Analysis of Cell Cycle Surveillance Mechanisms in Meiosis

by

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Summary

Numerous DNA double-strand breaks (DSBs) are introduced into the genome in the course of meiotic recombination. This poses a significant hazard to the genomic integrity of the cell. Studies in a number of organisms have unveiled the existence of surveillance mechanisms or checkpoints that couple DNA repair and microtubule integrity to meiotic cell cycle progression. Through their action, aberrant meiocytes are delayed in their meiotic progression to facilitate repair of meiotic DSBs, or are culled through programmed cell death, thereby protecting the germline from aneuploidies that could lead to spontaneous abortions, birth defects and cancer predisposition in the offspring. Two such surveillance mechanisms are analyzed in this thesis. The first is the meiotic recombination checkpoint, which delays meiotic cells in G2/prophase if recombination intermediates remain unrepaired. The extent of the delay is modulated by protein phosphatase 1 (PP1), whose activity allows cells to overcome the checkpoint dependent delay in a process called adaptation. In this work, experiments in the budding yeast *Saccharomyces cerevisiae* are described that show that premature adaptation is prevented by the FK506-binding protein Fpr3, which associates with and counteracts PP1 in vivo. The checkpoint activity of Fpr3 can be inhibited by the small molecule inhibitor rapamycin and requires the proline isomerase domain of Fpr3, but not its catalytic activity. The second surveillance mechanism analyzed here is a spindle checkpoint independent arrest response of meiotic cells to microtubule perturbation. This arrest is caused by down-regulation of the meiotic transcriptional program and occurs at one of two possible stages, in meiotic G1 prior to entry into the meiotic program, or in meiotic G2/prophase after pre-meiotic DNA replication. Both mechanisms described in this work may be conserved in other organisms, including mammals. The findings presented herein are incorporated into a general model of the surveillance mechanisms of meiotic recombination.

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Chapter 1

Introduction.

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Hochwagen, A., and Amon, A. (). Checking your breaks: surveillance mechanisms of meiotic recombination.

The Significance of Meiosis

The incidence of chromosomal abnormalities in human embryos is dramatically high; according to recent estimates the fraction of fertilized human oocytes that contain the wrong number of chromosomes lies between 10 and 30 per cent (Hassold and Hunt, 2001). This exceeds the error rate of most other sexually reproducing organisms by several orders of magnitude. The consequences are a prevalence of spontaneous abortions and genetic diseases such as Down's syndrome, the most common form of mental retardation. What underlies this high error rate remains obscure, but many chromosomal abnormalities can be traced to errors that occurred during meiosis (Hassold and Hunt, 2001), the cell division program leading to the formation of sperm and egg. Meiosis is a still poorly understood process that is exceedingly difficult to study in humans for many reasons, not the least of which is that in human females meiosis can take decades to complete. However, in recent years research using model organisms has helped elucidate many of the basic meiotic mechanisms at the molecular level. The observation that many meiotic factors are conserved through evolution suggests that similar mechanisms are also operative in humans.

One major finding that resulted from the study of meiosis was the discovery of a number of meiotic surveillance mechanisms, or checkpoints. These mechanisms monitor cellular and in particular chromosomal integrity as the cells progress through meiosis, allow for the coordination of distinct meiotic processes, and arrest or eliminate cells when things go awry. The present work will focus on the

control and molecular basis of several such surveillance mechanisms using the budding yeast *Saccharomyces cerevisiae* as a model organism.

Meiosis – An Overview

The characteristic division pattern of meiosis consists of two divisions without intervening DNA replication, during which first homologous chromosomes (meiosis I) and then sister chromatids (meiosis II) are segregated away from each other. As a consequence, gametes are produced that contain half the genome complement number of the adult organism. Ploidy is doubled again when two gametes fuse during fertilization. Thus, meiosis is the essential counterpart of fertilization, allowing ploidy to be held constant from one generation to the next. However, this unusual division pattern also introduces a number of constraints that do not exist when a chromosomes separate during mitosis (Marston and Amon, 2004; Petronczki et al., 2003). First, homologous chromosomes differ fundamentally from sister chromatids, because unlike sister chromatids, which are held together by cohesin complexes, homologous chromosomes are not *a priori* linked to each other. Thus, for correct alignment of chromosomes during metaphase I, connections between homologs need to be established, which occurs in the course of a process of controlled DNA breakage and homolog-directed repair, called meiotic recombination. Second, the connections between chromosomes need to be lost in a stepwise manner, such that homologs separate first, whereas sister chromatids remain connected until meiosis II. Finally, the

kinetochores of sister chromatids need to be co-oriented during the first division, to ensure that they are segregated to the same spindle pole during meiosis I.

Meiotic Entry

Since the meiotic products are often highly specialized cell types – sperm and egg in higher eukaryotes, highly stress-resistant spores in the yeasts – the decision to enter the meiotic cell cycle rather than to continue proliferation is strictly regulated and depends predominantly on external stimuli. This observation is true for higher eukaryotes, where germ cells require (as yet unidentified) signals from the surrounding somatic tissues of the gonads to enter the meiotic program (Bullejos and Koopman, 2004; Pepper et al., 2003; Zhao and Garbers, 2002). It is also true for the single-celled yeasts, for which the major signal for entry into meiosis is nutrient limitation (Figure 1). In budding yeast, starvation signals are integrated at the promoters of *IME1*, a transcription factor that controls the expression of early meiotic genes, and *IME2*, a protein kinase that controls both the initiation of premeiotic DNA replication and exit from meiotic G2/prophase (Honigberg and Purnapatre, 2003). Since budding yeast can grow in both haploid and diploid form, they use a/α mating type heterozygosity as a means to ensure that only diploid cells enter meiosis. In haploid cells, the transcriptional repressor Rme1 prevents entry into the meiotic program under nutrient limiting conditions by inhibiting *IME1* expression. By contrast, the combined activity of the mating-type specific transcription factors a1 and alpha2 represses the expression of *RME1*

in diploid cells, thus allowing *IME1* expression and entry into the meiotic program (Covitz et al., 1991).

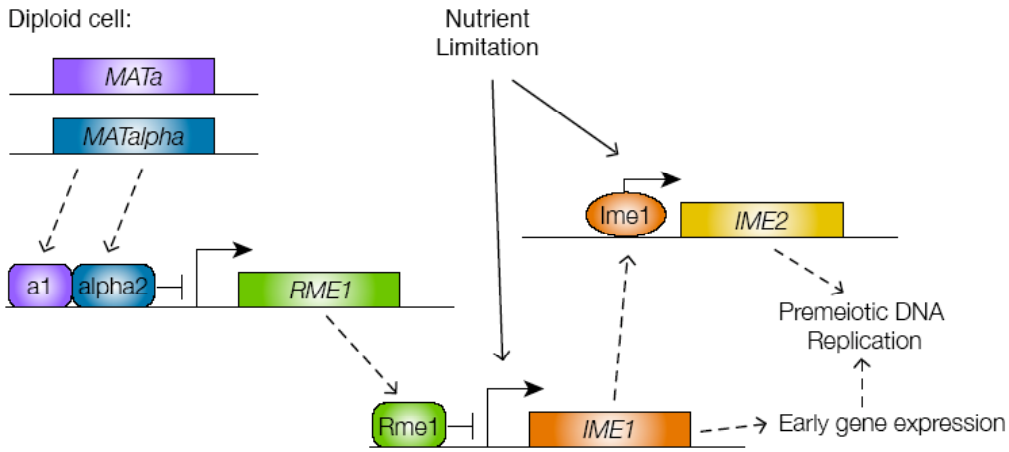

Figure 1: Induction of the meiotic program.
In diploid cells, expression of the transcriptional repressor Rme1 is repressed by the mating type specific transcription factors a1 and alpha2, permitting the nutrient limitation dependent expression of the transcription factor Ime1. The expression of the kinase Ime2 depends on both nutrient limitation and Ime1. Both Ime2 and Ime1-dependent early gene expression are necessary for the induction of premeiotic DNA replication and subsequent meiotic events.

