach to study this. If a candidate gene were involved in transducing the RIS, then removing that gene would result in no transduction of the RIS, hence an earlier first division. Using this approach we excluded members of the S phase (Mec1 and Rad53) and DNA damage checkpoints (Rad9, Rad24, as well as Rad53 and Mec1) as transducers of the RIS. Rachel Gast previously showed that members of the spindle checkpoint are not required for transduction of the RIS, however, her very clear result that the spindle checkpoint protein Mad3 is not required for the delay of the reductional division was different than the conclusion drawn by the Dawson lab (Cheslock, et al., 2005). In order to confirm Gast's conclusion that Mad3 does not transduce the RIS, I examined transcription of *NDT80* as well as transcription of a reporter for Ndt80 activity in a *mad3* strain. *mad3* transcription was only minimally earlier than in wild type, but not nearly as early in a rec102 strain supporting Rachel's conclusion that Mad3 does not transduce the RIS. Finally, I present evidence consistent with a hypothesis that the exclusively meiotic kinase Mek1 is responsible for transducing the RIS. *mek1* mutants start the reductional division early. Furthermore, overexpressing *MEK1* in both wild type and *spo11* strain backgrounds results in an earlier reductional division.

Introduction

In order for chromosomes in *Saccharomyces cerevisiae* to segregate correctly during meiosis, cells must engage in a seemingly drastic step of creating ~200 DSBs (Burgess, 2002; Keeney, 2001; Martini and Keeney, 2002; Paques and Haber, 1999). In *Saccharomyces cerevisiae*, these DSBs are repaired with a 5-fold bias toward interhomolog recombination as opposed to repair from sister chromatids (Gerton and Hawley, 2005; Schwacha and Kleckner, 1997). This promotes the pairing of homologous chromosomes allowing correct segregation during the reductional division. Recombination must be timed to occur precisely between DNA replication and the reductional division. If the sequence of events in meiosis is to occur correctly, cells likely have a signaling network in place to monitor and coordinate these events. This introduction will focus of genes and events in *S. cerevisiae*, though many of the genes that I will discuss have homologs in many eukaryotes and many of the processes I will present are conserved.

Checkpoints are points during the cell cycle where the cell can arrest or slow progression if there is damage to the cell. Additionally, checkpoint proteins coordinate many major events in the wild type cell such as mitotic exit. According to Hochwagen and Amon (2006), checkpoint components are defined as fulfilling four criteria: 1) a signal, 2) detection of this signal by sensory proteins, 3) activation of signal transduction pathways and 4) translation of the signal into an output by modifying checkpoint targets.

During vegetative growth even one unrepaired DSB will arrest cell cycle progression (Fogel and Mortimer, 1971). The DNA damage checkpoint senses this damage and signals to downstream effectors to stop the cell cycle (Harrison and Haber, 2006; Paulovich and Hartwell, 1995; Weinert et al., 1994). There is significant evidence that the events of meiosis are also monitored by a system of checkpoints some of which are similar to that of mitosis (Carballo and Cha, 2007; Hochwagen and Amon, 2006).

Checkpoints in mitosis

Three main checkpoints important in monitoring chromosomal behavior during thr mitotic cell cycle: the DNA damage checkpoint, the S phase checkpoint, and the spindle checkpoint (Harrison and Haber, 2006; Lew and Burke, 2003; Sclafani and Holzen, 2007). There is similiarity between some of the components of these checkpoints and they can impinge on similar targets. However, the three checkpoints differ in signal input (Pasero et al., 2003). The DNA damage checkpoint monitors DSBs and other DNA damage, the S phase checkpoint monitors replication fork progression and structure and helps to ensure that all origins of replication fire only once and the spindle checkpoint monitors correct attachment of spindle fibers between spindle pole bodies (SPBs) and kinetochores and mitotic exit. A detailed description of these three checkpoints can be found in Chapter 1. This chapter will specifically focus on the genes Mec1 and Rad53 which are required for the S phase checkpoint response and the DNA damage checkpoint response, Rad24 and Rad9 which are proteins that monitor the DNA damage checkpoint and Mad3, a protein required for the spindle assembly checkpoint.

Checkpoints in meiosis

Checkpoints have been shown to be important for monitering the chromosomal events of meiosis(Carballo and Cha, 2007; Hochwagen and Amon, 2006). The DNA damage, spindle and S phase checkpoints function during meiosis with a few key differences. The DNA damage checkpoints and S phase and spindle checkpoints present in meiosis are reviewed in Chapter 1. In addition to these three checkpoints, the Pachytene checkpoint (sometimes called the recombination checkpoint), is a checkpoint that causes mononucleate cell arrest before the reductional division in response to an excess of unprocessed ssDNA or certain types of SC defects. The Pachytene checkpoint is unique to meiosis though it shares many of the same members with mitotic checkpoints.

The Pachytene checkpoint

The Pachytene checkpoint has largely been characterized in cells that have a mutation in the gene *DMC1* (Hochwagen and Amon, 2006). As described in Chapter 1, these cells have an abundance of ssDNA, and therefore arrest (Bishop et al., 1992). It has been proposed that the Pachytene checkpoint senses this excess os ssDNA (Carballo and Cha, 2007). Rad17, Rad24, Mec1, Mec3, Ddc1 and Mec1 have all been shown to be required for arrest in *dmc1* cells (*e.g.*., a *rad24 dmc1* strain no longer arrests (Hochwagen and Amon, 2006; Lydall et al., 1996; Roeder and Bailis, 2000). A key difference between the DNA damage checkpoint in mitosis and the Pachytene checkpoint is that Rad9 and Rad53 are not required for the arrest of a *dmc1* cell (Hochwagen and Amon, 2006). Tel1 is also not necessary for the Pachytene checkpoint.

Mek1 is a meiosis-specific kinase required for the the Pachytene checkpoint (Bailis and Roeder, 2000; de los Santos and Hollingsworth, 1999; Niu et al., 2005; Wan et al., 2004) . Mek1 is a paralog of Rad53 and it has been proposed that Mek1 substitutes for Rad53 in the Pachytene checkpoint (Carballo and Cha, 2007). Mek1 interacts with Hop1 and Red1, though the details of this interaction are still being studied (de los Santos and Hollingsworth, 1999; Niu et al., 2005; Wan et al., 2004). Data from Holiingsworth's lab suggests that even though Mek1 forms a complex with Hop1 and Red1, it does not phosphorylate either protein. Both Hollingsworth and Roeder propose that Red1 is required for arrest in a *dmc1*cell and that Hop1 may be as well (Bailis and Roeder, 2000; de los Santos and Hollingsworth, 1999; Niu et al., 2005; Wan et al., 2004). An alternate interpretation is that *dmc1*-induced ssDNA can only be recognized in the context of the SC. No phosphorylation targets of Mek1 have yet been discovered.

The SC is also monitored by the proteins of the Pachytene checkpoint (Roeder and Bailis, 2000). *zip1*, *zip2* and *zip3* mutants all arrest in certain strain backgrounds, and delay the first and second division in most others (see Chapter 3 for details). Rad17,

Rad24, Mec1, Mec3, Ddc1, Hop1, Red1 and Mek1 are all required for this arrest. Details of this were presented in Chapter 1.

Is the RIS transduced by checkpoint proteins?

DSB formation and the reductional division of meiosis are major events that must be properly ordered. In *Saccharomyces*, cells starting the first division of meiosis without initiating recombination (*e.g*, *rec104* mutants), produce aneuploid, inviable meiotic products. Unlike *dmc1* mutants, recombination initiation mutants do not arrest, likely because there is no hyperresected DNA to trigger the Pachytene checkpoint. In fact, the reductional division of meiosis starts earlier in recombination initiation mutants (Galbraith et al., 1997; Jiao et al., 1999; Malone et al., 2004). This implies that wild type cells have a normal delay of the first division of meiosis. Our lab has shown that this delay is requires the presence of a subset of recombination initiation proteins. We have called this subset of proteins the Recombination Initiation Signal (RIS). Two SC mutants, *hop1* and *red1* also start MI earlier than wild type, although tit is not certain whether Hop1 and Red1 are members of the RIS or act downstream of the RIS (discussed further in Chapter 6). *NDT80*, a gene necessary for the first division of meiosis, appears to be the target of the RIS (Malone, et al., 2004 and Chapter 4). It is unlikely that RIS proteins influence *NDT80* transcription directly; rather there are likely one or more intermediary proteins between the RIS and *NDT80* transcriptional control. We hypothesized that checkpoint proteins might be responsible for transducing the RIS. We evaluated members of the DNA damage and S phase checkpoints to see if they were involved in either sensing or transducing the RIS. To test this, we examined the MI kinetics in various checkpoint mutants. We also evaluated the role of the meiotic kinase Mek1 in transducing the RIS. If a protein involved in transducing the RIS is eliminated, these strains should start MI early similar to RIS mutants.

Rachel Gast, a Masters student in our lab, evaluated the role of the spindle checkpoint in transducing the RIS. Murray's and Dawson's research suggested that the spindle checkpoint may coordinate the initiation of recombination and the start of the reductional division (Cheslock et al., 2005; Shonn et al., 2000; Shonn et al. 2003). Murray's lab proposed that the normal delay in wild type of the start of the reductional division is due to interhomolog tension created by recombination intermediates physically linking homologous chromosomes (Shonn et al., 2000; Shonn et al., 2003). Eliminating recombination *(e.g.*, in *spo11*), permits homologous chromosomes to segregate prematurely. The Murray lab proposed that the spindle checkpoint senses this tension and coordinates the start of the reductional division. They hypothesize that *spo11* cells would have no recombination intermediates physically linking homologous chromosomes; thus they would have no interhomolog tension. The flaw in this hypothesis is that recombination initiates before MI spindles form, hence the spindle checkpoint could not be present. Cheslock et al. (2005) later examined nuclear, SPB and spindle kinetics in *mad3* mutants and found that these strains were "post-prophase I" earlier than wild type. They also found that the presence of a single chromosome from a heterologous *Saccharomyces* species in an otherwise wild type genetic background delayed the start of MI and that *MAD1-3* was necessary for this delay (Cheslock et al., 2005). However, this delay argues that the spindle checkpoint can detect unrecombined chromosomes in the context of 15 other pairs of chromosomes that have recombination. It does not give insight into the role of the SAC in cells with absolutely no recombination at all between any homologs.

Gast examined the MI kinetics in strains that had either or both branches of the spindle checkpoint eliminated (Fig. 1-11). She found that *mad2,* and *bub2* and *mad2 bub2* strains started the reductional division at the same time as wild type strains. These results indicate that the spindle checkpoint is not required for sensing or transducing the RIS. When she examined the timing of the first division in a *mad3* strain, she also

observed a reductional division that started at the same time as in wild type. Taken together, these data led her to reject the hypothesis that the spindle checkpoint is involved in coordinating the initiation of recombination and the first division of meiosis. These results are not surprising as the initiation of recombination occurs before MI spindles form and hence the spindle checkpoint cannot yet exist.

Gast's *mad3* observations were contrasted to the Dawson lab's observation that *mad3* strains were "post-prophase" earlier. This difference was perplexing to us as well as some of our grant reviewers. Cheslock *et al.'s* (2005) figure does not clearly distinguish whether all three or only one of the metrics they used (nuclear, spindle or SPB) led them to the observation that *mad3* strains start MI earlier than in wild type. Gast examined DAPI-stained nuclei. In later work, Gast examined the timing of SPB and MMI spindle formation. Her preliminary results indicated that *mad3* was not different than wild type cells. To confirm that Mad3 does not transduce the RIS, I examined transcription of *NDT80*, a transcription factor necessary for spindle elongation and SPB duplication in *mad3* mutants. If Mad3 were involved in coordinating the first division, and especially if it plays a role in transducing the RIS, then *NDT80* should be expressed and active earlier in these strains.

[Some of the work in this chapter was performed in collaboration Honor's undergraduate student Morgan Pansegrau. Pansegrau performed the analysis of rad24 and rad9. I made the strains for the experiments. The remaining experiments were performed by me.]

Materials and methods

Yeast strains used in experiments

The yeast strains used for the experiments described in this chapter are derived from the homothallic diploid K65-3D ultimately derived from S288C. K65-3D is homozygous for the following markers: *HO, lys2-1, tyr1-1, his7-2, can1^{r}, ura3-13, ade5,*

met13-d, trp5-2, leu1-12, ade2-1. Complete descriptions of all strains are detailed in the Appendix. The *rec104* deletion strain used in this chapter is *rec104-Δ1* (Galbraith and Malone, 1992). The *rec102, spo11, sm1l, rad24 and rad9* and *mad3* mutations used are deletion strains containing an insertion of the *G418*^{r} gene. These strains are precise deletions of the entire coding region of these genes and were obtained from the Research Genetics deletion collection. They are null mutations. The proper designation of G418^{r}-deleted *mad3* mutant strains is *mad3Δ::G418*^{r}, but I will refer to this strain and others like it as *mad3* in this chapter. The deletions are described by the *Saccharomyces* Genome Deletion Project (http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.htmL). The *mec1* and *rad53* mutations were made by inserting *HIS7* to completely replace the coding region of that gene. These strains were made by PCR amplifying the *HIS7* gene from a strain wild type at that locus with 50bp tails on the PCR primers that contained homology to the regions surrounding the gene I desired to delete. All deletion and deletion/insertion mutations were tested by both genetic and Southern analysis using at least two restriction enzymes that would give different sizes and number of bands in the deletion and wild type copy of the gene (Southern, 1975). *G418*^{r} -deleted strains were selected using YPD medium (2% dextrose, 1% yeast extract, 2% bacto-peptone, 1.8 % agar) containing 200mg/L of G418.

All of the strains used in this chapter are homozygous diploids created by sporulating and dissecting a heterozygous strain and then selecting segregants containing the desired mutations. Strains containing a *G418*^{r} insertion could be selected by growth on rich medium containing 200mg/L of G418. The *rec104-Δ1* mutation was detected in strains by using PCR with the primers "*rec104* colony F (5'GAGCTGTTCGGGTATTGCGT3') and "*rec104* colony R" (5'GAAAGTATCAGTTCTATGGACAGTTC3').

Plasmids used in experiments

The plasmid pKF1 was constructed to study the effects of overexpression of *MEK1*. pKF1 contains the full length of the *MEK1* gene cloned into the high copy vector YEp24 with 188bp upstream of the start codon and 168 bp downstream from the stop codon. The *MEK1* fragment was engineered by using PCR to generate a 1.9 kb fragment that contained BamHI and SalI restriction sites at the 5' and 3' ends, respectively. This was cloned into the BamHI and SalI sites of the vector. The completed pKF1 vector is 9.5 kb. YEp24 was used as an empty vector control. (Fig. 5-1)

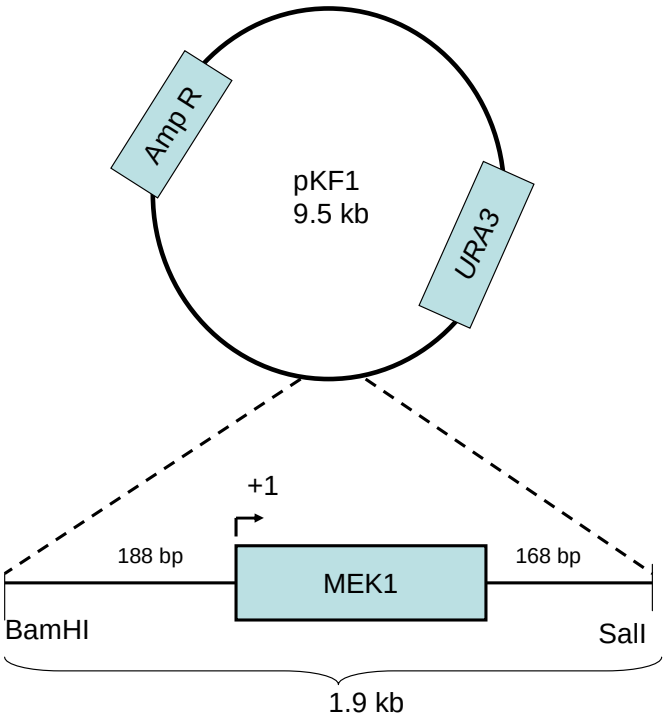
Media and growth conditions

All media not described below is described in Chapter 2. Sporulation and growth conditions are described in Chapter 2.

Liquid sporulation and timepoints

Liquid sporulation and cell harvesting are as described in Chapters 2 and 4. Strains bearing the pKF1 URA3-containing plasmid were grown as described in Chapter 4.

Figure 5-1: Map of pKF1. pKF1 contains the full length of the *MEK1* gene cloned into the high copy vector YEp24 with 188bp upstream of the start codon and 168 bp downstream from the stop codon. The *MEK1* fragment was engineered by using PCR to generate a 1.9 kb fragment that contained BamHI and SalI restriction sites at the 5' and3' ends, respectively. This was cloned into the BamHI and SalI sites of the vector.


Amp R
pKF1
9.5 kb
URA3
+1
188 bp
MEK1
168 bp
BamHI
SalI
1.9 kb

Microscopy

DAPI staining and analysis were as described in Chapter 2.

RNA isolation and northern analysis

RNA preparation and analysis were performed as described in Chapter 4. Normalization of *SPS4* and *NDT80* transcription levels were also as described in Chapter 4.

Results

Mad3 does not affect the transcription of *NDT80*

Rachel Gast's work supported the hypothesis that the spindle checkpoint was not a part of the RIS pathway. She showed that cells containing *mad2, mad3, bub2* and various double mutation combinations all start the first division of meiosis at a time indistinguishable from wild type cells. These results were not consistent with published results by the Dawson lab. Dawson argued that *mad3* mutants entered anaphase I earlier than wild type strains by plotting a combination of MI spindle formation, SPBs and nuclear division which they called 'post-anaphase I cells (Cheslock et al., 2005). To help determine whether Mad3 transduces the RIS, I examined the transcription of *NDT80* and *SPS4* (a reporter for *NDT80)* activity in *mad3,* and *rec102* mutants. Ndt80 is required necessary for the formation of MI spindles. Strains lacking *NDT80* arrest in pachytene with no spindles present (Hepworth et al., 1998). If *mad3* cells really entered Anaphase I earlier, then *NDT80* should be expressed and active earlier in these *mad3* mutant strains.

NDT80 transcription begins at 4 hours in wild type strains in meiosis (Fig. 5-2 A and B). The reporter gene *SPS4* is expressed about one hour later (Fig. 5-2 A and C). The RIS mutant *rec102* begins transcription of *NDT80* at 3 hours followed by transcription of *SPS4* about one hour later. *SPS4* transcription starts slightly earlier in *mad3* mutants than in WT strains, but the differences are small and not as dramatic as the

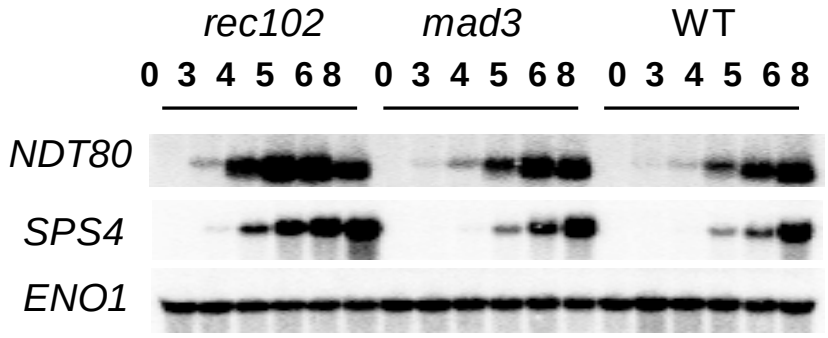
difference between WT or *mad3* and *rec102*. Normalized transcription of *NDT80* is 6.9 in *mad3* at 5 hours compared to in WT (Fig. 5-2 D). This is much lower than the 24.9 observed in *rec102*, however. *SPS4* transcription is 14.1 at 6 h in *mad3,* which is slightly higher than the 8.6 observed in WT; both values are considerably lower than the *SPS4* transcription of 41.4 in *rec102* cells at 6 hours (Fig. 5-3 E). These results suggest that Mad3 is at most minimally involved in transducing the RIS. Given Rachel Gast's experiments showing that *mad3* mutant strains start the reductional division at a time indistinguishable from WT, I propose that Mad3 is not involved in transducing the RIS at all. Taken together, these results raise some questions about the Dawson labs results (see Discussion).

Mec1 and Rad53 do not relay the RIS

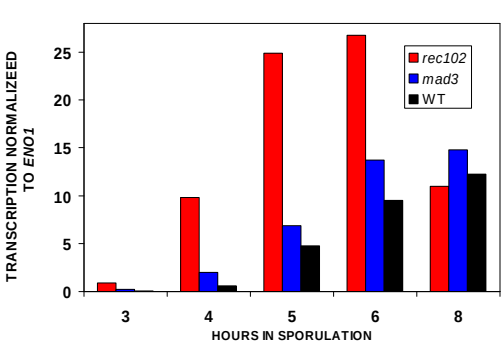
Mec1 and Rad53 are both essential for the DNA damage and S phase checkpoints during vegetative growth, though their role in meiosis is somewhat different. Both of these proteins are required for the meiotic S phase checkpoint and for monitoring DNA damage that occurs as a consequence of replication defects (Hochwagen and Amon, 2006; Lydall, 2003). Only Mec1 has been documented to have a role post-recombination initiation. Mec1 is required for both the Pachytene checkpoint and is one of several proteins that have been shown to help promote repair from the homologous chromosome as opposed to the sister chromatid (correct partner choice) during meiosis (Carballo and Cha, 2007; Hochwagen and Amon, 2006). Because both Rad53 and Mec1 have been shown to have an important role in monitoring the state of DNA, we evaluated their role in transducing the RIS. To do this, I studied the MI kinetics of *mec1* and *rad53* mutants. If either Rad53 or Mec1were involved in sensing or transducing the RIS, removing that protein should have a similar effect to removing a component of the RIS. As always, *rec104* and wild type strains were included as controls. *rec104* mutants start the reductional division 1-1.5 hours earlier than wild type.

Figure 5-2: The transcription/ activity of *NDT80* in *mad3* mutants. [A] The genotype of the strain examined is shown at the top. Numbers below the genotypes indicate the time in sporulation that RNA was isolated. The probes used for the Northerns are shown to the left. [B]The amount of *NDT80* transcription (corrected for loading by *ENO1*) is shown vs. time. The black bar represents transcription in WT cells, the red bar transcription in *rec102* cells, and the white bar transcription in *mad3* cells. Correction for loading is performed by dividing the amount of transcription of *NDT80* by the amount of transcription of *ENO1* for each lane. [C] Same as [B], but transcription of *SPS4*. [D] Quantification of transcription of *NDT80*. All values are shown normalized to *ENO1*. Transcription relative to wild type was calculated by dividing the transcription for a particular timepoint by the transcription level of *NDT80* at the same timepoint. This process is fully described in the methods section of Chapter 4. [E] Same as [D] but showing quantification of transcription of *SPS4*. Sporulation for this experiment was WT, 69%; *rec102* 16%; *mad3,* 70%. Only one experiment was performed.

A



B *NDT80* transcription



C *SPS4* transcription

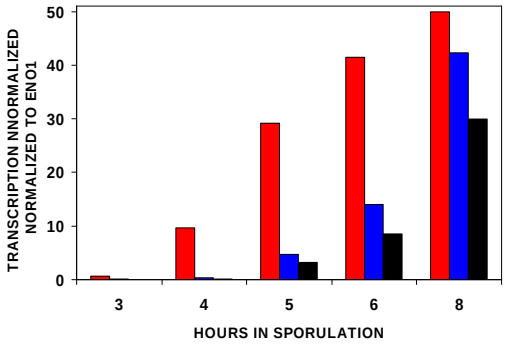


Figure 5-2, continued:

D. Quantification of *NDT80* Transcription in *mad3*

Strain Name	Relevant Genotype	Hours in Sporulation	transcription normalized to *ENO1*	transcription relative to wild type
SEN 2-1-7D	*rec102*	3	n.d. *	-
		4	10.	-
		5	25	5.2
		6	27	2.7
		8	11	0.92
RCG5-4-3B	*mad3*	3	n.d. *	-
		4	2.0	-
		5	6.9	1.4
		6	14	1.4
		8	15	1.3
K65-3D	WT	3	n.d. *	-
		4	n.d. *	-
		5	4.8	1.0
		6	10.	1.0
		8	12	1.0

*No transcription detected

Figure 5-2, continued:

E. Quantification of *SPS4* Transcription in *mad3*

Strain Name	Relevant Genotype	Hours in Sporulation	transcription normalized to *ENO1*	transcription relative to wild type
SEN 2-1-7D	*rec102*	3	1	-
		4	10	9.7
		5	29	4.6
		6	41	1.7
		8	50	1.7
RCG5-4-3B	*mad3*	3	n.d.*	-
		4	n.d*	1.7
		5	5	1.6
		6	14	1.4
		8	42	1.4
K65-3D	WT	3	n.d.*	-
		4	n.d.*	1.0
		5	3	1.0
		6	9	1.0
		8	30	1.0

*No transcription detected

mec1 and *rad53* null mutations are lethal in vegetative cells (Weinert et al., 1994). In order to study these mutants, either *ts* mutants can be used or a strain background containing the *smL1* (*suppressor of mec1 lethality*) mutation can be used (see Chapter 1). I chose the latter approach. Many *Saccharomyces* strain backgrounds naturally contain a *smL1* mutation (Zhao et al., 2001; Zhao et al., 2000), though ours does not (Kelley Foreman, data not shown). In the following, *rad53 smL1* and *mec1 smL1* strains will be referred to as *rad53* and *mec1*, respectively.

rad53 strains grow slowly during vegetative growth. I have calculated the doubling time of *rad53* strains to be 165 minutes compared to 120 minutes for WT in YPA pre-sporulation medium. *rad53* strains grow the slowest of any of the strains that I have worked with in the Malone lab. During meiosis, however, *rad53* strains were only slightly impaired. Sporulation was reduced to 53% compared to 65% in wild type and spore viability was only decreased to 78% compared to 92% for wild type (Table 5-1). In contrast, *mec1* strains are not particularly impaired during normal vegetative growth. I found that *mec1* strains doubled in 120 minutes in YPA (identical to WT). During meiosis, however, *mec1* strains are very impaired. Sporulation and spore viability were considerably decreased (29% spore viability and 50% sporulation) (Table 5-1).

When I examined the MI kinetics of *rad53* strains, I found no significant difference between the time wild type strains started the first division and when *rad53* strains started MI (Fig. 5-3). Both *rad53* and wild type strains entered MI at approximately 5 hours after the start of sporulation. These data support a conclusion that Rad53 is not involved in the RIS pathway. In these experiments, I used a *rec104 sm1l* strain and a wild type SML^+ strain as controls. The presence of a *sm1 l* mutation in the strain background does not alter a *rec104* strain's kinetics with respect to wild type. As is true in all other experiments that I have performed in the Malone lab, *rec104* mutants start the first the first division of meiosis 1-1.5 hours earlier than wild type whether *SML1*

is present or not. I conclude that a *smL1* mutation has no detectable affect on MI kinetics.

Unlike *rad53* strains, *mec1* strains do not start the reductional division at the same time as wild type strains, but are actually delayed by about an hour compared to wild type (Fig. 5-4 A and C). Many *mec1* cells fail to do either first or the second division as well (Fig. 5-4 C and D). This illustrates that while Mec1 may be important for later events during meiosis, it is not required for the transduction of the RIS (see Discussion). In this experiment, I chose not to use a *smL1* mutation in the genetic background of my controls because the *rad53* experiments suggested that *smL1* does not alter the MI kinetics of meiosis.

Rad24 and Rad9 do not transduce the RIS

Rad24 and Rad9 comprise separate branches of the DNA damage checkpoint that sense DNA lesions during vegetative growth (Fig. 1-9). These two proteins are important during meiosis, as well, for sensing DNA damage that occurs prior to programmed DSB formation. Rad24 has the additional role of being required for the Pachytene checkpoint and late recombination events during normal meioses.

We hypothesized that Rad24 and Rad9 might be required for coordinating recombination and the reductional division. Both sporulation and viability are slightly reduced in *rad24* mutants, though the defect is not as severe as in *mec1* strains (Table 5-1). When we examined the MI kinetics of these strains we found that the reductional division was slightly delayed in *rad24* strains compared to wild type (about 5.5 hours compared to 5 hours) (Fig. 5-5). The reductional division in *rad9* strains; however, started at a time indistinguishable from wild type cells (approximately at 5 hours) (Fig. 5-6). These data imply that Rad24 and Rad9 are not required for transducing the RIS. This work has been published in *Eukaryotic Cell* (Malone et al., 2004).

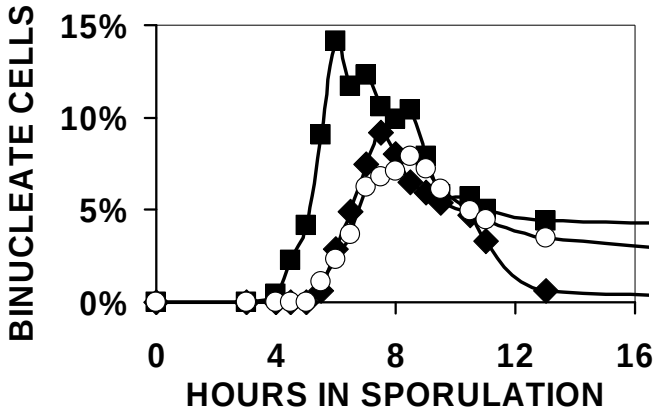
Table 5-1: Spore viability of strains used in this chapter.

Strain Name	Malone lab stock number	Relevant genotype	Spor. (%)	Spore viab. (%)	Number analyzed
KF 2-3-2D	M3500	*rad9*	72 ± 3	90	99
EG1-1-6C	M3372	*rad24*	48 ± 5	85	85
KF3-2-3C	M3604	*rad53 smL1*	53 ± 3	78	80
KF 3-5-2A	M3730	*mec1 smL1*	50 ± 4	29	80
EG1-1-2B	M2997	*rec104*	28 ± 6	0*	400
SEN 2-1 7D	M3577	*rec102*	29 ± 8	0*	400
BCJ 2-1-2C	M3888	*mek1*	31 ± 5	3	192
BCJ 2-1-2C [YEp24]	M3911	*mek1*[YEp24]	28 ± 4	0*	148
BCJ 2-1-2C [pKF1]	M3910	*mek1*[pKF1]	70 ± 6	88	92
K65-3D hom *spo11*[pKF1]	M3908	*spo11* [pKF1]	28 ± 3	0*	168
K65-3D hom *spo11* [YEp24]	M3909	*spo11*[YEp24]	26 ± 2	0*	144
K65-3D[pKF1]	M3907	WT [pKF1]	80 ± 7	92	100
K65-3D[YEp24]	M3906	WT[YEp24]	79 ± 3	89	104
K65-3D	M156	WT	75 ± 3	97	1220

Note: Sporulation percentages are the average of two or more cultures. Spore viability was determined by dissecting at least two independent diploids. *rad9*, *rad24* strains were dissected by Demelza Koehn. *rec104* , *rec102* and WT (K65-3D) strains were dissected by Kai Jiao. *For recombination initiation mutants (*i.g.*, *spo11*) whole asci were set out using a dissecting needle rather than dissected.

Figure 5-3: The first meiotic division in *rad53* mutants. The divisions in WT cells are shown as diamonds (♦) and are controls for normal timing. The control for early timing of the first division is a *rec104* strain indicated by squares (■). The *rad53* mutant is shown as open circles (○); two independent *rad53* experiments were performed, each with two cultures. [A] The first division is indicated by the appearance of binucleate cells. [B] MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells.[C] and [D] are the same as [A] and [B], but show an independent experiment.

A. First division in *rad53* (Exp. 1)



B. M1 + MII in *rad53* (Exp1.)

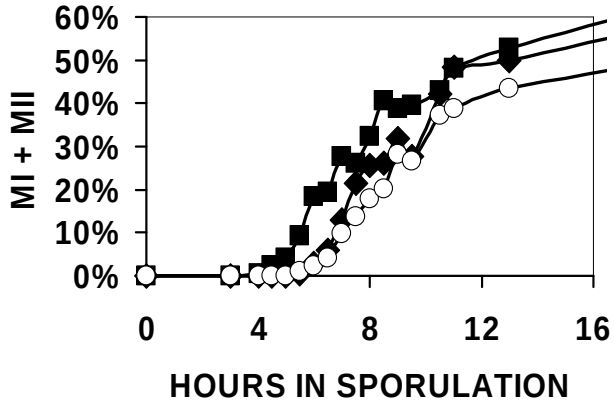
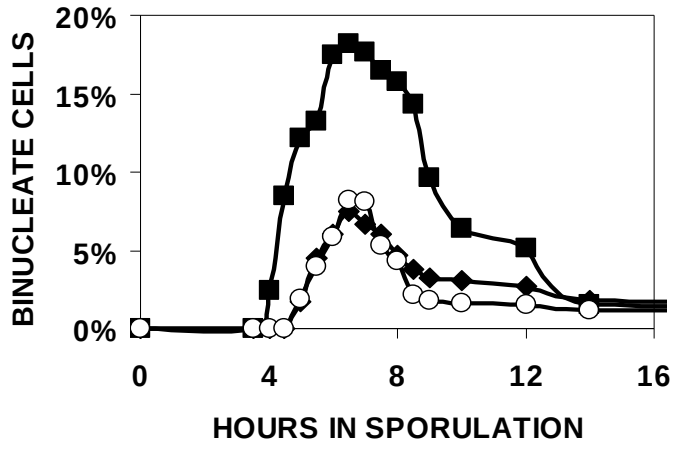


Figure 5-3, continued:

C. First division in *rad53* (Exp. 2)



D. MI + MII in *rad53* (Exp. 2)

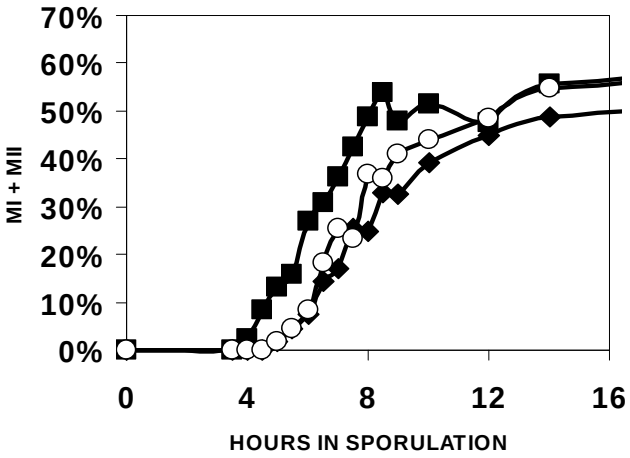
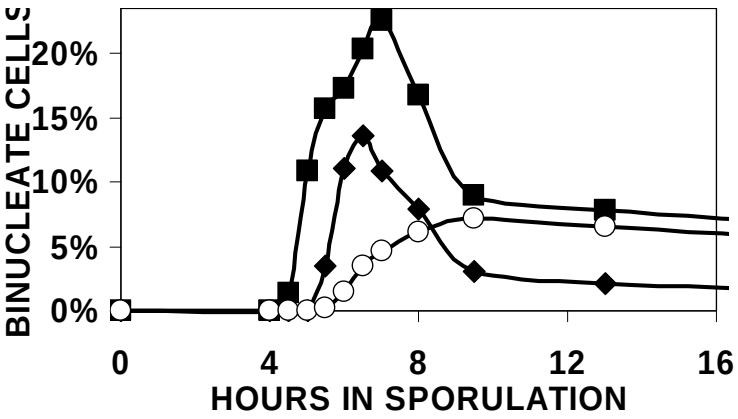


Figure 5-4: The first meiotic division in *mec1* mutants. The divisions in WT cells are shown as diamonds (♦) and are controls for normal timing. The control for early timing of the first division is a *rec104* strain indicated by squares (■). The *mec1* mutant is shown as open circles (○); two independent *mec1* experiments were performed, each with two cultures. [A] The first division is indicated by the appearance of binucleate cells. [B] MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells. [C] and [D] are same as [A] and [B], but an independent experiment.