Meiotic entry and progression are controlled in part by successive waves of transcription that coordinate the stage-specific expression of meiotic genes (Chu et al., 1998; Primig et al., 2000). Ime1 controls the first wave of meiotic gene expression, which includes predominantly genes required for premeiotic DNA replication, such as *IME2*, *MUM2*, *CLB5* and *CLB6* and meiotic recombination such as *SPO11*, *RED1*, *ZIP1*, and *DMC1* (Chu et al., 1998; Primig et al., 2000;

Smith et al., 1990). In a two-step process this leads to the activation of S phase cyclin dependent kinases (CDKs) complexes, first through the induction of the activating cyclin subunits *CLB5* and *CLB6*, and second through activation of Ime2, which promotes the degradation of the CDK inhibitor Sic1 (Dirick et al., 1998). Active Clb5-CDK and Clb6-CDK complexes then trigger the initiation of premeiotic DNA replication (Benjamin et al., 2003; Dirick et al., 1998; Stuart and Wittenberg, 1998).

Premeiotic DNA Replication

Premeiotic DNA replication appears to initiate largely at the same origins of replication that are used during mitotic proliferation (Collins and Newlon, 1994). Moreover, meiotic and mitotic origin firing, at least in the yeasts, requires many of the same proteins, including the origin recognition complex (ORC), the putative replicative helicase Mcm2-7, and the helicase loading factor Cdc6 (Hochwagen et al., 2005a; Lindner et al., 2002; Murakami and Nurse, 2001; Ofir et al., 2004). One meiosis-specific replication factor, Mum2, has been identified in budding yeast, but its role in premeiotic DNA replication is not understood (Davis et al., 2001). Nevertheless, origin firing differs somewhat between mitotic and meiotic cell cycles. In both cases, the activity of S phase Clb5/6-CDKs is necessary for firing; however, while B-type (Clb1-4)-CDKs can substitute for a lack of Clb5/6-CDKs in mitosis (Kuhne and Linder, 1993; Schwob and Nasmyth, 1993), this does not occur in meiosis (Dirick et al., 1998; Stuart and Wittenberg, 1998). This may be because the major mitotic B-type cyclin *CLB2* is not

expressed in meiosis, and *CLB1*, *CLB3*, and *CLB4* are not induced until after cells leave the extended meiotic G2/prophase phase (Dahmann and Futcher, 1995; Grandin and Reed, 1993). An additional difference between mitotic and meiotic DNA replication may be the requirement for the kinase complex Cdc7/Dbf4. While absolutely required for the firing of origins of replication in mitosis, *CDC7* appeared to be dispensable for premeiotic DNA replication (Hollingsworth and Sclafani, 1993; Schild and Byers, 1978). However, this apparent lack of requirement may also have been due to leakiness of the temperature-sensitive *CDC7* allele used in those studies. The cohesin complexes connecting sister chromatids after premeiotic DNA replication also differ from their mitotic counterparts. In particular, the cleavable subunit of cohesin, Scc1/Mcd1, is replaced by Rec8 in meiotic cohesin complexes. This replacement is necessary both for correct meiotic recombination and for the stepwise loss of cohesion during the subsequent meiotic divisions (Klein et al., 1999; Toth et al., 2000).

Meiotic Recombination

Sister chromatids never exist independently of each other. From the moment they are produced during premeiotic DNA replication to the moment they are separated during meiosis II, they are always linked by sister chromatid cohesion. In contrast, homologous chromosomes are not connected to each other as cells enter the meiotic program. It is during the lengthy meiotic G2 phase that meiotic recombination establishes the links between homologs (Figure 2). Throughout this work we will use the term “G2/prophase” instead of the commonly used meiotic

"prophase". Prophase is a cytologically defined stage, during which chromosome morphology becomes apparent. However, on a molecular level, CDK activity is low during meiotic "prophase", a feature that, in mitotic cells, indicates that the cells are in G2 phase rather than in prophase (Dahmann and Futcher, 1995; Grandin and Reed, 1993). To account for this difference, we will refer to the period of low CDK activity that follows premeiotic DNA replication, as G2/prophase.

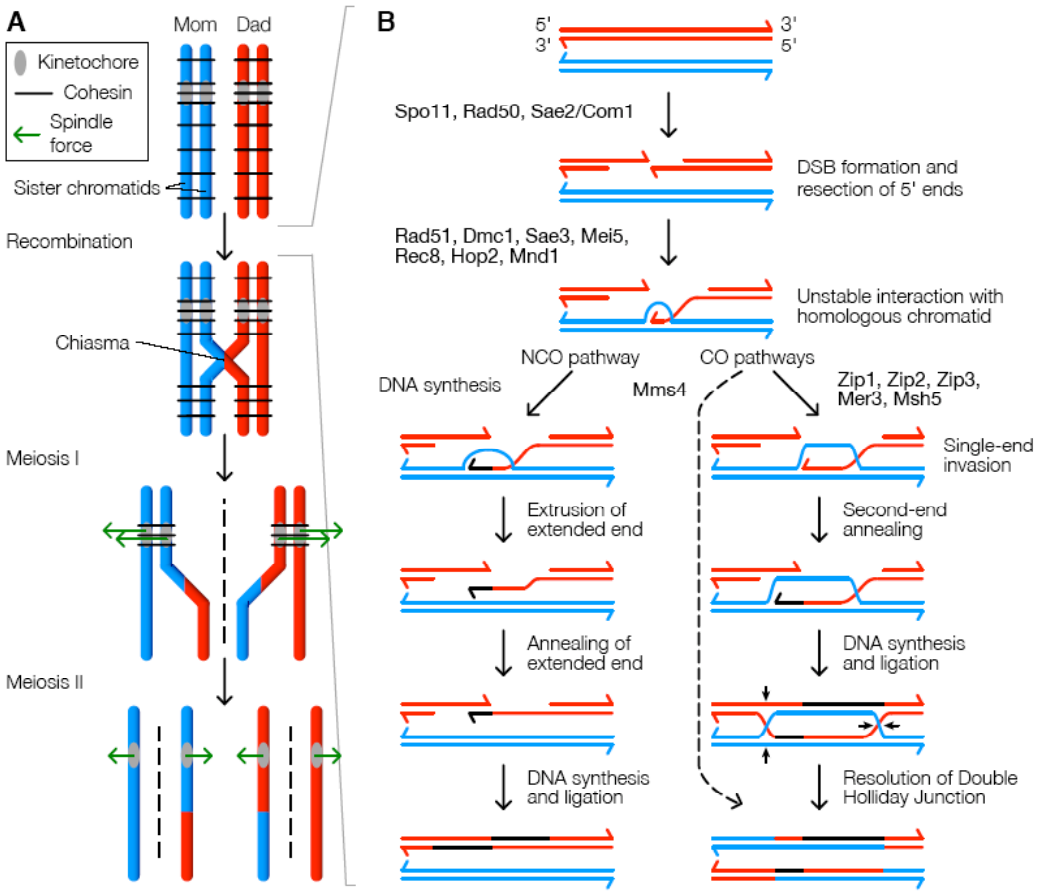

Figure 2. Meiosis.

(A) Meiotic chromosome segregation: Following loss of cohesins at chromosome arms during meiosis I, homologous chromosomes segregate to opposite poles. Subsequently, sister chromatids segregate to opposite poles during meiosis II.
(B) Meiotic recombination. DSBs can be processed to result in two types of recombination products, crossovers (COs) where flanking sequences are exchanged, and non-crossovers (NCOs) where flanking sequences are in the parental configuration. Unlike COs produced by the major *ZIP1* dependent pathway (solid arrows), COs produced by the less active *MMS4*-dependent pathway (dashed arrows) may not be formed via a double Holliday junction intermediate and do not exhibit interference – i.e. COs produced by the latter pathway are randomly distributed (Argueso et al., 2004; Hollingsworth and Brill, 2004). Recombination factors, whose inactivation results in a checkpoint response, are indicated next to the stage of recombination for which they are required. Adapted from (Bishop and Zickler, 2004).

Concomitant with premeiotic DNA replication, a number of protein complexes assemble onto chromosomes, including the recombination factors Hop2 and Mnd1, and the meiotic chromosome structure components Red1 and Hop1 (Blat et al., 2002; Smith and Roeder, 1997; Tsubouchi and Roeder, 2002; Zierhut et al., 2004). Within this context, the topoisomerase-like enzyme Spo11 introduces double strand breaks (DSBs) into the DNA (Keeney, 2001). Break formation by Spo11 requires a large set of accessory factors and the activity of Clb5/6-CDK (Arora et al., 2004; Hochwagen et al., 2005a; Kee et al., 2004; Keeney, 2001; Pecina et al., 2002; Prieler et al., 2005). Following DSB formation, Spo11 is nucleolytically cleaved off the break ends in a manner dependent on the Mre11/Rad50/Xrs2 complex, and DSBs are resected in the 5' to 3' direction to

expose 3' single-stranded overhangs (Alani et al., 1990; Neale et al., 2005) (Figure 2B). Single-stranded DNA (ssDNA) is incorporated into nucleoprotein filaments containing among other proteins the RecA-like strand invasion factors Rad51 and Dmc1 (Bishop, 1994). These filaments then engage in the search for homologous repair templates with a strong bias towards the homologous chromosomes rather than the sister chromatid (Schwacha and Kleckner, 1997). Template selection also requires factors such as the chromosome-associated kinase Mek1 that block the sister chromatid as a possible repair template (Niu et al., 2005; Wan et al., 2004). As DSBs are processed, a proteinaceous structure, the synaptonemal complex (SC), forms along meiotic chromosomes in many organisms (Page and Hawley, 2004; Zickler and Kleckner, 1999). Typically, the SC assembles around pairs of homologous chromosomes. However, in some mutant situations, such as yeast *hop2* mutants and *Msh5-/-* mice, synapsis can also occur between chromosomes that are not homologous (Leu et al., 1998; Mahadevaiah et al., 2001). Components of the SC, notably budding yeast Zip1, Zip2, and Zip3 proteins, as well as Mer3 helicase and the Msh4/Msh5 complex are required to ensure that recombination intermediates stably invade the homologous chromosomes and mature into crossovers (Borner et al., 2004; Kleckner et al., 2004). Crossover formation is the crucial step in the establishment of physical links between homologous chromosomes, which are manifested cytologically as chiasmata (Figure 2A).

The Meiotic Divisions

Once meiotic recombination is complete, cells prepare for the first meiotic division by inducing a new wave of gene expression that depends on the transcription factor Ndt80 (Chu and Herskowitz, 1998; Hepworth et al., 1998). Expression of the B-type cyclins Clb1, Clb3, and Clb4 leads to a dramatic increase in mitotic CDK activity and activates the formation of the meiosis I spindle (Dahmann and Futcher, 1995; Grandin and Reed, 1993). At the same time the kinetochores (the chromosomal microtubule attachment sites) of sister chromatids become modified by the monopolin complex, which relocalizes from the nucleolus to the kinetochores as cell exit meiotic G2/prophase (Rabitsch et al., 2003). The monopolin complex, together with the Polo kinase Cdc5 and the meiotic protein Spo13 is required for co-orientation of sister kinetochores during meiosis I, possibly by preventing microtubule attachment to one of the two sister kinetochores (Lee and Amon, 2003; Lee et al., 2004; Rabitsch et al., 2003; Toth et al., 2000; Watanabe, 2004; Winey et al., 2005). Once chromosomes are correctly attached to the meiosis I spindle, cohesin complexes are cleaved along chromosome arms by the protease Separase, leading to segregation of homologous chromosomes (Buonomo et al., 2000). In contrast, centromeric cohesin is protected from cleavage during meiosis I in a manner dependent on the kinetochore-associated protein MEI-S332/shugoshin (Kerrebrock et al., 1995; Kitajima et al., 2004; Lee et al., 2005; Marston et al., 2004; Watanabe, 2005). Only after homologs have separated to opposite poles of the spindle, do the monopolin complex and shugoshin leave the centromeric and pericentromeric

regions of sister chromatids and thus allow sister separation during meiosis II (Figure 2A). Therefore, a precise sequence of events is required for the establishment of the meiotic chromosome segregation pattern, starting with premeiotic DNA replication and ending only after sister chromatid cohesion has been lost at second meiotic division. This extraordinary choreography of events is in part controlled by the action of surveillance mechanisms, or checkpoints. The second part of this introduction will attempt to summarize our current understanding of these mechanisms.

Meiotic Surveillance Mechanisms

The sequence of events surrounding meiotic recombination is highly stereotyped. For example, DSB formation always occurs after DNA replication, cells exit from meiotic G2/prophase only after all DSBs have been repaired, and anaphase I is only initiated once chromosomes are correctly aligned on the metaphase I spindle. Research conducted in the past decade has uncovered some of the coupling mechanisms, so-called checkpoints, responsible for this temporal coordination between meiotic recombination and cell cycle progression.

Throughout this work we will use the term "checkpoint" to describe a mechanism that couples two events, which would occur in an uncoordinated manner in the absence of this mechanism. In this manner, a checkpoint comprises the following components: a signal (1), which is detected by signal sensors (2), which in turn activate signal transduction pathways (3) that translate the signal into an output by

modifying checkpoint targets (4). On the molecular level different checkpoints can share sensors and signal transduction pathways, and can impinge on the same targets. Here, we define checkpoints as distinct, if they differ in the signal and at least one of the above components.

The Double-Strand Break Checkpoint

DSB formation is coupled to the completion of premeiotic DNA replication, presumably to prevent aberrant replication across unrepaired DSBs or double Holliday junctions. Recent work in *S. cerevisiae* and *S. pombe* suggests that meiotic cells monitor the progression of the replication fork and permit DSB formation only once the replication fork has passed. If replication forks are stalled early in S phase by using mutations in ribonucleotide reductase (RNR) or the RNR inhibitor hydroxyurea (Borde et al., 2000; Tonami et al., 2005), DSBs are not formed. Furthermore, Borde and colleagues showed that the coupling of DSB formation to DNA replication is a local chromosomal phenomenon. A delay in replication on one arm of chromosome III selectively delayed DSB formation on that arm without influencing the kinetics of DSB formation on other chromosomes or even on the other (normally replicating) arm of chromosome III (Borde et al., 2000).