A. First division in *mec1* (Exp. 1)



B. MI + MII in *mec1* (Exp. 2)

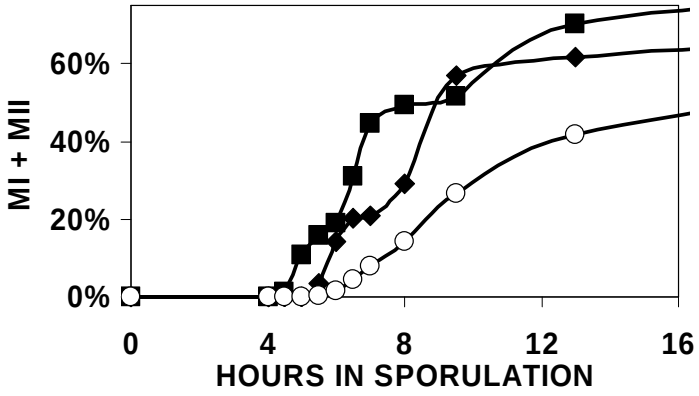
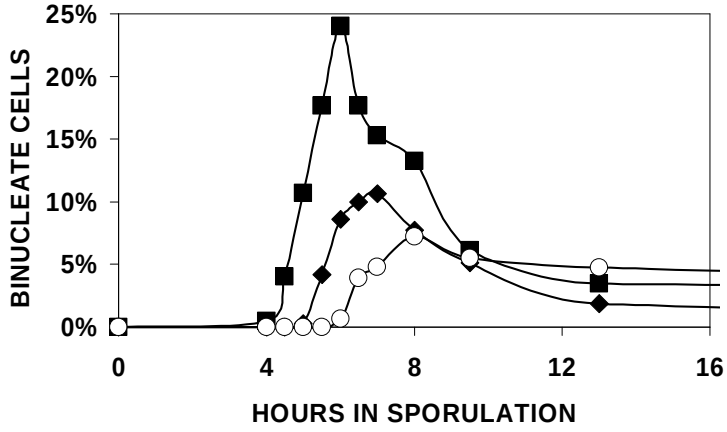


Figure 5-4, continued:

C First division in *mec1* (Exp. 2)



D MI + MII in *mec1* (Exp. 2)

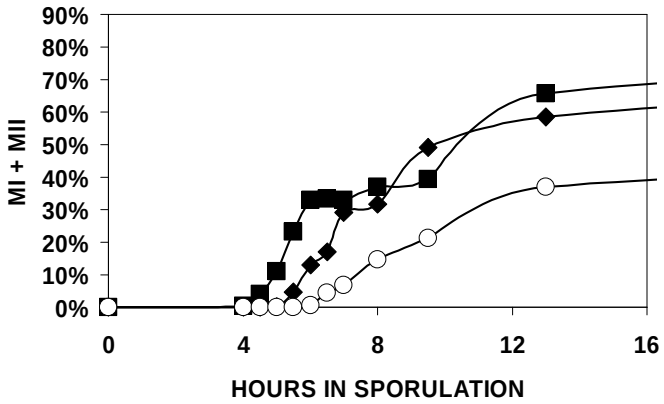


Figure 5-5: The first meiotic division in *rad24* mutants. The divisions in WT cells are shown as diamonds (♦) and are controls for normal timing. The control for early timing of the first division is a *rec104* strain indicated by squares (■). The *rad24* mutant is shown as open circles (○); two independent *rad24* experiments were performed, each with two cultures. [A] The first division is indicated by the appearance of binucleate cells. [B] MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells. Results from one of two experiments are shown. The second experiment showed identical results

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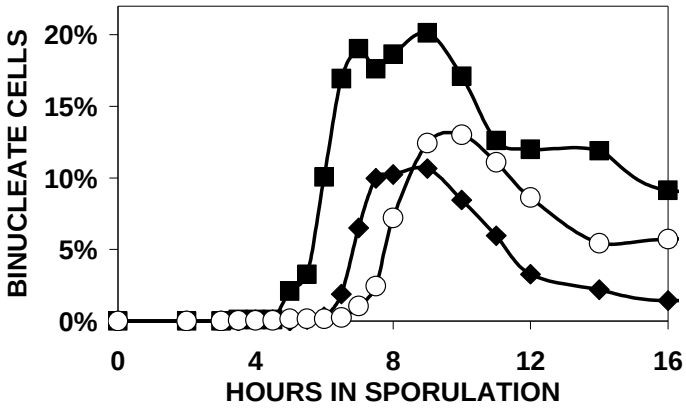
A. First division in *rad24*



B. MI + MII *rad24*

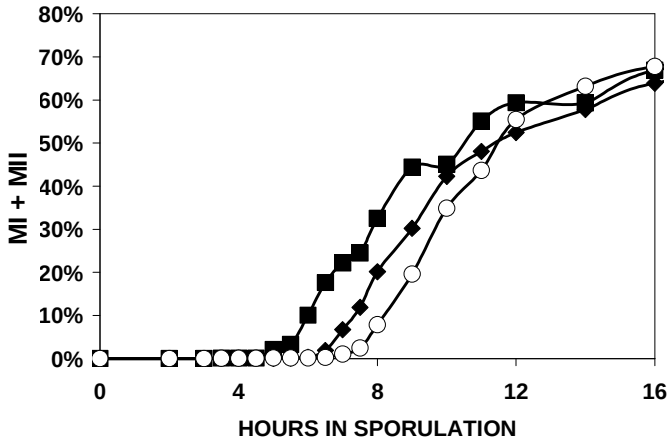
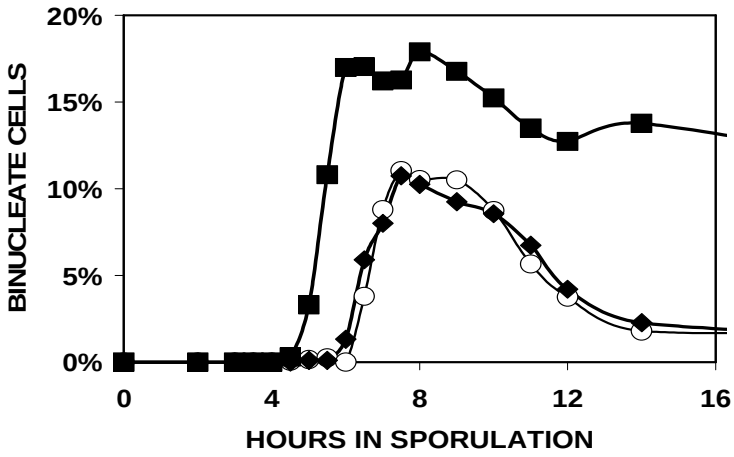
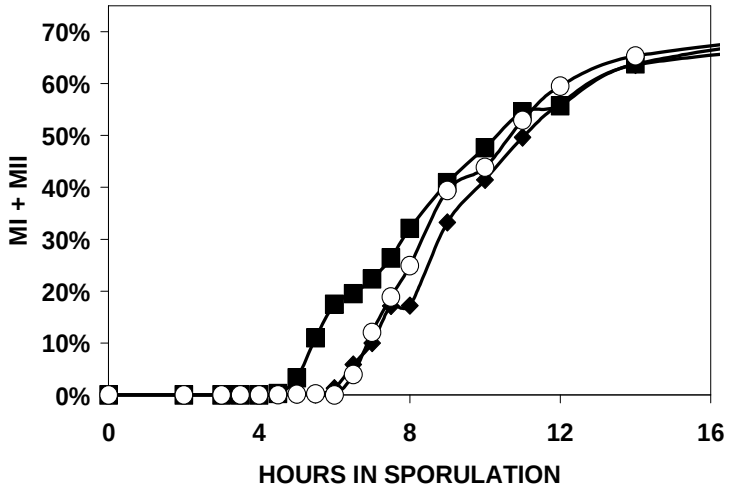


Figure 5-6: The first meiotic division in *rad9* mutants. The divisions in WT cells are shown as diamonds (♦) and are controls for normal timing. The control for early timing of the first division is a *rec104* strain indicated by squares (■). The *rad9* mutant is shown as open circles (○); two independent *rad9* experiments were performed, each with two cultures. [A] The first division is indicated by the appearance of binucleate cells. [B] MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells. One of two experiments is shown. The second experiment showed similar results.

A. First division in *rad9*



B. MI + MII in *rad9*



Mek1 is a candidate for transducing the RIS.

Results obtained by several members of the Malone lab have now eliminated the mitotic DNA damage, spindle and S phase checkpoints as potential transducers of the RIS. Several members of the Pachytene checkpoint have also been excluded. I next examined a meiotic kinase, Mek1, as a candidate for transducing the RIS. Mek1 is a Rad53 paralog that has been shown to be important in partner choice and the Pachytene checkpoint (Carballo and Cha, 2007). Mek1 has been shown to interact with the RIS proteins Hop1 and Red1 so it was an attractive candidate for being a part of the RIS signaling cascade. *mek1* mutants have reduced sporulation levels (31%) similar to *rec104* mutants (28%) and produce nearly all inviable spores (3% spore viability) (Table 5-1). Hollingsworth's lab reports similar low spore viability (1.1%) (Niu et al., 2007).

When I examined the MI kinetics of *mek1* mutants, I found that these strains started the first division of meiosis at the same time as *rec104* mutants (Fig. 5-7). *rec104* and *mek1* mutants started the reductional division at about 4 hours into meiosis while a wild type strain started MI about an hour later. These results are the first evidence suggesting that a kinase may be involved in the RIS cascade.

Mek1 acts downstream of the RIS

There are two alternate interpretations of the observation that *mek1* mutants start MI earlier than normal. One interpretation is that *mek1* mutants start MI earlier because Mek1 is a transducer (effector kinase) of the RIS. A second interpretation is that *mek1* strains start MI earlier because it is a part of the RIS itself. To distinguish between these two hypotheses, I overexpressed *MEK1* in *spo11* and wild type strain backgrounds. Overexpressing several components of the RIS does not alter MI kinetics (Doug Pittman, Anne Galbraith and R. Malone, unpublished results). If Mek1 were a part of the RIS, then overexpressing *MEK1* should alter when these strains start the reductional division. In contrast, if Mek1 is an effector kinase downstream of the RIS, then over-expressing

Figure 5-7: The first meiotic division in *mek1* mutants. The divisions in WT cells are shown as diamonds (♦) and are controls for normal timing. The control for early timing of the first division is a *rec104* strain indicated by squares (■). The *mek1* mutant is shown as open circles (○); two independent *mek1* experiments were performed, each with three cultures. The average sporulation values in this experiment were: WT, 71%; *rec104*, 31%; *mek1*, 28%. [A] The first division is indicated by the appearance of binucleate cells. [B] MI + MII indicates the cumulative appearance of binucleate and tetranucleate cells

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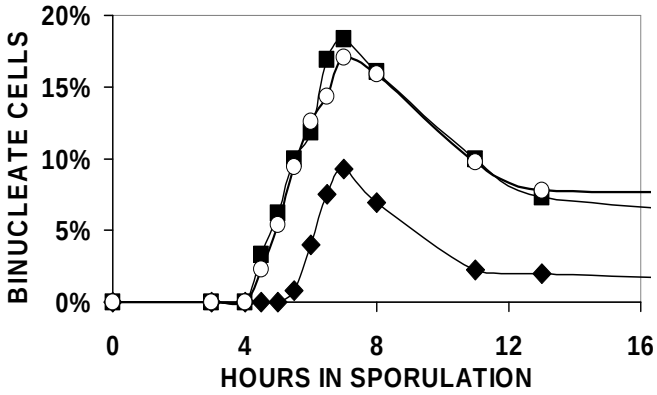
A. First division in *mek1* (Exp. 1)



B. MI + MII in *mek1* (Exp. 1)

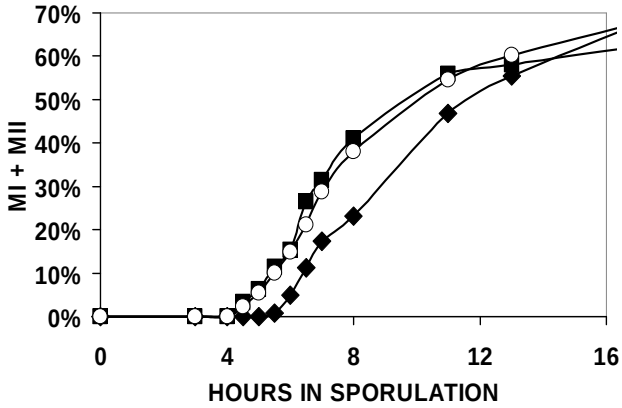
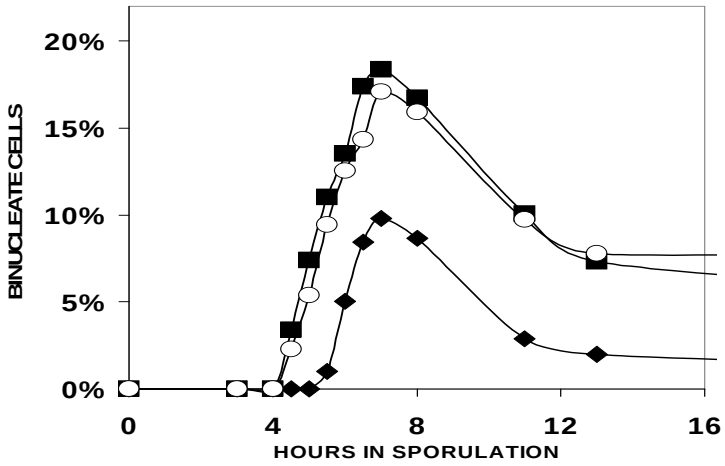
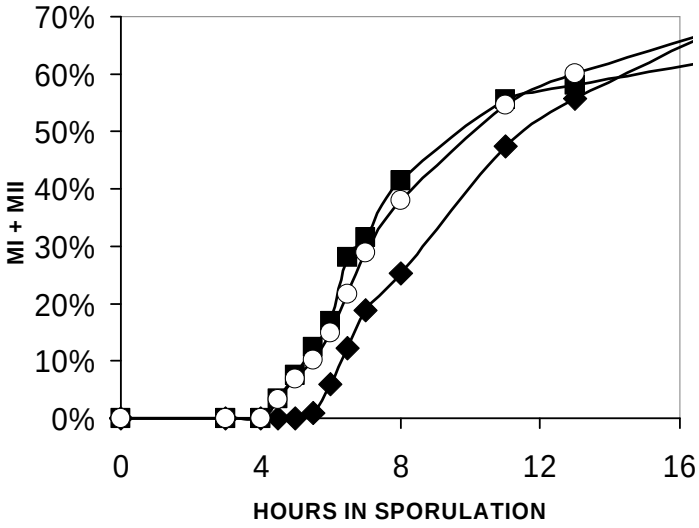


Figure 5-7, continued:

C. First division in *mek1* (Exp. 2)



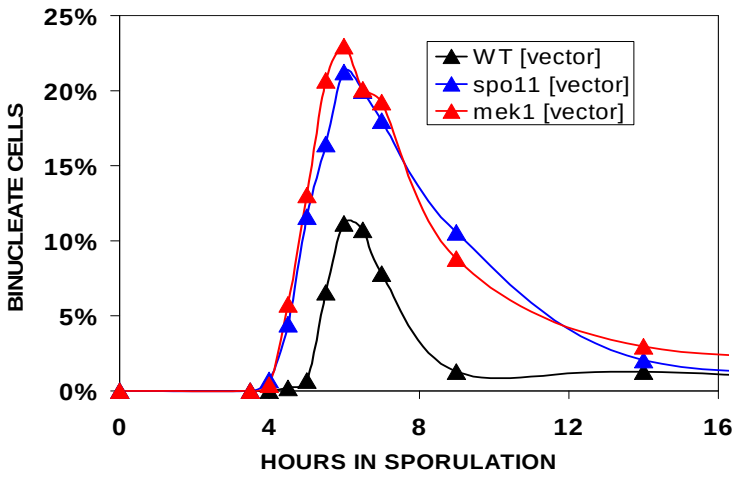
D. MI + MII in *mek1* (Exp. 2)



MEK1 should lead to establishment of a checkpoint signal. In other words, if Mek1 were a part of the RIS, then spo11 strains would have delayed MI, similar to wild type. The pKF1 overexpression construct that I created for this experiment contains a full length copy of the *MEK1* gene expressed from its native promoter cloned into the high-copy 2μ vector. Fig. 5-8 A and D show the vector controls used in this experiment. The *mek1* [vector] and *spo11* [vector] strains, as predicted, start the reductional division earlier than wild type strains carrying the empty vector. Viability and sporulation levels were similar to experiments performed without plasmids (Table 5-1). Adding the *MEK1* overexpression vector to a *mek1* mutant complements the viability and sporulation defects seen in *mek1* mutants (Table 5-1) (73 % sporulation and 88% viability). Over-expressing *MEK1* in *mek1* strains caused MI to be slightly, but reproducibly delayed (~30 min.) compared to WT strains containing the control vector (Fig. 5-8 B and E). This delay is similar to the MI timing observed when over-expressing *MEK1* in a wild type strain background (Fig. 5-8 B and E). Each experiment was done twice with two independent cultures of each strain containing the overexpression plasmid. These data are suggestive that Mek1 is downstream of the RIS. To fully demonstrate this, it was important to examine *MEK1* overexpression in a RIS mutant background. I found that *spo11* [MEK1 O/E] strains started the first division of meiosis at the same time as wild type strains indicating that overexpression of *MEK1* is epistatic to *spo11* (Fig. 5-8 C and F). This suggests that Mek1 acts downstream in the RIS signaling cascade rather than being a part of the RIS. Overexpression of *MEK1* cannot complement all phenotypes of a RIS mutant, however. *spo11* [MEK1 o/e] strains still produce inviable spores and have reduced sporulation rates due to a failure to recombine (0% viability and 23% sporulation) (Table 5-1).