Interestingly, the mechanisms that ensure this coupling are only active once DNA replication has been initiated. If the firing of origins of replication is prevented, for example by inactivating the *S. cerevisiae* pre-replication complex component

CDC6 (Hochwagen et al., 2005a) or its *S. pombe* homologue *CDC18* (Murakami and Nurse, 2001), cells form almost wild-type levels of DSBs, and after a delay repair these DSBs. Thus, the mechanism blocking premature meiotic DSB formation may require the presence of replication forks. This notion is highly reminiscent of the S phase and DNA damage checkpoint controls that couple mitosis to DNA replication. Cells preparing for mitosis are able to detect active and/or stalled replication forks and delay entry into mitosis accordingly. On the other hand if DNA replication is never initiated, cells initiate mitosis with unreplicated chromosomes (Kelly et al., 1993; Piatti et al., 1995; Tercero et al., 2003; Toyn et al., 1995; Whittaker et al., 2000) presumably due to the absence of a signal that engages the S phase and/or DNA damage checkpoints.

The possibility of a checkpoint sensing the presence of replication forks and delaying meiotic DSB formation is supported by the finding that inactivation of *RAD3* (*Atr*, Table 2) allows meiotic cells to form DSBs in the presence of stalled forks in *S. pombe* (Figure 3). A number of other DNA damage checkpoint components, including Rad1, Rad9, Rad17, Rad26, Hus1 and Cds1, are also required for this double-strand break checkpoint (Tonami et al., 2005). In *S. cerevisiae*, *Atr* (*MEC1*) does not appear to be required for the meiotic DSB block in response to stalled replication forks (Borde et al., 2000). However, we speculate that a checkpoint similar to the *S. pombe* double-strand break checkpoint, maybe dependent on redundant activities of both Mec1 and Tel1 (a checkpoint kinase closely related to Mec1), also exists in *S. cerevisiae*. Such a

checkpoint would presumably produce a global inhibitory signal preventing DSB formation once premeiotic DNA replication has been initiated (Figure 3). That block would then be inactivated locally by the passing replication fork, maybe by producing chromatin states permissive to DSB formation (Murakami et al., 2003).

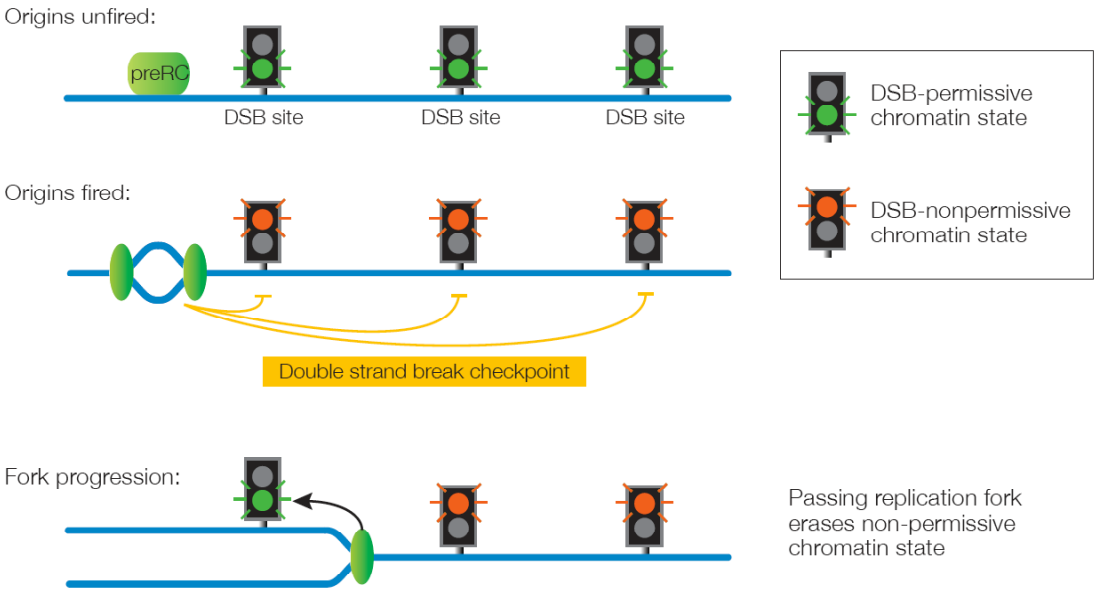

Figure 3. The double strand break checkpoint.

Prior to the initiation of DNA replication (pre-RC – pre-replicative complex), potential sites of DSB formation are permissive for DSB formation (indicated by green traffic lights). Once origins of replication have fired a global signal prevents DSB formation at all potential sites (indicated by red traffic lights). Passage of the replication fork (indicated as green oval) erases the checkpoint signal and resets potential sites of DSB formation to the permissive state.

Checkpoints Monitoring DSB Repair

Once DSBs are introduced, entry into meiosis I is delayed until the completion of meiotic DSB repair. This coupling mechanism becomes apparent in mutants defective in DSB repair. If recombination intermediates persist, meiotic cells arrest or undergo programmed cell death. Such a checkpoint response can be observed in many organisms, including budding yeast, *S. pombe*, *C. elegans*, *Drosophila*, and mouse (Barlow et al., 1997; Gartner et al., 2000; Ghabrial and Schupbach, 1999; Roeder and Bailis, 2000; Shimada et al., 2002). However, over the past years evidence has accumulated indicating that the response to DSB repair defects is far from homogeneous. First, both the exact arrest point and the duration of the delay frequently vary depending on the nature of the defect. This could be explained by different severities of the respective defects, and hence quantitative differences in the signaling of a single checkpoint. However, increasingly, checkpoint proteins are being identified that are only required for the response to a particular type of repair defect and are dispensable for others. Thus, it appears that the recombination checkpoint or pachytene checkpoint needs to be thought of as a set of distinct pathways. Below, we attempt to define these checkpoint pathways, notably the meiotic DNA damage checkpoint, the *rad50S* checkpoint, the recombination checkpoint, and the *zip1* checkpoint.

The Meiotic DNA Damage Checkpoint

Broken DNA ends and in particular the resulting ssDNA (coated with the ssDNA-binding protein RPA) activate the DNA damage checkpoint during the mitotic

cell cycle (Garvik et al., 1995; Lydall, 2003; Zou and Elledge, 2003). Evidence that the DNA damage checkpoint is also active prior to the meiotic division comes from the study of budding yeast *cdc13* mutants. At the restrictive temperature, temperature-sensitive *cdc13* mutants accumulate large amounts of ssDNA at the telomeres (Garvik et al., 1995). During the mitotic division, this triggers the DNA damage checkpoint and leads to a cell cycle arrest at metaphase depending on the DNA damage checkpoint factor Rad9 (Lydall, 2003). Inactivation of *CDC13* prior to the meiotic divisions leads to an arrest in G2/prophase that also depends on Rad9 (Weber and Byers, 1992). Rad9 is an adaptor protein in the checkpoint kinase cascade that allows the checkpoint kinase Mec1 to phosphorylate and activate the protein kinase Rad53 (Rouse and Jackson, 2002) (Figure 4). Inactivation of the RecQ family helicase Sgs1 likely also triggers the meiotic DNA damage checkpoint. *sgs1* mutants exhibit chromosome instability and a delay in meiotic G2/prophase even in the absence of Spo11-induced DSBs, suggesting a general defect in DNA metabolism that is sensed by the checkpoint. The delay of *sgs1* mutants depends on the DNA damage checkpoint components Rad24, Ddc1, and Mec3 (Figure 4A) (Rockmill et al., 2003). Rad24 recognizes ssDNA independently of Mec1, and loads a PCNA-like clamp consisting of Rad17, Ddc1, and Mec3 onto broken ends, which is required for full activation of Mec1 (de la Torre-Ruiz et al., 1998; Rouse and Jackson, 2002) (Figure 4B). These five factors (Mec1, Rad24, Rad17, Ddc1, and Mec3) are central DNA damage sensors common to all meiotic (and mitotic) checkpoints (see below). What distinguishes the meiotic DNA damage checkpoint from the

recombination checkpoint and the *zip1* checkpoint is a functional requirement for Rad9 (and presumably Rad53), which is not needed for the latter two checkpoints (Lydall et al., 1996; Roeder and Bailis, 2000). Furthermore, the chromosome structure proteins Red1 and Mek1, which play an important role in the *rad50S* checkpoint, recombination checkpoint, and *zip1* checkpoint, are dispensable for the meiotic DNA damage checkpoint (Rockmill et al., 2003; Xu et al., 1997) (Figure 4). Thus, even though both meiotic recombination and DNA damage (or stalled replication forks) lead to the formation of DSBs, which are repaired through ssDNA intermediates, different surveillance mechanisms are responsible for detecting these DNA lesions and halting cell cycle progression.

A role for the meiotic DNA damage checkpoint in detecting non-recombination induced DNA lesions appears to be conserved across species. Radiation-induced DNA damage triggers programmed cell death in mouse spermatocytes as well as in oocytes of *C. elegans* hermaphrodites. In both cases, germ cell apoptosis depends on p53, a key regulator of DNA damage dependent apoptosis in mitotic cells (Gartner et al., 2000; Odorisio et al., 1998), suggesting that a meiotic DNA damage checkpoint is also active in mice and worms.

The *rad50S* Checkpoint

Unlike the DNA damage checkpoint described above, the checkpoints described in the following sections appear to respond to particular meiosis-specific recombination intermediates. *rad50S*-like mutations, a set of non-null alleles of

RAD50, as well as null mutations in *SAE2*/*COM1*, result in a repair defect early during recombination (refer to Table 1 in which the mutants are grouped according to which checkpoint they activate). In these mutants, Spo11 remains covalently attached to the ends of DSBs and breaks are not resected (Alani et al., 1990). *rad50S*-like mutants delay in G2/prophase for several hours. (Note however, that eventually these cells enter meiosis despite the persistence of breaks, which may be a form of adaptation, see below). A *rad50S* checkpoint may also be active in mice. Although spermatocytes of $Rad50^{S/S}$ mice do not enter a permanent meiotic block, they exhibit increased apoptosis resulting in testes that are progressively depleted of mature spermatocytes (Bender et al., 2002).

Table 1: Speculative classification of budding yeast mutants exhibiting a checkpoint dependent G2/prophase delay[1].

Meiotic DNA damage checkpoint	*rad50S* checkpoint	Recombination checkpoint	*zip1* checkpoint
cdc13	*rad50S*	*dmc1*	*mms4*
sgs1	*com1/sae2*	*sae3*	*zmm* mutants[2] (23°C
rad51?		*mei5*	and 33°C)
		mnd1	
		hop2	
		rec8	
		zmm mutants[2] (33°C)	

[1] It is possible that some of the indicated mutants activate more than one checkpoint.
[2] *zmm* mutants are *zip1*, *zip2*, *zip3*, *mer3*, and *msh5* (Borner et al., 2004)

Like all other checkpoints, the *rad50S* checkpoint requires the DNA damage sensors Mec1 and Rad24 (Usui et al., 2001). However, as ssDNA does not appear to be exposed in *rad50S*-like mutants, it is unclear how these proteins recognize

the recombination intermediates. Based on the observations that neither *rad50S* mutants lacking the protein kinase *TEL1* nor *mre11-58* mutants (which also accumulate Spo11-linked DSBs) exhibit a delay, it has been suggested that the Mre11/Rad50/Xrs2 (MRX) complex and Tel1 are the primary sensors of protein-linked DSBs (Usui et al., 2001). Consistent with this, Tel1 and the MRX complex appear to be exclusively required for the *rad50S* checkpoint (Usui et al., 2001).

The checkpoint signal is then relayed through Rad9 (and presumably Rad53), which is similar to the meiotic DNA damage checkpoint but further distinguishes the *rad50S* checkpoint from the recombination checkpoint and the *zip1* checkpoint. Unlike the meiotic DNA damage checkpoint, however, the chromosomal structure proteins Mek1, Red1 and Hop1 are also required for *rad50S* checkpoint function (Usui et al., 2001; Woltering et al., 2000; Xu et al., 1997) (Figure 4B). Mek1 is a meiosis-specific paralog of the protein kinase Rad53 that also exhibits Mec1-dependent phosphorylation (Bailis and Roeder, 2000). It is possible that Mek1 substitutes for some of Rad53's functions in the context of meiotic recombination intermediates. In this context it is interesting to note, that Red1 also undergoes Mec1-dependent phosphorylation, and is required for the phosphorylation of Mek1 (Bailis and Roeder, 2000). Furthermore, Mek1 binds to phosphorylated Red1 with its phospho-specific FHA domain (Wan et al., 2004). In this way, Red1 may act as an adaptor between Mec1 and Mek1, similar to role of Rad9 in the activation of Rad53. Rad53 is recruited to phosphorylated Rad9 through its FHA domain, which allows Mec1 to phosphorylate Rad53 in the

mitotic DNA damage checkpoint (Rouse and Jackson, 2002; Sweeney et al., 2005).

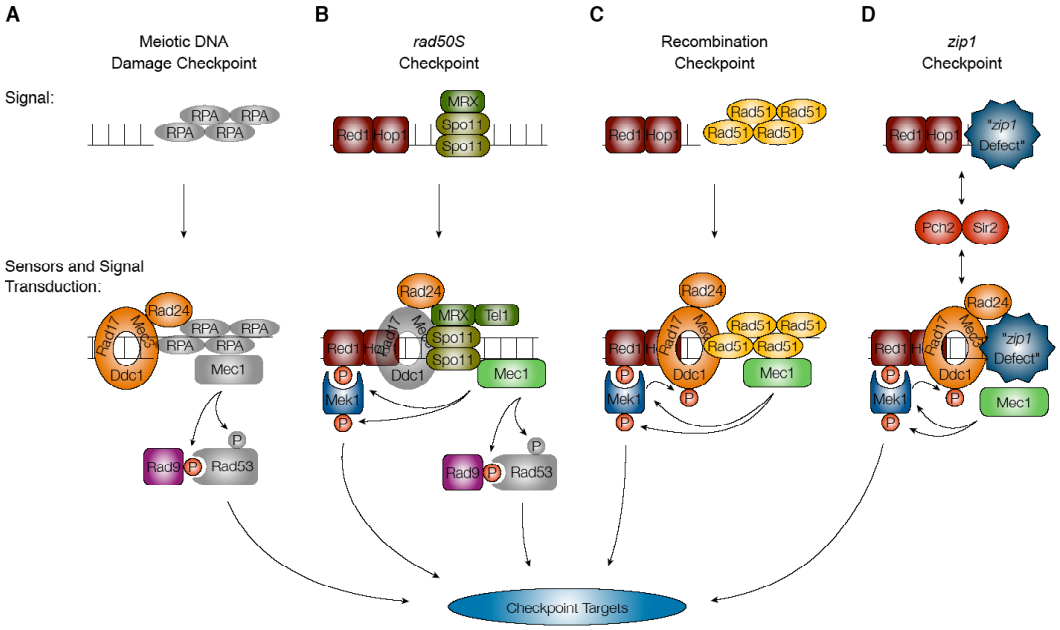

Figure 4. Surveillance of recombinatorial repair in budding yeast.
(A) Meiotic DNA damage checkpoint, (B) *rad50S* checkpoint, (C) Recombination checkpoint, (D) *zip1* checkpoint. The potential signal activating each checkpoint is depicted at the top. The proteins comprising each checkpoint pathway are listed below. Shared components are indicated by boxes overlapping the corresponding checkpoints. Components that have been demonstrated to act in a particular checkpoint are depicted in color; predicted checkpoint components are depicted in grey. P indicates phosphorylation.