Figure 5-8: Overexpression of *MEK1*: MEK1 was over-expressed from the high copy vector pKF1. The empty vector YEp24 was used a control. Data from multiple cultures in two experiments are shown. Panels A-C show data from one experiment; panels D-F show an independent experiment doen on a different day. The data from each experiment is separated into three panels for clarity. For comparison, some of the cultures have been shown on multiple graphs. For instance, the WT [vector] represented on panels A, B and C of experiment 1 is the same culture. Wild type is shown in black, *spo11* is shown in blue and *mek1* is shown in red. Strains containing the control vector are shown as triangles and solid lines and represent only one culture. Strains containing the overexpression vector are shown with solid circles and broken lines and are an average of two cultures. One culture each of WT [vector], *spo11* [vector] and *mek1* [vector] was grown. The average of two cultures is shown for each of *spo11* [pMEK1 O/E], *mek1*[pMEK1 O/E] and WT[pMEK1o/E]. The average final sporulation for each of these cultures is 26%, 70% and 83%, respectively. Data from the three strains containing the control vectors is shown in [A]. Data comparing wild strains with and without the overexpression vector is shown in [B] and data comparing *spo11* with and without the overexpression vector is shown in [C]. [D], [E] and [F] are the same as [A], [B], and [C], but are an independent experiment.

A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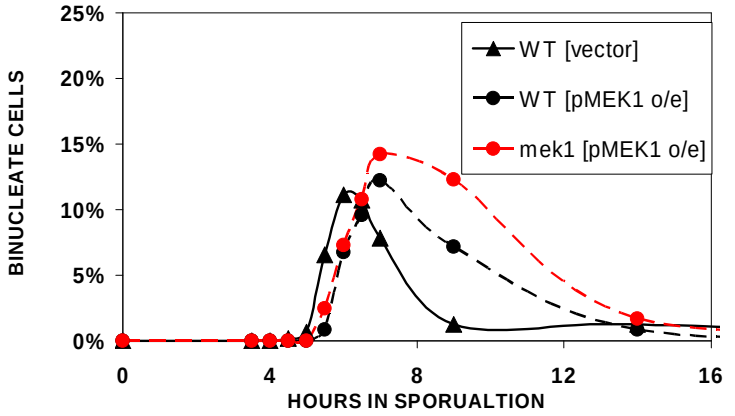
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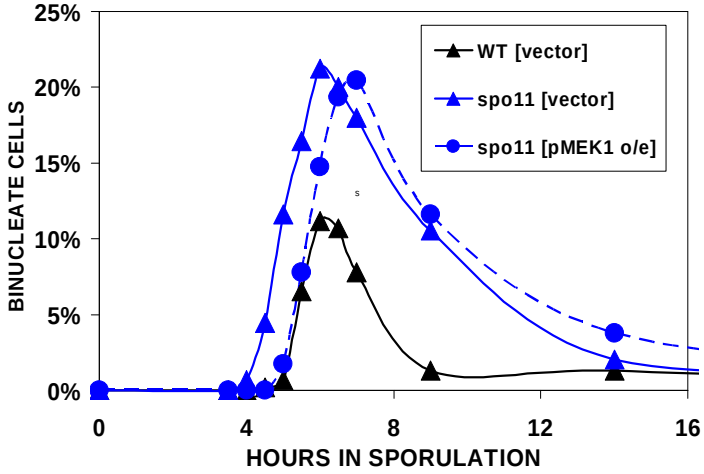
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HOURS IN SPORULATION
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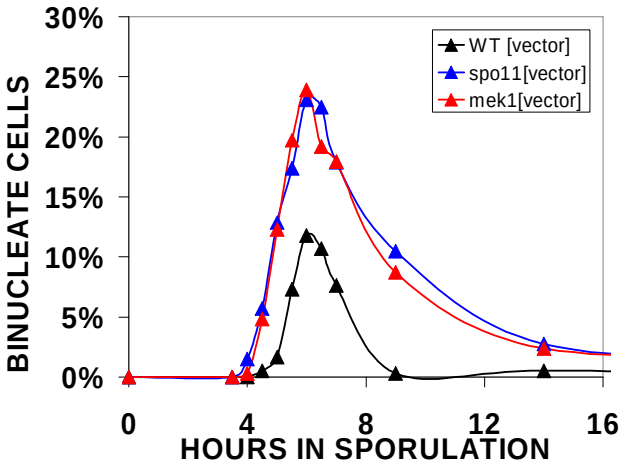
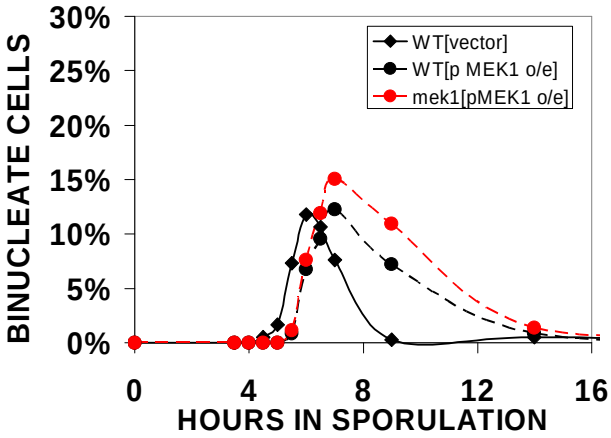
spo11 [vector]
spo11 [pMEK1 o/e]

Figure 5-8, continued

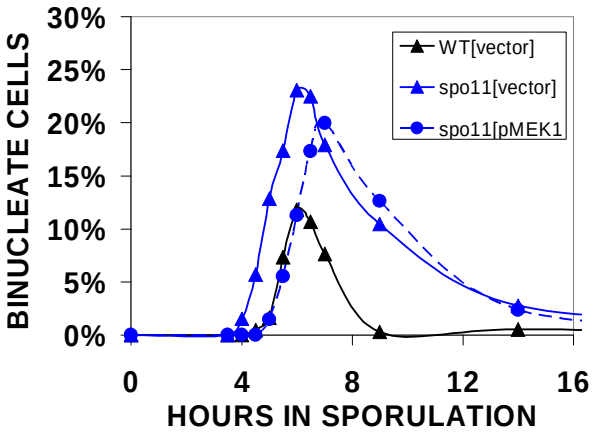
D



E



F



Discussion

The major events of meiosis must be properly coordinated to insure viability of the resulting meiotic products. Checkpoint proteins have been shown to play a key role in regulating these events. Carballo and Cha (2007) make a distinction between the regulation of a normal (unperturbed) cell cycle and an abnormal cell cycle such as seen in a *dmc1* mutant. Checkpoint proteins have been implicated in regulating both perturbed and unperturbed meioses. One example of a checkpoint protein's involvement in a normal meiosis is in promoting recombination between homologs as opposed to sister chromatids or ectopic recombination (Carballo and Cha, 2007; Grushcow et al., 1999; Thompson and Stahl, 1999). The coordination of recombination and MI by the RIS is another example of the regulation of a normal meiosis. We hypothesized that checkpoint proteins might be involved in regulating the start of the reductional division.

During a normal meiosis the reductional division must occur only after recombination in order for correct segregation of homologs to occur. We have shown that the presence of 8 of 10 recombination initiation proteins help coordinate the start of the first division of meiosis. We evaluated three meiotic checkpoints, the spindle checkpoint, the S phase checkpoint and the DNA damage checkpoint to see if they transduced the RIS. I also tested the meiosis-specific *RAD53* paralog *MEK1*.

The spindle checkpoint does not transduce the RIS

Rachel Gast's work showed that neither the Mad2 nor the Bub2 branch of the spindle checkpoint transduce the RIS. Gast also showed that *mad3* mutants do not start the first division of meiosis earlier than wild type. As discussed briefly in Results, these results are inconsistent with work from the Dawson Laboratory (Cheslock et al., 2005). Although they used nuclear staining by DAPI as one of their methods to determine the timing of the first division, they also examined spindles and spindle pole bodies. They used tubulin staining to analyze spindles and Tub4 staining to analyze SPBs. The data

from all three methods of analysis were combined into a single metric, which was plotted in their graphs. By this method, cells were either "post prophase" or "post anaphase" (Cheslock et al., 2005). We assume that "post prophase" indicated one DNA mass, short spindles, and two SPBs and that "post anaphase" indicated at least two DNA masses, an elongated MI spindle or two short spindles, and two or four SPBs. Gast wanted to determine if the altered timing of the first division which they found was due solely to differences in the timing in the appearance of duplicated spindle pole bodies and/or elongated spindles. She performed preliminary studies on the kinetics of spindle and SPB formation and found that there is no significant difference between *mad3* mutants and wild type cells.

I took an alternate approach to determining if Mad3 is involved in transducing the RIS. Ndt80 is necessary for formation of MI spindles; *ndt80* mutants arrest in pachytene without spindles present (Hepworth et al., 1998; Xu et al., 1995). If Mad3 was necessary for transducing the RIS, *NDT80* and the reporter for Ndt80 function *SPS4* would be transcribed earlier than in wild type. I found that *NDT80* and *SPS4* transcription were slightly earlier than in wild type, but not nearly as early as in a *rec102* mutant (Fig. 5-2). It could be argued that these results suggest that Mad could be playing a minor role in transducing the RIS. I did not repeat this experiment, however, and analyzed only one culture. In contrast, Gast assayed more than 8 cultures in three independent experiments. Furthermore, the difference between wild type and *mad3* are very small compared to the difference between WT and *rec102* (see Fig. 4-2). Considering Gast's results, I conclude that *mad3* is not required for transduction of the RIS. Furthermore, this conclusion makes sense because the RIS occurs before spindle formation. Without presence of MI spindles, the spindle checkpoint has nothing to monitor.

Our evidence indicates that *NDT80* is the target of the RIS (Chapter 4). The spindle checkpoint monitors spindle-kinetechore attachment, spindle SPB interaction and perhaps spindle tension, although the latter is currently being debated (Burke and

Stukenberg, 2008; Lew and Burke, 2003). Because the target of the RIS is the transcription of *NDT80*, the gene necessary for spindle formation, it is clear that the RIS acts *before* the spindle formation. The spindle checkpoint cannot yet be active.

Cheslock *et al.*'s (2005) observation that *mad3* mutants *start* anaphase I 2-3 hours earlier than wild type is somewhat perplexing. Both the Dawson laboratory and our laboratory use an S288C-derived strain background. However, the possibility that a small difference in the strain backgrounds could contribute to these different phenotypes seen in the *mad3* mutant cannot be ruled out. Another possibility is differences in the treatment of the cells during the meiotic time course. Cheslock's group (2005) grew their cells in pre-sporulation media to a density of 5 X 10^7 cells/mL whereas we sporulated our cultures at a concentration of 3-4 X 10^7 cells/mL. They did not indicate how many generations of growth occurred in the presporulation media; our strains grow 3-4 generations in the presporulation media. Laboratories often have differences in protocols; for example, the length of time for centrifuging or how many washes are done. Several small differences in the meiosis time course conditions could have had an additive effect on the factors used to analyze the reductional division. I must conclude that the timing differences observed by Dawson and colleagues cannot be in nuclear divisions assayed by DAPI staining. Given the modest affect of of a *mad3* mutation on *NDT80* transcription, I can only speculate that in their experiments, *mad3* strains showed earlier MI spindle formation.

The S phase checkpoint does not transduce the RIS

Mec1 and Rad53 are large PI 3-kinase family proteins that transduce the response to lesions associated with replication defects and DNA damage (Carballo and Cha, 2007; Friedel et al., 2009). During meiosis, Mec1 has been shown to be important for the Pachytene checkpoint response to an excess of single-stranded DNA created in *dmc1* mutants (Carballo and Cha, 2007). An important target of the Pachytene checkpoint is

NDT80 (Tung et al., 2000). *dmc1*-arrested cells do not accumulate phosphorylated Ndt80 in their nuclei. Both the arrest and the failure to phosphorylate Ndt80, can be bypassed by disabling members of the Pachytene checkpoint such as *MEC1*. Because *NDT80* transcription is a target of the RIS, we hypothesized that Mec1 might transduce the RIS. Because Rad53 is an important kinase that senses a variety of DNA defects, we also examined its role in transducing the RIS.

To study the role of Rad53 and Mec1 in transducing the RIS, we looked at the MI kinetics in mutants of these genes. We found that *rad53* strains started the reductional division at the same time as wild type strains, while *mec1* strains were delayed by about an hour compared to wild type. This delay is perhaps because of Mec1's demonstrated role in recombination partner choice (Carballo and Cha, 2007; Thompson and Stahl, 1999). Because neither of these mutants started the reductional division early like in *rec104* strains, we conclude that these two proteins do not transduce the RIS.

I was somewhat surprised that Mec1 does not transduce the RIS. Mec1 has been shown to phosphorylate the RIS member protein Hop1 in response to the initiation of DSBs by Spo11 (Carballo et al., 2008). Tel1 has also been shown to be required to phosphorylate Hop1. One would predict that cells lacking Mec1 would fail to phosphorylate Hop1 with the ultimate result of an earlier first division. We have shown that hop1 mutants start the first division early. There are two possible reasons why this phenotype is not seen. Tel1 is somewhat functionally redundant to Mec1, so Hop1 could be phosphorylated in a *mec1* mutant. However it seems unlikely that Tel1 is transducing the RIS. Heather DeBey, an undergraduate in our lab, created *tel1* mutant strains and found that they sporulated as highly as wild type strains (74% in *tel1* vs. 77% for wild type) (Heather De Bey, personal comm.). Carballo and Cha (2007) report similar sporulation results as unpublished data. It may be that be that both Mec1 and Tel1 need to be mutated in order to see an early first division. Such a strain would be difficult to study, however. Carballo and Cha (2007) report that *mec1 tel1 smL1* mutants arrest

during pre-meiotic S phase (cited as unpublished data in Carballo, et al. 2007). An alternative explanation is that Mec1 *is* involved in transducing the RIS, but an earlier first division is not observed because of the increased ectopic recombination (or sister) and decreased inter-homolog recombination seen in *mec1* mutants. It could be that this strain would have early MI phenotypes if not for the fact that chromosomal division is impaired due to altered later meiotic events that are mediated by Mec1. To more confidently know whether Mec1 is relaying the RIS, one could examine the transcription of the target *NDT80.* If Mec1 is involved in transducing this signal, one would see earlier transcription of *NDT80* in *mec1* mutants. Relaying the signal to delay MI would be a separate function of Mec1 other than the later stages of meiosis. In this way Mec1 would have two roles in a normal meiosis: an inhibitory role in delaying *NDT80* transcription until the RIS is in place and a positive role in promoting later recombination events. This normal role of Mec1 is different from the Pachytene checkpoint which requires hyperresected DNA such as is found in *dmc1* mutants. Hyperresected DNA does not occur during wild type meiotic recombination.

It is interesting to note that Rad53 has not been documented to be important in determining partner choice. Recently, it has shown that while Rad53 is activated in response to chemically-induced DSBs in meiosis, it does not become activated in response to programmed, naturally-occurring DSBs (Cartagena-Lirola et al., 2008). This possibly explains why we see a delay in the reductional division in *mec1* cells, but not in *rad53* cells. In mitosis Rad53 is a downstream target of Mec1, while in meiosis this appears not to be the case.

The DNA damage checkpoint does not transduce the RIS

dmc1 mutants accumulate large of ssDNA that cause an arrest at the mononucleate stage of meiosis (Grushcow et al., 1999). This arrest can be bypassed by presence of a *rad24* mutation, but not a *rad9* mutation. Kleckner and colleagues proposed that the normal amount of ssDNA created during DSB processing caused a transient delay to allow time for homologous repair of DSBs before the reductional division occurred. To test this hypothesis, we examined the kinetics of MI in *rad24* mutants. If Rad24 is detecting ssDNA created during recombination, then eliminating this protein should result in an earlier reductional division. We observed that *rad24* mutants had a slightly later (certainly not earlier) reductional division than in wild type (Fig. 5-9). This result has been reported by others (Shinohara et al., 2003). *rad17* strains are also reported to have a slightly later MI (Shinohara et al., 2003; Wu and Burgess, 2006). Because we did not observe an earlier reductional division, we conclude that normal amounts of ssDNA created during DSB resection are not coordinating the timing of the reductional division via the DNA damage checkpoint. Carballo and Cha (2007) agree with this conclusion. Members of the Pachytene checkpoint may only be sensing an abnormal amount of ssDNA such as that seen in DSB processing mutants. The proteins of the RIS do coordinate the timing of the reductional division. When one of these proteins is removed, the reductional division starts earlier. These results show that the RIS is not transduced by Rad24.

Because Rad24 was not shown to be required for transducing the RIS, we examined the MI kinetics in a mutation in *rad9.* Rad9 acts in an independent pathway from Rad24 to sense DNA damage. We found that the start of the reductional division in *rad9* mutants is indistinguishable from wild type cells.

Rad24 and Rad9 sense DNA damage in separate pathways [Fig. 1-9 and (de la Torre-Ruiz et al., 1998)]. During mitosis, it is necessary to eliminate both branches of

this pathway to completely abolish the DNA damage checkpoint. To test whether disabling both pathways is necessary to eliminate RIS transduction, Demelza Koehn, a PhD student in our lab, examined the MI kinetics in a *rad9 rad24* double mutant. She found that these strains started the reductional division at the same time as wild type. She also concluded that the DNA damage does not transduce the RIS.

One caveat to the above conclusion is that Rad24 has been demonstrated to have a role in promoting homologous recombination over recombination between sister chromatids and in facilitating late recombination events (Grushcow et al., 1999; Shinohara et al., 2003). We have observed that *rad24* strains start the first division of meiosis about 30 minutes later than in wild type. Shinohara *et al*. (2003) have reported similar results. The processing and repair of DSB ends has been shown to be delayed in *rad24* cells (Aylon and Kupiec, 2003). Because this repair occurs at a later time in *rad24* cells, this may explain the subsequent delay of the reductional division in these cells. Just as with Mec1 and Tel1, it is possible that Rad24 is involved in transducing the RIS but is delayed in division due to incorrect later steps partner choice and suppression of ectopic recombination. As I have suggested above, this could be resolved by examining the transcription of *NDT80* in *rad24* mutants. If Rad24 is actually transducing the RIS, then removing Rad24 will cause an earlier transcription of the target *NDT80*. Because no role for Rad9 has been defined beyond DNA damage repair associated with S phase, it is unlikely that *NDT80* transcription will be any different in these strains.