Interestingly, aside from their checkpoint function, Mek1, Red1 and Hop1 are also directly involved in the control of repair template choice and serve as

structural components of the SC (Bailis and Roeder, 1998; Niu et al., 2005; Schwacha and Kleckner, 1997; Wan et al., 2004; Woltering et al., 2000). This raises the possibility that the meiotic chromosomal context is important for sensing unprocessed DSBs and/or relaying the checkpoint signal. Which aspect of chromosome structure, if any, is important for checkpoint signaling is an important question to be addressed.

The Recombination Checkpoint

The recombination checkpoint has been investigated mostly in cells lacking factors required for the initial strand invasion step of meiotic recombination, such as *DMC1*, *HOP2*, and others (Figure 2B, Table 1), which, unlike *rad50S*-like mutants, are competent to remove Spo11 from the ends of DSBs. However, because of a failure to engage in interhomolog repair, these mutants accumulate large amounts of hyperresected DSBs and exhibit a delay in G2/prophase that is substantially more pronounced than that caused by activation of the *rad50S* checkpoint (Bishop et al., 1992; Gerton and DeRisi, 2002; Leu et al., 1998).

The hyperresection of DSBs observed in homology search mutants leads to large amounts of Rad51-coated ssDNA and it has been suggested that the Rad51 nucleoprotein filament may constitute a signal recognized by the recombination checkpoint (Lydall et al., 1996; Shinohara et al., 1997). Consistent with this interpretation, a *rad50S* mutation (which prevents formation of the Rad51 filament) strongly reduces the delay of *dmc1* mutants (Bishop et al., 1992).

Furthermore, in the absence of Rad51, the delay of *dmc1* mutants is substantially reduced (Shinohara et al., 1997). Indeed, *rad51* mutants which also accumulate large amounts of ssDNA, exhibit only a modest (and presumably meiotic DNA damage checkpoint dependent) delay in meiotic G2/prophase (Shinohara et al., 1997)(unpublished observations), supporting a signaling role for the Rad51 filament. Similar arguments can also be made for the Dmc1 nucleoprotein filament. *hop2* and *mnd1* mutants, which accumulate both Rad51 and Dmc1 filaments exhibit a more pronounced cell cycle arrest than *dmc1* mutants (Leu et al., 1998). In support of the notion that Dmc1 and Rad51 filaments constitute additive signals, lack of *DMC1* reduces the G2/prophase delay of *hop2* mutants to the level of *dmc1* single mutants (Tsubouchi and Roeder, 2003). On the other hand, however, lack of *RAD51* does not alleviate the arrest of *hop2* mutants (Tsubouchi and Roeder, 2003). It is, therefore, also possible that the absence of *RAD51* and *DMC1* prevents the recombination intermediates of *dmc1* and *hop2* mutants, respectively, from being processed into structures that are detected by the checkpoint.

The recombination checkpoint shares components with the *rad50S* checkpoint and the DNA damage checkpoint, including Mec1, Rad24, Rad17, Mec3 and Ddc1 (Hong and Roeder, 2002; Lydall et al., 1996; Roeder and Bailis, 2000) (Figure 4C). In their absence, *dmc1* mutants do not experience a G2/prophase delay and initiate the first meiotic division despite a large number of unrepaired DSBs (Grushcow et al., 1999; Hong and Roeder, 2002; Lydall et al., 1996). In

contrast to the *rad50S* checkpoint and the meiotic DNA damage checkpoint, however, neither Rad9 nor Tel1 play a role in the recombination checkpoint (Lydall et al., 1996; Usui et al., 2001).

Similar to the *rad50S* checkpoint, a macromolecular assembly of the meiotic chromosomal proteins Hop1, Red1 and Mek1 is thought to provide a framework for the activation of the recombination checkpoint (Bailis and Roeder, 1998; Bailis et al., 2000; Hollingsworth and Ponte, 1997; Woltering et al., 2000; Xu et al., 1997). Their correct localization to chromosomes appears to depend in part on the histone methyltransferase Dot1 (San-Segundo and Roeder, 2000). The recombination checkpoint response of *dmc1* mutants is completely eliminated in cells lacking *HOP1*, *RED1*, or *MEK1* and much reduced in the absence of *DOT1* (Hochwagen et al., 2005a; San-Segundo and Roeder, 2000; Xu et al., 1997). The kinase activity of Mek1 is necessary to maintain the arrest of *dmc1* mutants (Bailis and Roeder, 1998; de los Santos and Hollingsworth, 1999; Wan et al., 2004) and both Ddc1 and Red1 exhibit Mek1-dependent phosphorylation (Bailis and Roeder, 1998; de los Santos and Hollingsworth, 1999; Hong and Roeder, 2002). However, recent experiments using kinase-specific ATP-analogues indicate that Mek1-dependent phosphorylation of Red1 is not direct (Wan et al., 2004). It is possible that Mek1 serves to hyperactivate Mec1 by phosphorylating Ddc1, thereby leading to further phosphorylation of Red1 (Figure 4C).

The recombination checkpoint is widely conserved. Mice lacking *Dmc1*, *Hop2*, or *Msh5* (and a growing list of other factors) experience a block in gametogenesis followed by widespread apoptosis of germ cells (de Rooij and de Boer, 2003). Also, inactivation of *Spo11* or *Mei1* (another factor likely required for DSB formation) in a *Dmc1*$^{-/-}$ or *Msh5*$^{-/-}$ mutant background result in the bypass of the cell cycle arrest (Barchi et al., 2005; Di Giacomo et al., 2005; Reinholdt and Schimenti, 2005), suggesting that, in mice as in yeast, a checkpoint detects DSBs and/or subsequent repair intermediates. To date, however, no components of the mouse recombination checkpoint have been identified. *Atm*$^{-/-}$mutants, which show a profound defect in the somatic DNA damage checkpoint, exhibit a meiotic arrest very similar to *Dmc1*$^{-/-}$ mutants (Barlow et al., 1996; Xu et al., 1996). This suggests that *Atm* has a direct role in DSB repair. *Atm* may still be involved in the checkpoint, but given that *Atm*$^{-/-}$ mutants arrest, other aspects of the checkpoint are clearly intact. The analysis of another likely checkpoint component, *Atr*, has been precluded by the fact that loss of *Atr* is embryonic lethal (Brown and Baltimore, 2000; de Klein et al., 2000). Nevertheless cytological evidence is consistent with a role for *Atr* in the recombination checkpoint (Keegan et al., 1996; Moens et al., 1999). A number of other somatic checkpoint factors have been implicated in the recombination checkpoint based on cytological data, including TopBP1 (Barchi et al., 2005; Perera et al., 2004) and Rad1 (Freire et al., 1998).

Table 2. Meiotic checkpoint proteins and their homologues.

Protein names in bold indicate factors whose meiotic checkpoint role has been demonstrated experimentally. Names in brackets indicate factors for which experiments did not identify a meiotic checkpoint role.