Mek1 is a candidate for transduction of the RIS

After eliminating three mitotic checkpoints as transducers of the RIS, I decided to examine a gene encoding a meiotic kinase. Mek1 is a PI 3-kinase family protein containing an FHA domain. Mek1 is paralogous to Rad53 and is induced 20 fold during early meiosis from an Ime1-dependent promoter (Hochwagen and Amon, 2006; Rockmill and Roeder, 1991). Despite their essential roles in the DNA damage checkpoint in

response to mitotic DSBs, Rad9 and Rad53 do not appear to be involved in controlling meiosis I progression in response to meiotic DSBs formed during meiosis (Carballo and Cha, 2007; Lydall et al., 1996). This control instead requires the meiosis-specific proteins Mek1, Red1, and Hop1. In particular, meiotic DSB formation leads to Mec1- or Tel1-dependent Hop1 phosphorylation, which is required for Mek1 activation (Carballo et al., 2008). Mek1has been shown to have a role in suppressing recombination between sister chromatids and is required for arrest of *dmc1* cells (Carballo et al., 2008). Mek1 interacts with the SC components, though the precise nature of that interaction is somewhat unclear (de los Santos and Hollingsworth, 1999; Niu et al., 2005; Wan et al., 2004). Hollingsworth's more recent studies suggest that though Mek1 forms a complex with Hop1 and Red1, it does not phosphorylate either protein. At the time of this writing, no Mek1 targets have been identified in *Saccharomyces*.

To test whether Mek1 is involved in transducing the RIS, I examined the MI kinetics of a *mek1* mutant. I found that *mek1* strains started the first division of meiosis at a time indistinguishable from *rec104* (Fig. 5-7). This result represents the first evidence for a kinase mutant having an earlier reductional division.

There are two alternate interpretations of this finding. One interpretation is that *mek1* mutants start MI earlier because Mek1 is a transducer (effector kinase) of the RIS. A second interpretation is that *mek1* strains start MI earlier because they are a part of the RIS itself. Mek1 has been shown to interact with Hop1 and Red1. Our lab has shown that Hop1 and Red1 mutants start MI early. Because Mek1 interacts with Hop1 and Red1, it may be a part of the signal that transduced by other factors. To distinguish between these two hypotheses, I over-expressed Mek1 in *spo11* and wild type strain backgrounds. If Mek1were a part of the RIS, then over-expressing *MEK1* should not affect when these strains start the reductional division. In contrast, if Mek1 were an effector kinase downstream of the RIS, then over-expressing *MEK1* might lead to a later MI in a wild type background and should reverse the earlier MI phenotype seen in the

RIS mutant *spo11*. Over-expressing a downstream protein of a checkpoint often activates the checkpoint in absence of the signal. For instance, overexpression of *RAD53* results in a delay in cell-cycle progression during mitotic growth in absence of damage (Kim and Weinert, 1997). The cell-cycle delay did not require any of the upstream checkpoint genes tested (e.g. *RAD9* or *MEC1*), indicating that the cell-cycle delay is either unrelated to the checkpoint responses, or that it occurs constitutively because *RAD53* acts further downstream of the checkpoint genes tested. I found that MI began slightly later than wild type in WT [MEK1 O/E] strains compared to wild type strains containing a control vector (Fig. 5-8 B and E). Furthermore, over-expressing *MEK1* in a *spo11* strain background resulted in a MI that started at a time indistinguishable from wild type with an empty vector (Fig. 5-8 C and F). These data indicate that Mek1 is downstream of the RIS and is an effector of this signal.

We have shown that the transcription factor *NDT80* is the downstream target of the RIS. It is unlikely that Mek1 directly influences the transduction of *NDT80.* To date, no phosphorylation targets of Mek1 have been published in *Saccharomyces* [(Niu et al., 2007) and Nancy Hollingsworth, personal comm.]. I hypothesize that the RIS would be transduced by the kinase Mek1 and this would lead to inhibition of *NDT80* transcription. Currently, no inhibitory phosphorylation of Ndt80b has been described; phosphorylation of Ndt80 activates this protein. The meiotic regulatory kinase Ime2 promotes activity of *NDT80* by phosphorylating the negative regulator of *NDT80* transcription Sum1 on Thr 306 at a consensus Pro-X-Ser/Thr site (Moore et al., 2007). In addition, post-transcriptional phosphorylation of Ndt80 by Ime2 has been shown to promote further *NDT80* transcription (Tung et al., 2000). Because the RIS delays *NDT80* transcription and activity, it is possible that Mek1 acts through an intermediate to delay *NDT80.* .

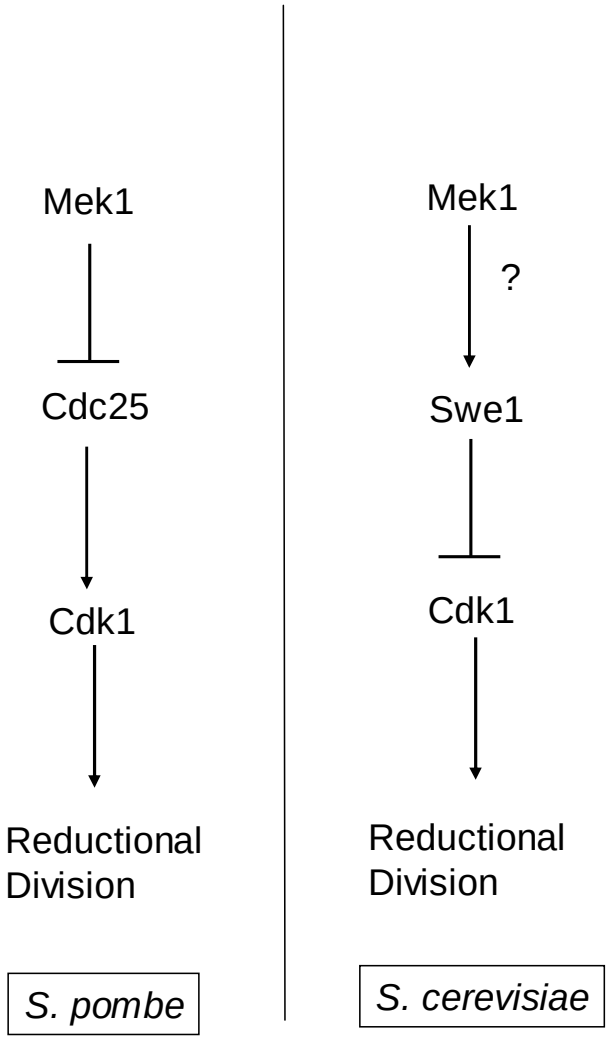
Unlike in *S. cerevisiae,* a Mek1 target in *S. pombe* has been identified, though the activity of Mek1 is somewhat different in *S. pombe* than in *S. cerevisiae* (Fig. 5-9). In both *S. pombe* and *S. cerevisiae,* an inhibitory tyrosine phosphorylation of Cdk1, encoded

by *CDC2* in *S. pombe* and *CDC28* in *S. cerevisiae*, is necessary for the Pachytene checkpoint (Leu and Roeder, 1999; Perez-Hidalgo et al., 2003, 2008). In *Saccharomyces*, hyper-phosphorylation of Cdk1 by an unknown kinase and subsequent stabilization of the inhibitory kinase Swe1 maintains an inhibitory phosphorylation of Cdk1. In *S. pombe* fission this inhibitory phosphorylation of Cdk1 results when the Cdk1-promoting phosphatase Cdc25 is phosphorylated and inactivated. Cdc25 is the fission yeast homolog of Mih1 in *Saccharomyces*. In *S. pombe* Mek1 has been shown to directly phosphorylate Cdc25 *in vitro* and this phosphorylation has been shown to be necessary for the Pachytene checkpoint response *in vivo* (Perez-Hidalgo et al., 2003, 2008). It is an attractive hypothesis that Mek1 phosphorylates Swe1 in response to the Pachytene checkpoint, thus producing a similar inhibitory phosphorylation of Cdk1 to arrest cells at pachytene. This will be discussed further in the final chapter.

Interestingly, overexpression of Mek1 leads to cell cycle arrest in *S. pombe* (Perez-Hidalgo et al., 2008), while in *S. cerevisiae*, my results show MI is only slightly delayed. This could reflect either experimental and/or organismal differences between *S. pombe* and *S. cerevisiae* or it could reflect differences in robustness of target response between the two yeasts. Mek1 may be the only or primary kinase required for control of the reductional demission in *S. pombe*, while other factors appear to be required for control of the start of the reductional division in *S. cerevisiae*. Further experiments are necessary to clarify this discrepancy.

In *S. cerevisiae* it has been shown that Mek1 must dimerize and auto-phosphorylate to become completely activated in *dmc1*mutant cells (Niu et al., 2007). Two conserved threonine residues in the putative activation loop of Mek1 have been shown to be important for this. *REC104* has been shown to be necessary for auto-phosphorylation of Mek1 on T327 and T321. Interaction with Red1 and the C domain of Hop1 is also required for this phosphorylation. Hollingsworth attributes the requirement for Rec104 in the dimerization and auto-phosphorylation of Mek1 via T327 and

Figure 5-9: Cdk1 regulation during MI in *S. pombe* in *S. cerevisiae*. In *S. pombe*, when the Pachytene checkpoint is activated, Mekl1 has been shown to phosphorylate the phosphatase Cdc25 (Perez-Hidalgo et al., 2003, 2008). This destabilizes Cdc25. Cdc25 dephosphorylates Cdk1 on an inhibitory Y residue. Dephosphorylating this Y allows Cdk1 to become activated and the reductional division to occur. In *S. cerevisiae*, I hypothesize that Mek1 phosphorylates Swe1. An unknown kinase has been shown to hyperphosphorylate and stabilize the kinase Swe1 (Leu and Roeder, 1999). Swe1 can phosphorylate Cdk1 on an inhibitory Y residue. Cdk1 activity is required for the reductional division in *S. cerevisiae*, similar to in *S. pombe*.


Mek1
Cdc25
Cdk1
Reductional Division
S. pombe
Mek1
?
Swe1
Cdk1
Reductional Division
S. cerevisiae

T321 to a requirement for the formation of DSBs. However they did not examine any other recombination initiation mutant.

I hypothesize instead that that the proteins of the RIS are what catalyzes auto-phosphorylation and dimerization of Mek1 rather than DSBs. To test this, one could examine phosphorylation of Mek1 in a *ski8* background. I predict that you would see phosphorylation of Mek1 thus demonstrating that proteins of the RIS, and not DSBs are important for activating Mek1. In addition it would be necessary to examine Mek1 auto-phosphorylation in a third recombination initiation mutant, such as *spo11*, to demonstrate that this auto-phosphorylation is not dependant on only Rec104.

Is the RIS a part of a checkpoint?

If there is a mutation in a checkpoint gene, a cell will fail to arrest when perturbed. An example of this is the spindle checkpoint. When wild type cells are treated with the spindle depolarizing drug benomyl, they arrest while spindle checkpoint mutants continue to divide in the presence of this drug. The Pachytene checkpoint in meiosis can recognize an excess of single stranded DNA as seen in *dmc1* cells. This is not a normal meiosis, rather it is an example of a perturbed meiosis. When *DMC1* is absent, the DNA surrounding a break is hyperresected leaving larger than normal 3' overhangs that are coated with Rad51 and RPA (Bishop, 1994; Carballo and Cha, 2007). The increased concentration of these two proteins on the DNA is sensed by Pachytene checkpoint proteins and the cell is arrested (Lisby et al., 2004). Carballo and Cha (2007) surmise that this response is actually related to the S phase checkpoint which also responds to an excess of RPA-coated ssDNA found when replication forks are stalled and leading and lagging strand synthesis are decoupled . While there is some resection of DSBs by the MRX complex that occurs in a normal meiosis, it appears to be insufficient to trigger the Pachytene checkpoint (Chapter 1).

In contrast to the Pachytene checkpoint system, the RIS pathway regulates a normal meiosis, so it does not fulfill the traditional definition of a checkpoint and we have never referred to it as a checkpoint in our publications. However, Hochwagen and Amon's (2006) definition of a checkpoint is somewhat more liberal than the traditional definition. They define a checkpoint as fulfilling four criteria: 1) a signal, 2) detection of this signal by sensory proteins, 3) activation of signal transduction pathways, 4) translation of the signal into an output by modifying checkpoint targets. By these criteria the RIS can be considered to fulfill many of the requirements to be considered a checkpoint. We have propsed that a subset of recombination initiation factors constitutes a signal. The precise mechanism of how this signal is sensed is unknown. I will speculate on this in the final chapter.

CHAPTER 6

SUMMARY, CONCLUSIONS AND FUTURE EXPERIMENTS

The Recombination Initiation Signal

Meiotic recombination is an essential process in the life cycle of almost all eukaryotes. There are still many questions in the field of meiotic recombination regarding how the recombination initiation proteins interact with each other and with chromatin to carry out the formation of DSBs. This is not surprising considering the complexity of a putative protein complex made up of at least ten proteins that interact with chromatin in a transient manner during meiosis. The events of recombination and the reductional division must be coordinated to ensure viable meiotic products. Our laboratory had previously demonstrated that seven of the eight recombination initiation proteins and two synaptonemal complex proteins that we had examined are involved in signaling to delay the reductional division (Galbraith et al., 1997; Jiao et al., 1999; Malone et al., 2004). If any one of these nine proteins is absent the first division starts at an earlier time. In this thesis, I presented data examining the remaining two recombination initiation genes, *SKI8* and *REC107*. We found that *rec107* mutants started the first division of meiosis earlier than wild type (Malone et al., 2004). However, *ski8* mutants start the reductional division at the same time as wild type (Malone et al., 2004). We have proposed that eight of the ten recombination initiation proteins (not including Mei4 or Ski8) form a signal which can delay the start of the reductional division. The two SC proteins Hop1 and Red1 may be a part of the RIS or may serve as downstream adaptor proteins for the RIS. This possibility will be described below.

One alternate explanation for the earlier first division observed in eight recombination initiation mutants is that pre-meiotic DNA synthesis is shortened in these mutants. Lichten's lab has shown that replication and recombination are coupled events, so this is not an unreasonable hypothesis [(Borde et al., 2000)] and Chapter]. If

formation of recombination initiation complexes is delayed because one protein is absent, the progression of S phase may also be delayed. In 2000, Cha *et al*. proposed that the early MI observed in some recombination initiation mutants is due to a shorter S phase. They reported that *spo11* mutants in the SK1 strain background have a shorter duration of pre-meiotic replication and proposed that this was the reason that *spo11* mutants started the first division of meiosis early. If Spo11 were absent then, the progression of S phase might be faster because the cell would not be able to assemble initiation complexes. Interestingly, they observed that the duration of S phase in *rec102* strains was indistinguishable from wild type, however. Both Rec102 and Spo11 are required to initiate meiotic recombination, so it is difficult to accept their conclusions regarding the duration of S phase in *spo11* cells. To investigate the role of recombination imitation genes in determining the length of S phase, Stuart Haring, a graduate student from our lab, used flow cytometry to determine the length of S phase in our S288C-derived strain background and found that the duration, time of entry and time of 50% of cells completing S phase in *rec102*, *rec104*, *rec114*, *spo11 rad50* or *ski8* mutants was not significantly different from wild type (Malone et al., 2004). Furthermore, Haring calculated that S phase was 51-59 minutes for all strains tested, including wild type cells, similar to the 59 minutes reported for *spo11* SK1 strains by Cha *et al.* (2000). This supports the conclusion that the length of S phase is not affected by recombination initiation mutants and does not support the hypothesis by Cha *et al.* (2000).

The paradox of *mei4* and *rec103*

One paradox which became apparent after the analysis of the ten recombination initiation proteins was the normal timing of the reductional division in the *ski8* and the *mei4* mutants. As discussed in Chapter 1, Keeney's lab has proposed thatSki8 serves as sdcaffolding for the assembly of a subcomplex containing Spo11, Rec102, and Rec104 (Arora et al., 2004; Kee et al., 2004). They also reported that Spo11 accumulation in the

nucleus is dependent on Ski8, and *vice versa*. In addition, Rec102 and Rec104 are proposed to require Ski8 for their recruitment to meiotic chromosomes (Kee et al., 2004). If Spo11 were dependent on Ski8 for proper localization and entry into the nucleus, and if Rec102 and Rec104 were dependent on Ski8 for proper chromatin association, then a *Ski8* mutant should show the same early MI timing as *spo11*, *rec102*, and *rec104* null mutants. It is formally possible that Spo11, Rec102, and Rec104 have functions independent of Ski8 and recombination and these functions relate to the kinetics of the reductional division; however, there is no evidence for this.

mei4 strains also start the reductional division early. This is perplexing because Mei4 is required for for Spo11 removal after DSB formation (Prieler et al., 2005). Furthermore, Rec114 and Mei4 proteins can associate with the hotspots in *spo11* mutants suggesting that the Rec107/Rec114/Mei4 subcomplex precedes the binding of Spo11 to chromatin (Sasanuma et al., 2008). Our data indicate that Mei4 is not a part of the RIS. If Mei4 were required only late in initiation, then that could explain it not being a part of the RIS. On the other hand Sasanuma *et al.* (2008) have shown that Mei4 can associate with DNA in the absence of Spo11. This implies that Mei4 is a part of the normal initiation complex prior to Spo11, and thus should be present on the DNA with the other RIS proteins. Evidence suggests that Ski8 is recruited after Mei4 and it is most likely present in a separate subcomplex from Mei4, though yeast-two hybrid experiments show that all ten recombination initiation proteins interact to some degree (Arora et al., 2004; Li et al., 2006b; Sasanuma et al., 2008). While Mei4 is present in the recombination initiation complex and is catalytically required for the formation of DSBs, removing Mei4 does not affect the ability of the other recombination initiation proteins in the RIS to send the signal to delay MI. One explanation could be that the protein-protein interactions required for DSB formation are likely not the same as those required for the RIS.

We have proposed that the proteins of the RIS and not DSBs create the signal that co-ordinates the timing of the first division. To test this hypothesis, Nick Lyons, an Honor's student from our lab examined the MI kinetics of *spo11* a point mutant strain which cannot make DSBs (Nick Lyons, Honor's Thesis). *spo11-Y135F* strains contain a mutation in the proposed catalytic residue necessary for DSB formation (Keeney et al., 1997). The Spo11-Y135F protein has been shown to localize to hotspot DNA, however (Prieler et al., 2005). Lyons showed that the reductional division in *spo11-Y135F* strains occurred earlier than wild type strains, similar to the MI timing in a *spo11Δ* mutant. Lyons also observed similar MI timing results with a *spo11*-D288A strain. This mutation occurs within a conserved structural motif called the Toprim domain which is required for meiotic recombination (Diaz et al., 2002). The Toprim domain has been implicated in binding a metal ion cofactor in topoisomerases and bacterial primases. Lyons also examined the MI kinetics in several strains containing point mutations in either *REC102, REC104* or *REC114* that eliminated recombination. He found that MI started at the same time in the RIS *rec102, rec104* and *rec114* point mutants as it did in a RIS null mutants. The results from these reductional division timing experiments of RIG point mutations do not support the hypothesis that the presence of the proteins is enough to delay MI in wild type cells. It must be noted, however, that Lyons did not examine whether the protein still localized to meiotic chromatin in the point mutant strains he examined. It is formally possible that, each of these mutations abolished binding to the DNA and this is why Lyons observed an early MI in all of the point mutants that he examined. This explanation does not, however explain his results with the *spo11-Y135F* mutant because it has been demonstrated that Spo11-Y135F protein is bound to the DNA. It is possible that a mutation at Y135 disrupts the binding of other RIS proteins causing them either to be non-functional or absent. This would result in an early reductional division.

Perhaps a better experiment would be to generate point mutations in *SKI8* or *MEI4* and look for strains that no longer start the first division at the same time as wild

type, but now start the first division early. Such a phenotype might occur if a protein required for the RIS were occluded by the Ski8 or Mei4 point mutant -containing protein. This would prevent downstream factors (such as Mek1 or perhaps Hop1 or Red1) from sensing or transducing the RIS. If you found such a point mutant, you could then perform suppressor analysis by screening for suppressors that would restore normal timing to the point mutant strain. This would reveal which regions of RIS proteins are required to send the signal. This screen could also potentially reveal other downstream factors required in the RIS pathway.

Statistical analysis of *rec104* and other mutants

The time when strains start the reductional division varies from experiment to experiment. Sporulation conditions and media can vary slightly depending on when and by whom the the media was prepared. Additionally, different experimenters can score the same data differently. While these differences have been observed, we have always observed that *rec104* mutants start the reductional division earlier than wild type by ~1.5 hours. In order to statistically compare *rec104* and other mutants between experiments, I have performed an analysis of the time at which MI begins in all of the *rec104* and wild type strains presented in this thesis. To do this, I drew a trend-line using least-squares analysis on the part of each MI graph when the percentage of binucleate cells was increasing approximately linearly (Table 6-1). By calculating the x-intercept of each line, I could then obtain the time at which a culture started the reductional division. I found that *rec104* strains started the first division at 4.1 ± 0.39 hours, while wild type strains started the reductional division at 5.2 ± 0.48 hours. The difference between the two strains is 1.18 ± 0.41 hours.

The above data can be used in statistically determining whether a mutant of interest starts the reductional division at a time indistinguishable from *rec104* cells or statistically different from a *rec104* mutant. Because my results obtained from studying

Table 6-1: Statistical analysis of timing *rec104* and wild type cells

Source Figure	*rec104* x-int.	*rec104* R^2	WT x-int.	WT R^2	diff. between *rec104* and WT
2-1	3.9	0.98	5.2	0.98	1.3
2-2, exp. 1	3.8	0.94	6.1	0.95	2.3
2-2 exp. 2	4.3	0.96	5.2	0.99	0.90
5-3 exp. 1	4.1	0.95	5.3	0.99	1.2
5-3 exp. 2	3.5	0.95	4.5	0.97	1.0
5-4 exp.1	4.0	0.96	4.9	0.91	0.9
5-4 exp.2	4.1	0.97	4.9	0.95	0.8
5-5	5.1	0.95	6.1	0.97	1.0
5-6	4.0	0.98	5	0.96	1.0
5-7 exp. 1	4.0	0.99	5.3	0.98	1.3
5-7 exp. 2	3.9	0.99	5.2	0.96	1.3
Mean ± st. dev	**4.1 ± 0.41**		**5.2 ± 0.48**		**1.2 ± 0.41**

Note: *rec104* and wild type MI timing data calculated using figures shown. The x incercepts of *rec104* and WT were determined using regressin analysis. The confidence of this analysis is shown using R^2.

ski8 and *mek1* mutants are of great importance to the major conclusions made in this thesis, I chose to test whether the timing of the reductional division in *ski8* or *mek1* cells is the same or different than the MI timing of *rec104* cells. In Chapter 2, I presented two independent experiments with a total of four cultures showing that *ski8* strains start the reductional division at the same time as wild type cells, rather than early like *rec104* cells. Using methods described above, I have calculated that *ski8* strains start the reductional division at 5.7 ± 0.48 hours (Table 6-2). The calculated timing difference between the *ski8* cultures and the wild type controls used in these experiments is minimal (0.01 ± 0.084 hours). I used a student t test to show that the difference in the timing of *rec104* mutants compared to wild type is significantly different than the difference between *ski8* and *rec104* mutants ($p = 1.1 \times 10^{-9}$).

In Chapter 5, I show two independent experiments with a total of six cultures showing that the reductional division begins early in *mek1* mutants. Using the methods described above, I calculated that *mek1* cells start the reductional division at 4.0 ± 0.096 hours (Table 6-3). The calculated difference between when *mek1* mutants and wild type mutants start the reductional division is 1.3 ± 0.090 hours. The student t test of the difference between *rec104* cells and wild type vs. *mek1* cell and wild type shows that these strains start the reductional division at a statistically indistinguishable time ($p = 0.50$).

Termination of the RIS

The RIS must be a transient signal in order to allow the eventual reductional division. Our work has not directly addressed how the RIS is terminated, but the enndonucleolytic properties of the Mre11/Rad50/Xrs2 (MRX) complex do suggest one possible mechanism. As mentioned in Chapter 1, after DSBs are formed, Spo11 (and presumably the proteins that interact with it, though this has not been tested) is endonucleolytically cleaved from the 5' end of DSBs generating an oliogonucleotide

Table 6-2: Statistical analysis of *ski8* mutants

Source Figure	*ski8* x-int.	*ski8* R^2	WT x-int.	WT R^2	diff. between *ski8* and WT
2-2 exp. 1	6.1	0.92	6.1	0.92	0.040
2-2 exp. 1	6.0	0.96	6.1	0.92	0.10
2-2 exp. 2	5.3	0.98	5.2	0.99	0.10
2-2 exp. 2	5.2	0.98	5.2	0.99	0.0
Mean ± st. dev	**5.7 ± 0.48**		**5.2 ± 0.48**		**0.01 ± 0.48**

Note: Regression analysis was performed using data from four *ski8* cultures. The wild type data shown is from that particular experiment.

Table 6-3: Statistical analysis of *mek18* mutants

Source Figure	*mek1* x-int.	*mek1* R^2	WT x-int.	WT R^2	diff. between *mek1* and WT
5-6 exp. 1	4.1	0.92	5.3	0.98	1.2
5-6 exp. 1	3.9	0.97	5.3	0.98	1.4
5-6 exp. 1	4.0	0.99	5.3	0.98	1.3
5-6 exp. 2	4.0	0.99	5.2	0.96	1.2
5-6 exp. 2	3.8	0.96	5.2	0.96	1.4
5-6 exp. 2	3.9	0.99	5.2	0.96	1.3
Mean ± st. dev	**4.0 ± 0.096**		**5.2 ± 0.48**		**1.3 ± 0.089**

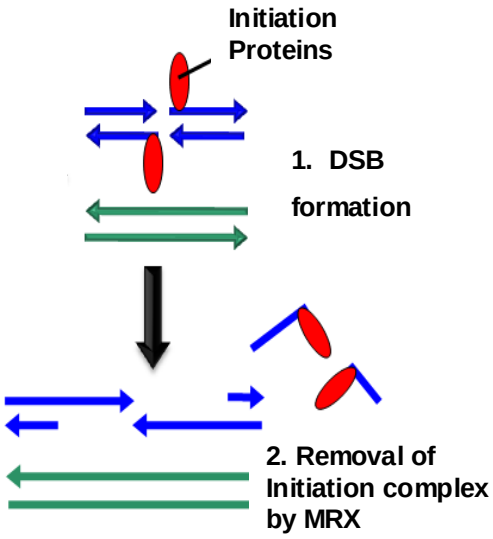
Note: Regression analysis was performed using data from six *mek1* cultures. The wild type data shown is from that particular experiment.

fragment with a free 3' end bound to Spo11 (Fig. 6-1). Once Spo11-containing complexes are freed from the context of meiotic chromatin, (including Hop1, Red1 and Mek1) it is unlikely that the RIS could be sent.

If endonucleolytically cleaving and removing Spo11-containing complexes were to terminate the RIS, then MRX mutants lacking endonuclease activity should arrest as mononucleate cells. *mre11S* and *rad50S* cells form but cannot resects DSBs (See Chapter 1). *com1/sae2* deletion mutants share a similar phenotype, suggesting that Com1/Sae2 performs a similar role to the late recombination function of the MRX complex (See Introduction of Chapter 2). Stuart Haring, a graduate student from our lab, extensively used *rad50S* strains. He observed that these strains sporulated very poorly (~1%) (Stuart Haring, personal comm.). Kleckner's lab also observed that rad50S strains have very reduced sporulation (~1%) and produce inviable spores (Alani et al., 1990). I stained some of the *rad50S* cells that Haring collected at 16 hours with DAPI and observed that the reductional division was very delayed in these cells. Only ~5% of cells at this timepoint had started the reductional division, though I recall that they were extremely difficult to score because the nuclei were degraded and distinct nuclei were difficult to ascertain (K. Foreman, unpublished observation). Haring mentioned that sporulation percentages were equally difficult to determine. This is different from a *rad50* deletion strain which starts the reductional division earlier than wild type cells (Jiao et al., 1999). Usui et al., (2001) report that cells containing the *rad50S* mutation, a *com1/sae2* deletion or a *mre11S* mutation are delayed several hours in the reductional division. Taken together, mutant cells that cannot remove Spo11 arrest or are very delayed.

Hochwagen and Amon (2006) have proposed the existence of a *rad50S* checkpoint. They propose that cells containing *rad50S, mre11S* point mutations or *com1/sae2* null mutations trigger a checkpoint response that causes cells to be delayed by several hours in the start of the reductional division. It is attractive to posit that this

Figure 6-1: Proposed mechanism for termination of the RIS. The endonuclease activity of the MRX complex has been shown to cleave Spo11 from DSBs.


Initiation
Proteins
1. DSB
formation
2. Removal of
Initiation complex
by MRX

checkpoint that Hochwagen and Amon have proposed is actually triggered by the continued presence of the RIS which has not been terminated. However, this hypothesis is contradicted by the observations of Usui et al. showing that the presence of a rad24 or mec1 mutations in either rad50S, mre11S or com1/sae2 strains allow the reductional division to start at the same time as in wild type.

We have shown that neither MEC1 nor *RAD24* is required for the RIS; *mec1* and *rad24* strains do not start the reductional division early (Chapter 5). This contradiction is another reason why I propose that Rad24 and especially Mec1 be re-evaluated in their role in transducing the RIS by examining the transcription of *NDT80* in these strains (proposed in Chapter 5). This will be discussed below.

The RIS is conserved in other strain backgrounds

Work by Kee and Keeney showed that if the recombination initiation protein Spo11 is absent, the first division begins at an earlier time in the SK1 strain background (Kee and Keeney, 2002). Data presented in Chapter 3 supports the hypothesis that the RIS is conserved in the SK1 strain backgrounds. In addition, Storlazzi *et al.* (2003) found that *spo11* mutants in *Sordaria macrospora* display a phenotype analogous to the early reductional division seen in *S. cerevisiae spo11* mutants suggesting that the RIS may be conserved in at least all ascomycete fungi. Both *S. cerevisiae* and *Sordaria* can detect the absence of even one protein of the RIS and in these cells the reductional division begins at an earlier time. In male mice, knockout of the mouse *SPO11* gene prevents formation of DSBs and SC during meiosis and leads to the meiotic arrest of spermatocytes at zygotene. This suggests that the RIS is not active in male mice. In female mice, however, *spo11* mutants show decreased follicle formation due to apoptosis, however many of the cells do finish the reductional division, though the timing of this is not clear (Di Giacomo et al., 2005). An attractive hypothesis is that there is a RIS active in mice oocytes that delays follicular maturation and triggers programmed cell death

when initiation proteins are absent. This would suggest that that the RIS is conserved even in some multi-cellular eukaryotes.

The RIS and Pds1 degradation

Andrew Murray's lab examined Myc-tagged Pds1 in wild type and *spo11* sporulating cells (Shonn, et al 2000). They observed that nuclear Pds1 was always degraded in wild type cells in Anaphase I. In contrast, 77% of *spo11* cells that they examined had had Pds1-Myc present even though they had an elongated spindle and separated nuclei. When I examined Pds1-HA, in *rec104* mutants, I also found that most cells were able to divide even though Pds1-HA was still present in the nucleus. Murray interpreted his observation to mean that the absence of interhomolog tension created by chiasmata was responsible for the nuclear division in *spo11* mutant cells without Pds1 degradation. Murray proposed that this tension is monitored by the spindle checkpoint and that this checkpoint delays the reductional division until chiasmata are linking homologous chromosomes. While the data presented in chapter 3 is in agreement with Murray's results, I do not agree with his interpretation. *ski8* and *mei4* mutant strains do not have DSBs, and thus cannot have any interhomolog tension due to chiasmata, yet these strains start MI earlier than wild type. We propose that it is the the RIS that delays the start of the reductional division and not the spindle checkpoint, as Murray hypothesizes. Because the RIS targets *NDT80*, the transcription factor necessary for spindle formation, the RIS signal is sent *before* the MI spindle is even present and *before* inter-homolog tension is even created (discussed below and in Chapter 4). The spindle checkpoint cannot occur before there is an MI spindle present.

The target of the RIS is *NDT80*

Data shown in chapter 4 establishes that *NDT80* is the target of the RIS, though the mechanism of transcriptional control remains unknown. We have shown that *NDT80* transcription is both necessary and sufficient to determine the start of the reductional

division. The control of *NDT80* is complex and occurs at the level of transcriptional and post-transcriptional events [(Ahmed et al., 2009; Pak and Segall, 2002b; Tung et al., 2000) and Chapter 1]. I propose that the RIS affects Sum1-mediated repression of *NDT80*. Sum1 represses the transcription of most middle sporulation genes (including *NDT80)* during mitosis and early meiosis by binding to MSEs (Pak and Segall, 2002b). Sum1 is bound to meiotic chromatin alsong with the histone deacetylase Hst1 and the tethering factor Rfm1 (McCord et al., 2003). The meiotic kinase Ime2 must phosphorlylate Sum1 in order for the MSE upstream of the *NDT80* gene to be de-repressed (Moore et al., 2007). If Sum1 is targeted by the RIS, then *sum1* mutants should start the reductional division early.

Furthermore, it is unknown whether the RIS affects the activity of Ime2. Ime2-dependent phosphorylation of Ndt80 is necessary for the full activation of Ndt80 (Benjamin et al., 2003). It will be necessary to examine the phosphorylation state of Ndt80 in wild type and a RIS mutant to answer the question.

Transduction of the RIS

The spindle checkpoint does not transduce the RIS

Rachel Gast's work has shown that neither the Mad2 (spindle Assembly Checkpoint) nor the Bub2 (Mitotic Exit Network) branch of the spindle checkpoint transduce the RIS. Gast also reproducibly showed that *mad3* mutants do not start the first division of meiosis earlier than wild type in. This result contrasted with work from the Dawson lab who showed that *mad3* strains were "post-prophase I" by examining a combination of MI spindles, SPBs and nuclear division, though they do not separate these three metrics in their graphs. (Cheslock et al., 2005). To test Gast's conclusion, I examined the transcription of *NDT80* and the reporter gene *SPS4* in *mad3* cells. If *MAD3* were necessary for transducing the RIS, *NDT80* and the reporter for Ndt80 function *SPS4* would be transcribed earlier than in wild type. I did not conclusively find this (See

Chapter 5). I conclude that Mad3 does not transduce the RIS. Combined with Gast's other results, I conclude that the spindle checkpoint does not transduce the RIS.

The S phase checkpoint does not transduce the RIS

Mec1 and Rad53 are large PI 3-kinase family proteins that respond to lesions associated with replication defects and are required for the S phase and DNA damage checkpoints. To study the role of Rad53 and Mec1 in transducing the RIS, we examined the MI kinetics of null mutants of these genes (Chapter 5). We found that *rad53* strains started the reductional division at the same time as wild type strains. *mec1* mutants are slightly delayed in starting MI, perhaps because of Mec1's demonstrated role in promoting meiotic recombination between homologs rather than between sister chromatids (partner choice) (Carballo and Cha, 2007; Thompson and Stahl, 1999). Because neither of these mutants started the reductional division early (as in *rec104* strains), we conclude that these three proteins do not transduce the RIS. Similar reductional division timing results have been reported by others; however, they do not take timepoints as frequently as we do, nor did they count as many cells (Borde et al., 2000; Lydall et al., 1996)

It is somewhat perplexing that Mec1 does not transduce the RIS because Mec1 has been shown to phosphorylate Hop1 in response to DSBs or the presence of Spo11, thus one would predict that *mec1* strains should start the reductional division early. While it is formall possible that mec1 cells do not start MI early because the protein Tel1 is partially redundant for Mec1, there is an alternative explanation. Mec1 could be involved in the RIS pathway, but an earlier first division is not seen because of the increased ectopic and sister recombination and decreased inter-homolog recombination seen in *mec1* or *tel1* mutants. Increased recombination between sister chromatids could lead to decreased homologous pairing which could slow the progress of the reductional

division. An earlier starting time of the reductional division could be obscured by this slowed progress of the reductional division.