S. cerevisiae	***S. pombe***	***C. elegans***	***Drosophila***	**Mouse**	**Function**
Checkpoint factors					
Rad24	**Rad17p**	HPR-17	Rad17	Rad17	RFC-like clamp loading factor
Rad17	**Rad1p**	**MRT-2**	Rad1	Rad1	PCNA-like clamp (Rad17/Mec3/Ddc1)
Mec3	Hus1p	**HUS-1**	Hus1-like	Hus1	PCNA-like clamp
Ddc1	**Rad9p**	**HPR-9**	Rad9	Rad9	PCNA-like clamp
Mec1	**Rad3p**	ATL-1	**Mei-41**	Atr	PI3kinase-like kinase
Mek1	**Mek1p**	-	-	-	Meiotic kinase (Rad53 paralog)
Red1	Rec10p	-	-	-	Meiotic chromosomal protein
Hop1	Hop1p	HIM-3	-	-	Meiotic chromosomal protein
Dot1	-	-	Gpp	Dot1L	Histone methyltransferase
Rad53	**Cds1p**	(CHK-2)	**Chk2**	Chk2	Kinase with FHA domain
Tel1	Tel1p	ATM-1	Tefu	Atm	PI3-kinase-like kinase
Rad9	Crb2p	BRC-1?	-	Brca1?	Adaptor protein with BRCT domain
Mre11	Rad32p	MRE-11	Mre11	Mre11	Nuclease, MRX complex component
Xrs2	Nbs1p	-	Nbs	Nbs1	MRX complex component
Rad50	Rad50p	RAD-50	Rad50	Rad50	MRX complex component
Sir2	Sir2p	several	Sir2	several	Histone deacetylase
Pch2	-	**PCH-2**	-	-	ATPase
Dpb11	**Cut5p**	-	Mus101	TopBP1	Protein with BRCT domain
Tel2	-	**RAD-5**	-	-	DNA binding protein
(Chk1)	Chk1p	CHK-1	(Grp)	Chk1	Protein kinase
Targets					
Cdc28	**Cdc2p**	CDK-1	Cdc2	Cdc2	Cyclin-dependent kinase
Swe1	(Wee1p)	several	**Wee1**	Wee1	Tyrosine kinase of Cdc28
(Mih1)	**Cdc25p**	CDC-25	Twe	several	Tyrosine phosphatase of Cdc28
Ndt80	-	-	-	-	Transcription factor
Sum1	-	-	-	-	Transcriptional repressor
-	-	**CED-1**	(p53)	**p53**	Transcription factor
several	several	several	Vas	several	Translation initiation factor
Adaptation					
Glc7	several	several	several	several	Protein phosphatase 1
Fpr3	Fkbp39p	several	several	several	FK506-binding protein

RFC – replication factor C, PCNA – proliferating cell nuclear antigen, PI3kinase – 3-phospho inositol kinase, FHA domain – forkhead associated domain, BRCT domain – Brca1 carboxy terminal domain, MRX complex – Mre11/Rad50/Xrs2 complex, Gpp – Grappa, Tefu – Telomere fusion, Grp – Grapes, Twe – Twine, Vas - Vasa

As observed in mouse gametogenesis, cells with meiotic DSB repair defects are removed by apoptosis in the female germline of *C. elegans* hermaphrodites (Colaiacovo et al., 2003; Gartner et al., 2000). The damage-dependent programmed cell death is induced in the pachytene stage of meiotic G2/prophase and requires the checkpoint factors MRT-2, HUS-1, HPR-9 and RAD-5 (Gartner et al., 2000; Stergiou and Hengartner, 2004)(Table 2). MRT-2, HUS-1, and HPR-9 likely act as a complex in parallel to RAD-5 (Hofmann et al., 2002). It is unclear whether the checkpoint kinase CHK-2 has a role in the worm recombination checkpoint. A mutation in *chk-2* does prevent apoptosis in oocytes lacking *rad-51*. However, this may be due to a defect in DSB formation rather than inactivation of the checkpoint (Alpi et al., 2003; MacQueen and Villeneuve, 2001). Not all worm repair mutants trigger checkpoint dependent apoptosis. No programmed cell death is elicited in oocytes lacking the SC components *him-3* or *rec-8*, despite defects in synapsis and an accumulation of RAD-51 foci (a cytological marker for unrepaired DSBs) (Alpi et al., 2003). Given that *him-3* is related to *HOP1* (Table 2), this may also indicate a checkpoint role for HIM-3.

The recombination checkpoint of *S. pombe* has long eluded detection, because most *S. pombe* repair mutants do not exhibit dramatic cell cycle delays, and even mutants completely deficient in DSB repair progress through meiosis (Catlett and Forsburg, 2003). Careful analysis of meiotic cell cycle kinetics, however, indicated that repair-deficient *meu13* (*hop2*) mutants delay entry into meiosis I by approximately 30 minutes (Perez-Hidalgo et al., 2003; Shimada et al., 2002). The

meu13 delay depends on the formation of DSBs and requires a set of conserved checkpoint factors, including Rad17, Rad9, Rad1, Rad3, Mek1, Cds1, and Cut5 (Perera et al., 2004; Perez-Hidalgo et al., 2003; Shimada et al., 2002), most of which are also involved in the recombination checkpoint in other organisms (Table 2).

Evidence for a recombination checkpoint in *Drosophila* oocytes comes from the analysis of *spnA*, *spnB*, *spnD*, and *okra* mutations, which disrupt several Rad51-like factors (Abdu et al., 2003; Ghabrial and Schupbach, 1999; Staeva-Vieira et al., 2003). These mutants exhibit defects in the formation of the karyosome, a chromosome structure specific for meiotic G2/prophase. Furthermore, the subsequent patterning of the eggshell is abnormal in these mutants due to a failure to accumulate wild-type levels of the patterning protein Gurken (Ghabrial et al., 1998). Both defects are suppressed in mutants of the Spo11 homolog *Mei-W68*, suggesting that they result from a defect in DSB repair. Furthermore, both karyosome and egg patterning defects depend on the checkpoint factors *Mei-41* and *Chk2*, and Chk2 is phosphorylated in a Mei-41-dependent manner in *spnB*, *spnD* and *okra* mutants (Abdu et al., 2002; Ghabrial and Schupbach, 1999). Several other DNA damage checkpoint factors including the Chk1-homolog *grapes*, and the *Mei-41* interacting factor *Mus304* are likely not involved in the recombination checkpoint (Abdu et al., 2002; Masrouha et al., 2003).

The *zip1* Checkpoint

The stable invasion of the homolog by a subset of DSBs that will later be repaired as crossovers requires the SC components Zip1, Zip2, and Zip3 as well as a set of other recombination factors (Figure 2B). In their absence, cells undergo a temperature-dependent delay in G2/prophase (Agarwal and Roeder, 2000; Borner et al., 2004; Chua and Roeder, 1998; Sym et al., 1993). The best-analyzed checkpoint response is the delay of *zip1* mutants, which requires Rad24, Rad17, Ddc1, Mec3, and Mec1, as well as Red1, Hop1 and Mek1 (Roeder and Bailis, 2000). The nature of the checkpoint signal in these mutants is unclear. However in contrast to the other checkpoints, lesion detection requires the ATPase Pch2 (San-Segundo and Roeder, 1999). Pch2 is specifically required for the *zip1* checkpoint, because inactivation of *PCH2* eliminates the cell cycle delay of *zip1*, *zip2*, and *mms4* mutants (de los Santos et al., 2001; San-Segundo and Roeder, 1999), but does not impair the arrest of *hop2*, *mnd1* and *sgs1* mutants (Rockmill et al., 2003; Roeder and Bailis, 2000; Zierhut et al., 2004). The bypass of the *dmc1* in the absence of *PCH2* appears to depend on the strain background (Hochwagen et al., 2005a; San-Segundo and Roeder, 1999). Pch2 localizes to the nucleolus, which appears to be important for Pch2 function and depends both on Dot1 and the histone deacetylase Sir2 (San-Segundo and Roeder, 1999; San-Segundo and Roeder, 2000). The exact checkpoint role of Pch2 in the *zip1* checkpoint is, however, unclear. Pch2 may be involved in the production or accumulation of the recombination intermediate detected by the *zip1* checkpoint, because *PCH2* also

plays a direct role in recombination (V. Börner, personal communication) (Hochwagen et al., 2005a).