A second explanation for the delayed MI seen in *mec1* strains is that Mec1 almost surely is required to phosphorylate histone H2A surrounding DSBs. This has been has observed during mitosis in yeast. Phosphorylation of mammalian histone H2AX (a homolog of H2A in yeast) occurs around DSB sites during meiosis (Unal et al., 2004). If this phosphorylation were absent, it may be more difficult for the cell to recruit necessary late recombination factors or cohesins. Unal et al., has proposed that Mec1-dependent phosphorylation of H2A surrounding DSBs serves as a platform for late recombination factors required for DNA repair. If this platform were absent, then an excess of ssDNA would form and not be repaired in a timely manner, thus slowing down the overall MI kinetics of *mec1* strains. If the breaks never become properly repaired this broken DNA would not segregate correctly into two nuclei explaining the decreased viability observed in *mec1* strains. This could also explain why *mec1* nuclei appear fragmented compared to wild type meiotc nuclei (K. Foreman, unpublished observation). In order to begin studying this, it would be necessary to ChIP the chromatin immediately surrounding a hotspot to see if H2A is phosphorylated in a Mec1 dependent manner. ChIP could also be used to see whether late recombination factors were delayed in recruitment to DSBs in *mec1* mutants.

To test whether Mec1 is relaying the RIS, one could examine the transcription of the target *NDT80* in *mec1* strains. If Mec1 were involved in transducing the RIS, you should see earlier transcription of *NDT80* in *mec1* mutants. Transducing the signal to delay MI would be a separate function of Mec1 and Tel1 than the later functions involved in partner choice or H2A phosphorylation. If these kinases were required in transducing the RIS, then Mec1 and/or Tel1 would have at least two roles in a normal meiosis. One role would be an inhibitory role in delaying *NDT80* transcription unrtil after the initiation

of recombination and a second positive role would be in promoting later recombination events.

The DNA damage checkpoint does not transduce the RIS

dmc1 mutants arrest at the mononucleate stage. This arrest can be bypassed by presence of a *rad24* mutation, but not a *rad9* mutation. To test whether RAD9 or RAD24 were required for the transducing the RIS, we examined the kinetics of MI in *rad24* and *rad9* mutants. We observed that *rad24* mutants had a slightly later (certainly not earlier) reductional division than in wild type. Rad9 acts in an independent pathway from Rad24 to sense DNA damage. We found that the start of the reductional division in *rad9* mutants is indistinguishable from wild type cells. It is possible that Rad9 does not have a role in meiosis beyond sensing the DNA damage occurring during S phase. Because Rad9 and Rad24 sense DNA damage in separate pathways (Chapter 1), Demelza Koehn examined the MI kinetics of a rad24 rad9 strain. She found that these strains started the reductional division earlier than wild type. Taken together, these data eliminate members of both the DNA damage checkpoint in transducing the RIS.

Like Mec1, Rad24 has been demonstrated to have a role in promoting homologous recombination over recombination between sister chromatids and in facilitating late recombination events [(Grushcow et al., 1999; Shinohara et al., 2003) and discussed in Chapter 1]. This perhaps explains why we observe that *rad24* mutants start the first division of meiosis slightly later than in wild type. The processing and repair of DSB ends has been shown to be delayed in *rad24* cells (Aylon and Kupiec, 2003). Because Rad24 has been shown to be required for meiotic progression, I propose that the transcription of *NDT80* be examined in *rad24* cells to test whether Rad24 serves two roles in meiosis. If *NDT80* were transcribed earlier in *rad24* cells, then this would indicate that the RIS is transduced by Rad24 and that the late MI is seen in *rad24* cells occurs because Rad24 is required for late recombination events. Because no role for

Rad9 has been defined beyond DNA damage checkpoint associated with S phase, it is unlikely that *NDT80* transcription will be any different in this strain, however, I propose that *NDT80* transcription be examined in *rad9* cells for completeness.

Mek1 is a candidate for transduction of the RIS

In Chapter 5, I present data demonstrating that Mek1, a PI 3-kinase family protein containing an FHA domain is a part of the RIS pathway. Our primary reasons for examining Mek1 as a candidate for the transduction of the RIS were that 1)*MEK1* is transcribed early in meiosis, the same time as the the genes that comprise the RIS, 2) Mek1 interacts with Hop1 and Red1, two proteins we propose are a part of the RIS pathway, 3) *MEK1* is homolougous to *RAD53*, and has been proposed by Hollingsworth's lab to substitute for *RAD53* as a part of the Pachytene checkpoint response (Niu et al., 2007) I found that *mek1* strains started the first division of meiosis at a time indistinguishable from *rec104*. This result represents the first evidence we have observed that a kinase/regulatory protein mutant has an earlier start of the reductional division.

There are two alternate interpretations of this finding. One interpretation is that *mek1* mutants start MI earlier because Mek1 is a transducer (downstream) of the RIS. A second interpretation is that *mek1* strains start MI earlier because Mek1 is a part of the RIS itself. To distinguish between these two hypotheses, I overexpressed Mek1 in *spo11* and wild type strain backgrounds. In wild type, cells over-expressing *MEK1*, the reductional division started slightly later than wild type cells. In *spo11*Δ cells over-expressing *MEK1*, the reductional division occurred at a time similar to wild type cells. These data support a conclusion that Mek1 is downstream of the RIS and is a transducer of this signal.

Mek1 interacts with Hop1 and Red1

In *S. cerevisiae* it has been shown that Mek1 must dimerize and auto-phosphorylate to become activated in *dmc1* arrested cells and subsequently the Pachytene checkpoint (Niu et al., 2007). Two conserved threonine residues in the putative activation loop of Mek1 have been shown to be important for this autophosphorylation. Hollingsworth attributes the requirement of Rec104 in the dimerization and auto-phosphorylation of Mek1 via T327 and T321 to a requirement for the formation of DSBs. I hypothesize that the proteins of the RIS are what catalyze autophosphorylation and dimerization of Mek1 rather than DSBs. To test this, one could examine phosphorylation of Mek1 in a *ski8* background. I predict that you would not observe auto-phosphorylation of Mek1 thus demonstrating that proteins of the RIS, and not DSBs are important for activating Mek1.

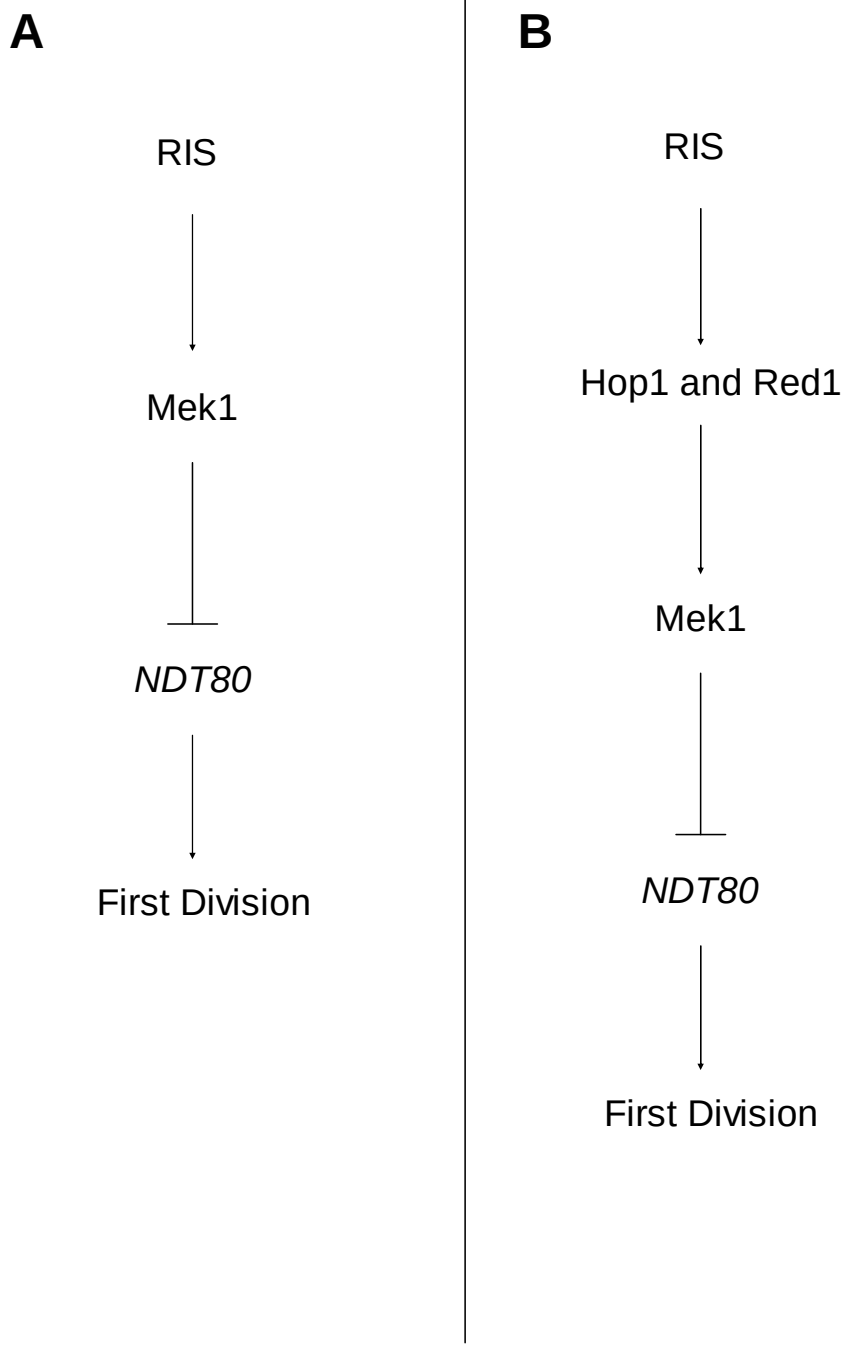
Mek1 has been shown to interact with the AE components Hop1 and Red1 in complex ways. These interactions are important for both partner choice and the Pachytene checkpoint. The C domain of Hop1 (Lysine 593) appears to promote dimerization of Mek1. The C domain also promotes dimerization of Mek1 and kinase activity. *hop1-K593 dmc1 MEK1* diploids bypass the Pachytene checkpoint and are unable to prevent recombination from sister chromatids, resulting in inviable spores due to non-disjunction. In cells where Mek1 is able to artificially dimerize (*hop1-K593A dmc1 GST-MEK1*, where Mek1 dimerizes because of the presence of GST), the *dmc1*-induced arrest is maintained (Niu et al., 2005). Hop1 is phosphorylated by Mec1 and Tel1 during prophase I, and this phosphorylation is also required partner choice (Carballo et al., 2008). Additionally, Mek1 kinase activity was reported to depend on Hop1 phosphorylation (Carballo et al., 2008). Whether this Hop1 phosphorylation is also linked to Mek1-dimerization is unknown, though in *mec1* mutants Mek1 is unphosphorylated, though it is likely because Hop1 is not phosphorylated in *mec1* strains rather than a result of a direct interaction between Mek1 and Mec1.

In addition to its interactions with Hop1, Mek1 also interacts with Red1. It was originally hypothesized that Mek1 was the kinase responsible for phosphorylating Red1, in part, because cells with a constitutively active Mek1 protein (*MEK1-C*) also arrested in pachytene (Bailis and Roeder, 2000). It was later reported by Hollingsworth and colleagues that Mek1 does not phosphorylate Red1 (Wan et al., 2004). The kinase responsible for phosphorylation of Red1 remains unknown, though Mek1 is necessary for Hop1-Red1 complex formation. Mek1 is thought to interact with Red1 through the R51 residue based on studies with the mutant *mek1-R51A*. Mek1 and Red1 co-IP in wild type cells, but Mek1 and Red1 were not co-IPed when the cells were mutant for *mek1-R51A* (Wan et al., 2004).

Hollingsworth has proposed that Mek1 substitutes for Rad53 as an effector kinase of the Pachytene checkpoint and that the role of Rad9 as an adaptor protein is substituted by Hop1 and Red1 (Niu et al., 2007). This is an intriguing idea because both Hop1 and Red1 have been shown to be important for the RIS pathway. If Hop1 and Red1 are adaptors (downstream) of the RIS rather than a part of the RIS, then overexpression of Hop1 and Red1 should be epistatic to a *spo11* mutation. Alternatively, if Hop1 and Red1 were a part of the RIS rather than downstream of the RIS, then *HOP1 RED o/e spo11 strains* should start MI at the same time as *spo11* mutants. These two models are shown in Figure 6-2.

In summary, dimerization and activity of Mek1 has been shown to be dependent on the presence Rec104. Hop1 and Mek1 have been shown to be required for Red1/Hop1 complex formation in addition to Mek1 dimerization (Fig. 6-2). These findings demonstrate a clear ability of Mek1 to interface with members of the RIS, however some questions remain. The kinase responsible for phosphorylation of Red1 remains to be identified and the nature of Mek1's interactions with the remaining RIS proteins is unknown. Furthermore, no target of Mek1 has yet been identified.

Figure 6-2: Two models for the role of Hop1 in the RIS. [A] In this model Hop1 and Red1 are a part of the RIS along with eight of the ten recombination initiation genes. The RIS is transduced by Mek1. The target of the RIS is the transcription of *NDT80*, which is required for the reductional division. [B] In this model Hop1 and Red1 are downstream of the RIS and act as adaptor proteins for the RIS. Mek1 is an effector kinase that transduces the signal.


A
RIS
Mek1
NDT80
First Division
B
RIS
Hop1 and Red1
Mek1
NDT80
First Division

Swe1: A candidate for a target of Mek1?

It is unlikely that Mek1 directly influences the transduction of *NDT80*. Currently, no inhibitory phosphorylation events have been shown to be required for either transcriptional or post-transcriptional regulation of *NDT80*. The meiotic regulatory kinase Ime2 promotes transcription of *NDT80* by phosphorylating Sum1, a negative regulator of *NDT80* transcription, on Thr 306 at a consensus Pro-X-Ser/Thr site (Moore et al., 2007). Subsequent phosphorylation of Ndt80 by Ime2 has been shown to promote further *NDT80* transcription (Sopko et al., 2002). Because the RIS delays *NDT80* transcription and activity, it is possible that Mek1 acts through an intermediate to delay *NDT80* transcription.

To date, no Mek1 phosphorylation targets have been identified (other than auto-phosphorylation). A Mek1 target in *S. pombe* has been identified, however, though activity of Mek1 is somewhat different in *S. pombe* than in *S. cerevisiae*. In both *S. pombe* and *S. cerevisiae*, an inhibitory tyrosine phosphorylation of the cyclin-dependent kinase Cdc28 (Cdc2 in *S. pombe*) is important for the Pachytene checkpoint (Leu and Roeder, 1999; Perez-Hidalgo et al., 2003, 2008). In *Saccharomyces*, hyper-phosphorylation by an unknown kinase and subsequent stabilization of the inhibitory kinase Swe1 maintains a phosphorylation of Cdc28 that inhibits Cdc28 function. In fission yeast this inhibitory phosphorylation results when the Cdc2-promoting phosphatase Cdc25 is phosphorylated and inactivated (Chapter 5, Fig. 5-13). Cdc25 is the fission yeast homolog of Mih1 in baker's yeast. In *S. pombe* Mek1 has been shown to directly phosphorylate Cdc25 *in vitro* and this phosphorylation has been shown to be necessary for Pachytene checkpoint response *in vivo* (Perez-Hidalgo et al., 2003, 2008). Because the activity of Cdc28 is important for the first division of meiosis (Benjamin et al., 2003), it is an attractive hypothesis that Mek1 phosphorylates Swe1 in response to the Pachytene checkpoint, thus producing a similar inhibitory phosphorylation of Cdk1 to arrest cells at pachytene. Swe1 could also be responsible for the mediating the normal

delay of MI relayed by the RIS. Segall's lab has shown that a *swe1* mutation partially overcomes a *dmc1* arrest and that transcription and activity of *NDT80* is partially restored in *dmc1 swe1* strains. Though there is no direct evidence that Cdc28 activity is required for *NDT80* transcription, it is clear that removing a kinase that inhibits Cdc28 activity can effect the transcription of *NDT80*.

Segall's results indicate that Swe1 is not entirely responsible for mediating the Pachytene checkpoint response, because only partial activity and transcription of *NDT80* is restored in *swe1 dmc1* mutants. A 1.5 h delay of MI as seen in wild type cells in response to the RIS is not as severe as an arrest caused in response to large regions of ssDNA DNA present in *dmc1* cells. Thus, Swe1 activity alone could be sufficient to cause the delay that we observe in wild type cells (a "delay" as opposed to a "strong block"). To test this hypothesis, one could examine the MI kinetics of a *swe1* mutant compared to wild type and *rec104* strains. *swe1* strains are viable in *Saccharomyces* (*Saccharomyces* Genome Database: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=SWE1) and can enter meiosis (see below). If Swe1 inhibits Cdc28 in response to the RIS, then a *swe1* mutant would start MI earlier than wild type. Furthermore, if Swe1 is indeed a part of the RIS pathway, then Cdc28 should be phosphorylated at the inhibitory tyrosine (Y19) in wild type strains prior to the first division, but not at all in *rec104* strains.

Both the Nickels lab and the Roeder lab have published studies on the effects of a *swe1* mutation in meiosis (Leu and Roeder, 1999; Rice et al., 2005). The Nickels lab has shown that *swe1* strains form mostly viable spores (77% or greater), but 12% of the asci that are formed contain more than four spores (multi-spore asci) (Rice et al., 2005). This is because Cdc28 must be phosphorylated by Swe1 (thus inhibiting Cdc28) to prevent re-replication of DNA during pre-meiotic S phase. In figure 2, they show that a *swe1* strain appears to start the reductional division at the same time as wild type, but they only take timepoints every four hours, so it is difficult to discern a more subtle effect such as would

occur if Swe1 were important for the RIS pathway. Furthermore, they report that transcription of *NDT80* and *SPS4* is not delayed in *swe1* strains, but they show no quantification of their data and they used RNA from cells sampled every four hours. Roeder's lab has also found that *swe1* strains produce high levels of viable spores (93%) (Leu and Roeder, 1999). Unlike the Nickels lab, Roeder's lab showed that *swe1* mutants may start the reductional division about an hour earlier than wild type, though Roeder does not mention this point in their text. Their study of meiotic nuclear kinetics is more complete than the study done by the Nickels lab; Leu and Roeder took timepoints every hour during the time interval when the reductional division starts. Roeder's findings would suggest that Swe1 can delay the reductional division in wild type cells and would support Swe1 as a candidate for a part of the RIS pathway. It should be noted, however, that both the Roeder and the Nickels lab used a different strain background than we do (BR and W303, respectively), so MI timing differences may be noted between their backgrounds and our S288C-derived background.

The B-type cyclins Clb5 and Clb6 are required for MI. Cyclins are the regulatory subunits of Cdc28 (and all Cdks) and are required for Cdc28 activity. Clb5 and Clb6 accumulate prior to the reductional division in an *NDT80*-dependent manner (Chu and Herskowitz, 1998). Both Clb5 and Clb6 are required for the reductional division. This Ndt80-dependent regulation of Cdc28 via control of *CLB5* and *CLB6* expression initially excludes Cdc28 as a part of the RIS pathway because the RIS pathway ultimately targets the transcription of *NDT80*. However, Clb5 and Clb6 are present and required for both the initiation of pre-meiotic S phase and DSB formation (Stuart and Wittenberg, 1998; Wan et al., 2008). Because Clb5 and Clb6 are present before *NDT80* is expressed, this does not eliminate Cdc28 as a part of the RIS pathway.

Swe1 is also an attractive candidate for a target of the RIS cascade because the effects of Swe1-mediated inhibition of Cdc28 are reversible. Reversibility is a key requirement for the RIS because the delay of MI imposed by the RIS is transient.

Recently, it has been shown that the polo-like kinase Cdc5 is necessary for resolution of Holliday junction, SC disassembly, Rec8 removal and exit from pachytene (Hollingsworth, 2008; Sourirajan and Lichten, 2008). *CDC5* transcription in meiosis is entirely dependent on *NDT80*. It has recently been shown that Cdc5 stimulates the degradation of Swe1 through an unknown mechanism during the G2/M transition of mitosis (Liang et al., 2009). I hypothesize that the low levels of *NDT80* transcription that initially occur in an *IME1*-dependent manner are sufficient to transcribe enough *CDC5* to destabilize Swe1 protein to allow a threshold of Cdc28 to become dephosphorylated by an unknown phosphatase to allow for the first division to occur. As the Swe1-mediated block of MI is only a delay of MI rather than a cellular arrest, presumably, only a small amount of *NDT80* transcription would need to occur to reverse this block (Fig. 6-2).

The paradox presented by this hypothesis is that *NDT80* is a target of the RIS and it is difficult to explain how a product dependent on that target (Cdc5) could be responsible for delaying the target itself. However, one can assume that the RIS only provides a delay of *NDT80* transcription and only low levels of Cdc5 would be required to destabilize sufficient amounts of Swe1 as to shift the equilibrium of Y19-phosphorylated inhibited form of Cdc28 toward the unphosphorylated active form of Cdc28. It been demonstrated that there are two primary transcriptional controls of *NDT80* transcription (Pak and Segall, 2002a) and (Chapter 3). Low levels of *NDT80* are transcribed in an *IME1*-dependent manner at an URS1 site. *NDT80* can then autoregulate its own transcription by binding to a MSE. Before Ndt80 can bind to a MSE, the transcriptional repressor, Sum1 must be phosphorylated by the regulatory kinase Ime2. Sum1 competes with Ndt80 at MSEs to repress middle meiotic promoters during vegetative growth and early meiosis. I propose that the RIS delays MSE- dependant *NDT80* transcription, perhaps by inhibiting Ime2 or by directly affecting Sum1. The RIS would not affect the low levels of *NDT80* transcription that occur in an Ime1-dependant manner. The delay imposed by the RIS would be overcome as the low levels of Ndt80-

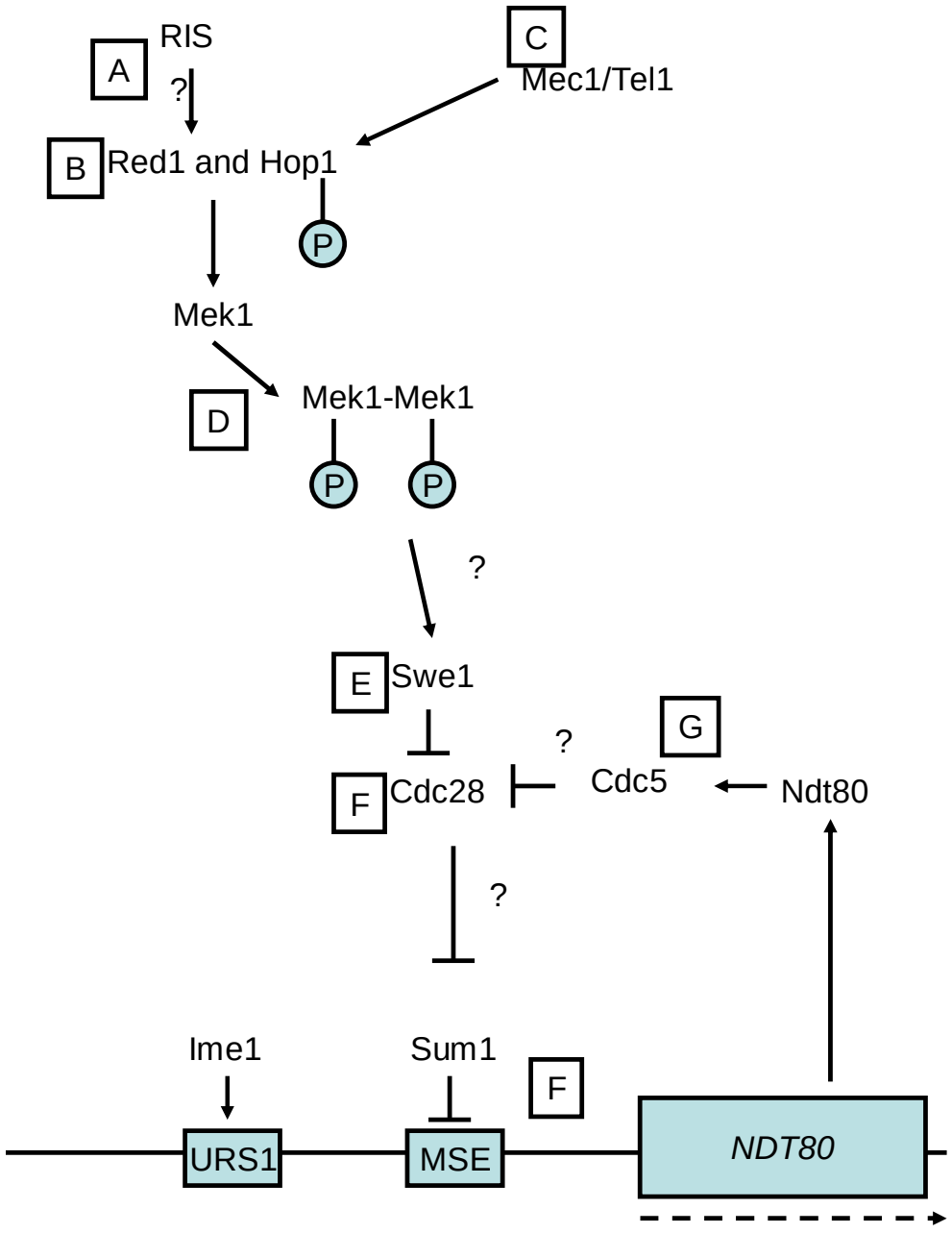
transcription mediated by Ime1 led to the transcription of a sufficient threshold of *CDC5*. Cdc5 could then destabilize Swe1. This would prevent phosphorylation of Y 19 on Cdc28.

Summary and Model

The initiation of recombination and the reductional division are two important events that the cell must monitor to ensure correct segregation of chromosomes at the proper time in meiosis. This coordination is accomplished by a recombination initiation signal comprised of eight of the ten recombination initiation proteins (Fig. 6-2A). Hop1 and Red1 could either act as adaptor proteins that sense the RIS or could be a part of the RIS itself (Fig. 6-1 and 6-2B). The RIS is most likely relayed by the meiosis-specific kinase Mek1 (Fig. 6-2D). Mek1 dimerizes and auto-phosphorylates in response to the presence of Rec104, Hop1 and Red1 and possibly other parts of the RIS (Fig. 6-2B). In particular, the Hop1 C domain is important for this activity and Hop1 must be phosphorylated by Mec1 and Tel1 for dimerization and auto-phosphorylation of Mek1. There are no known targets of Mek1, but one possibility is that Mek1 phosphorylates Swe1 (Fig. 6-2E), either directly or indirectly. Swe1 is an inhibitory kinase that phosphorylates Cdc28 on Y19. Swe1 activity reduces the amount of transcription of *NDT80,* a transcription factor necessary for the reductional division. It is unclear how Cdc28 affects *NDT80* transcription, though one hypothesis is that Cdc28 activity either directly or indirectly affects the control of transcription of *NDT80* (Fig. 6-2F) that requires MSE binding by Ndt80 itself. Cdc28 could be required for Ime2 to phosphorylate Sum1, an inhibitor of Ndt80 transcription. The RIS would not affect low levels of *NDT80* transcription that occur in an Ime1-dependent manner. Low levels of Ndt80 transcription would be sufficient to cause enough transcription of Cdc5 Cdc5 can destabilize Swe1 and shut off the RIS (Fig. 6-2G). . As I proposed earlier in this chapter, the RIS would be ultimately terminated by MRX-mediated endonucleolytic cleavage of

DNA near the DSB-site, thus removing covalently attached Spo11 and the other RIS proteins from the break sites.

Figure 6-3: A model for the relay of the RIS. Speculative parts of the model are indicated with question marks (?). [A]The RIS is composed of 8 recombination initiation proteins. [B]The SC proteins Hop1 and Red1 may act as adaptor (downstream) proteins that sense the RIS, or they may be a part of the RIS itself. [C] In this model, Mec1 and Tel1 phosphorylate the C domain of Hop1. The Hop1 C domain, Red1 and presence of at least some of the other RIS members are necessary for [D] dimerization and autophosphorylation of Mek1. This activates Mek1. [E] Mek1 then phosphorylates the inhibitory kinase Swe1 which, in turn, phosphorylates Cdc28 on Y19. [F] Cdc28 is necessary for full levels of *NDT80* transcription through an unknown mechanism. Low levels of *NDT80 t*ranscription (represented by the broken arrow) occur in an Ime1-dependent manner and are unregulated by the RIS. The RIS inhibits MSE-dependent transcription of *NDT80*. [G]The RIS can be reversed by the effects of low levels of *NDT80* transcription that can mediate transcription of *CDC5*. *CDC5* can destabilize Swe1, thus shutting off the RIS.


A
RIS
?
C
Mec1/Tel1
B
Red1 and Hop1
P
Mek1
D
Mek1-Mek1
P
P
?
E
Swe1
?
G
F
Cdc28
Cdc5
Ndt80
?
Ime1
Sum1
F
URS1
MSE
NDT80

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APPENDIX

YEAST STRAINS USED

Table A-1: *Saccharomyces cerevisiae* strains

Strain name	Malone stock #	Genotype
K65-3D	M156	*MAT-A/MAT-α lys2-1 tyr1-1 his7-2 can^R ura3-13 ade5 met13-d trp5-2 leu1-12 ade2-1*
K65-3D-104Δh	M2334	isogenic to K65-3D, except *rec104-Δ1/REC104*
K65-104-4A	M2549	isogenic to K65-3D, except *rec104-Δ1*
EG1-1-2B	M3371	isogenic to K65-3D, except *rec104-Δ1*
RCGmad2Δ	M3563	*MAT-α mad2Δ::kan^r his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0* Research Genetics Background BY4741
SEN2-1-7D	M3577	isogenic to K65-3D, except *rec102Δ::kan^r*
SKY250	M3655	*MAT-α ho::LYS2 lys2 ura3 leu2::hisG rec102Δ::URA3* SK1 Background
SKY251	M3656	*MAT-A ho::LYS2 lys2 ura3 leu2::hisG rec102Δ::URA3* SK1 Background
SKY299	M3657	*MAT-A ho::LYS2 lys2 ura3 leu2::hisG rec104Δ* SK1 Background
SKY300	M3658	*MAT-α ho::LYS2 lys2 ura3 leu2::hisG rec104Δ* SK1 Background
RCG5-6	M3716	*ho::LYS2 lys2 ura3 leu2::hisG*. Diploid created by mating M3717 and M3718 SK1 Background
RCG5-3c9-9D	M3717	*MAT-A ho::LYS2 lys2 ura3 leu2::hisG*. Dissected from RCG5-3c9 (diploid made from cross of M3655 and M3657) SK1 Background
RCGmad3Δ	M3718	*MAT-α mad3Δ::kan^r his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0* Research GeneticsBackground BY4741
RCG7-1	M3727	isogenic to K65-3D, except*mad3Δ::kan^r/MAD3 rec104-Δ1/RC104*

Table A-1, continued

KF1-1(A)	M3748	isogenic to K65-3D, except *xrs2*Δ*::kan*^{r}*/XRS2 rec104-*Δ*1/REC104 mei4*Δ*::URA3/ MEI4*
KF1-1-2C	M3388	isogenic to K65-3D, except *xrs2*Δ*::kan*^{r}*/ xrs2*Δ*::kan*^{r} *rec104-*Δ*1/ rec104-*Δ*1*
KF1-1-7A	M3384	isogenic to K65-3D, except *xrs2*Δ*::kan*^{r}*/ xrs2*Δ*::kan*^{r}
KF2-4	M3481	isogenic to K65-3D, except *mer2*Δ*::kan*^{r}*/MER2 rec104-*Δ*1/ REC104 mei4*Δ*::URA3/ MEI4*
KF2-7	M3483	isogenic to K65-3D, except *mre11*Δ*::kan*^{r}*/MRE11 rec104-*Δ*1/ REC104 mei4*Δ*::URA3/ MEI4*
KF2-3(A)	M3485	isogenic to K65-3D, except *rad9*Δ*::kan*^{r}*/RAD9 rec104-*Δ*1/ REC104 mei4*Δ*::URA3/ MEI4*
KF2-3-4A	M3499	isogenic to K65-3D, except *rad9*Δ*::kan*^{r}*/ rad9*Δ*::kan*^{r}
KF2-3-2D	M3500	isogenic to K65-3D, except *rad9*Δ*::kan*^{r}*/ rad9*Δ*::kan*^{r}
KF2-4-3A	M3507	isogenic to K65-3D, except *mer2*Δ*::kan*^{r}*/ mer2*Δ*::kan*^{r}
KF2-4-5D	M3508	isogenic to K65-3D, except *mer2*Δ*::kan*^{r}*/ mer2*Δ*::kan*^{r}
KF3-1(A)	M3600	isogenic to K65-3D, except *rad53*Δ*::HIS7/RAD53 rec104-*Δ*1/ REC104*
KF3-2(A)	M3601	isogenic to K65-3D, except *rad53*Δ*::HIS7/RAD53 rec104-*Δ*1/ REC104 smL1*Δ*::kan*^{r}*/ SML1*
KF2-11	M3496	isogenic to K65-3D, except *rec103*Δ*::kan*^{r}*/REC103 rec104-*Δ*1/ REC104 mei4*Δ*::URA3/ MEI4*
KF2-11-1B	M3497	isogenic to K65-3D, except *rec103*Δ*::kan*^{r}*/ rec103*Δ*::kan*^{r}
KF2-11-4A	M3498	isogenic to K65-3D, except *rec103*Δ*::kan*^{r}*/ rec103*Δ*::kan*^{r}
KF3-2-3A	M3603	isogenic to K65-3D, except *rad53*Δ*::HIS7/ rad53*Δ*::HIS7 smL1*Δ*::kan*^{r}*/ smL1*Δ*::kan*^{r}
KF3-2-3C	M3604	isogenic to K65-3D, except *rad53*Δ*::HIS7/ rad53*Δ*::HIS7 smL1*Δ*::kan*^{r}*/ smL1*Δ*::kan*^{r}
KF3-2-10A	M3605	isogenic to K65-3D, except *smL1*Δ*::kan*^{r}*/ smL1*Δ*::kan*^{r}
KF3-2-15C	M3606	isogenic to K65-3D, except *smL1*Δ*::kan*^{r}*/ smL1*Δ*::kan*^{r}
SMS4-1(B)	M3631	isogenic to K65-3D, except *PDS1-HA*$_3$*/PDS1 rec104-*Δ*1/ REC104*

Table A-1, continued

K65-3D [pHOP1NDT80]	M3675	isogenic to K65-3D, except transformed with the plasmid pHOP1-NDT80
K65-3D [pHOP1NDT80]	M3676	isogenic to K65-3D, except transformed with the plasmid pHOP1-NDT80
BCJ2-1 (A)	M3886	isogenic to K65-3D, except *mek1*Δ*::kan*^{r}*/REC103 rec104-*Δ*1/ REC104*
BCJ2-1-3C	M3888	isogenic to K65-3D, except *mek1*Δ*::kan*^{r}*/ MEK1*
K65-3D[YEp24]	M3906	K65-3D transformed with the plasmid YEp24
K65-3D[pKF1]	M3907	K65-3D transformed with the plasmid pKF1
K65Homspo11[YEp24]	M3908	K65-Homspo11 transformed with the plasmid YEp24
K65-Homspo11[pKF1]	M3909	K65-Homspo11 transformed with the plasmid pKF1
K65-Homspo11	M3159	isogenic to K65-3D, except *spo11*Δ*::kan*^{r}*/ spo11*Δ*::kan*^{r}