A Synapsis Checkpoint?

Not all meiotic checkpoints respond to DSB-derived recombination intermediates. Some mutant mice, such as *Spo11*$^{-/-}$ or *Mei1*$^{-/-}$ mice, also exhibit meiotic blocks in the absence of DSBs (Baudat et al., 2000; Libby et al., 2002; Romanienko and Camerini-Otero, 2000), suggesting that some aspect of synapsis or lack thereof may constitute another checkpoint signal. The block in *Spo11*$^{-/-}$ and *Mei1*$^{-/-}$ spermatogenesis and oogenesis occurs at a later stage than the block in *Dmc1*$^{-/-}$ or *Msh5*$^{-/-}$ meiocytes (Ashley et al., 2004a; Barchi et al., 2005; Di Giacomo et al., 2005; Reinholdt and Schimenti, 2005), supporting the notion that the defects of *Spo11*$^{-/-}$ and *Mei1*$^{-/-}$ germ cells are potentially detected by a distinct checkpoint. Recently, *PCH-2* has been implicated in the *C. elegans* checkpoint response to unsynapsed chromosomes (cited in (McDougall et al., 2005)). In budding yeast, absence of DSBs does not cause a checkpoint response, and may in fact accelerate meiotic progression (Malone et al., 2004), suggesting that the absence of synapsis is not detected in this organism.

Signal Integration

Some recombination mutants may activate more than one checkpoint. A striking example of additive checkpoint activation comes from the analysis of *zmm* mutants, a class of mutants in budding yeast that includes *zip1*, *zip2*, *zip3*, *mer3*,

and *msh5*. These mutants are proficient in strand invasion at 23°C, albeit with a delay, but fail to form single-end invasion intermediates at 33°C. Concomitantly, *zmm* mutants only delay in G2/prophase at 23°C but completely arrest at 33°C (Borner et al., 2004), presumably because the failure to process Rad51 and Dmc1 filaments at 33°C activates the recombination checkpoint in addition to the *zip1* checkpoint. Given that several of the checkpoint components (e.g. Mec1) are shared between different checkpoints, we speculate that these factors may serve as signal integrators that translate the inputs of the various checkpoints into a corresponding cell cycle delay.

Checkpoint Targets

The activated checkpoint factors transmit their signal to downstream targets that control cell cycle progression, DNA repair, programmed cell death and, in some cases, development. Most studies concerning checkpoint targets have been conducted in the context of the recombination checkpoint and the *zip1* checkpoint. Whether the different checkpoints activate distinct targets has thus far not been investigated.

Cell cycle progression:

The major cell cycle targets of the recombination checkpoint and *zip1* checkpoint are cyclin-dependent kinases, protein kinases composed of a catalytic kinase subunit (CDK) and a regulatory cyclin subunit. CDKs, when associated with cyclin A or B in higher eukaryotes, or Clb1, 3, or 4 in budding yeast, drive cells

into meiosis (reviewed in (Marston and Amon, 2004)). Two mechanisms keep CDKs inactive in response to checkpoint activation. First, CDK is inhibited in a checkpoint-dependent manner by the dual specificity protein kinase Wee1, which phosphorylates CDK on a crucial threonine and tyrosine (T14, Y15). In budding yeast *hop2* mutants, Swe1 (budding yeast Wee1, Table 2) is hyperphosphorylated and stabilized, and inactivation of *SWE1* allows the partial bypass of checkpoint dependent delay (Leu and Roeder, 1999; Pak and Segall, 2002). In *Drosophila spnB* (rad51-like) mutants, Wee1 is modified in a Chk2-dependent manner (Abdu et al., 2002) indicating that similar to budding yeast, cell cycle arrest also occurs by modulating Wee1 activity. The *S. pombe* recombination checkpoint, on the other hand, does not regulate CDKs throughWee1. Rather, CDKs remain phosphorylated on Y15 during the *hop2* delay, because of Mek1-dependent inhibition of Cdc25, a CDK-Y15 phosphatase (Perez-Hidalgo et al., 2003; Shimada et al., 2002). In contrast, Cdc25 (Mih1) does not play a checkpoint role in *S. cerevisiae* (Leu and Roeder, 1999). Nevertheless, this suggests that CDK is a conserved meiotic checkpoint target.

In budding yeast, checkpoints activation also keeps the transcript (and protein) levels of the B type cyclins low (Chu and Herskowitz, 1998; Hepworth et al., 1998). The promoters of meiotically expressed B type cyclins contain a short DNA element called the middle sporulation element (MSE), which is found in many other so-called “middle genes” whose expression is induced once cells exit from meiotic G2/prophase and enter meiosis I. Meiotic cyclin expression is

controlled by two transcription factors, Ndt80 and Sum1. Ndt80 is a transcriptional activator that binds to the MSE and induces middle gene expression (Chu and Herskowitz, 1998; Hepworth et al., 1998). Sum1 is a transcriptional repressor that recognizes a DNA element that overlaps with the MSE, and thereby competes with Ndt80 for MSE binding at a subset of middle genes (Lindgren et al., 2000; Pierce et al., 2003; Xie et al., 1999). Both Ndt80 and Sum1 are under checkpoint control. *NDT80* expression levels are kept low and overexpression of *NDT80* allows a partial bypass of the *dmc1* G2/prophase delay (Pak and Segall, 2002; Tung et al., 2000). Furthermore, the extensive phosphorylation of Ndt80 is reduced, albeit not eliminated, in a checkpoint-dependent manner in *dmc1* or *zip1* cells (Hepworth et al., 1998; Shubassi et al., 2003; Tung et al., 2000). Ndt80 phosphorylation has been shown to depend in part on the meiotic kinase Ime2 and the Polo kinase Cdc5 (Benjamin et al., 2003; Clyne et al., 2003), but it is unclear whether these kinases are involved the checkpoint-dependent phosphorylation changes of Ndt80. Sum1, on the other hand, appears to be regulated at the level of protein stability. The level of Sum1 protein transiently drops as meiotic cells progress from G2/prophase into meiosis I, despite increasing levels of *SUM1* mRNA (Lindgren et al., 2000). Moreover, Sum1 protein remains at high levels while cells are delayed in G2/prophase by the checkpoint, and *SUM1* is required for the checkpoint arrest of *dmc1* mutants (Lindgren et al., 2000; Pak and Segall, 2002). The checkpoint factors controlling both Ndt80 phosphorylation and the drop in Sum1 protein levels remain to be identified.

DSB repair:

At least in budding yeast, the meiotic checkpoints also induce DSB repair. For example, Rfa2, a subunit of the ssDNA-binding protein complex RPA is hyperphosphorylated in *dmc1* mutants. This phosphorylation is dependent on *MEC1* and DSBs, and is thought to be required for DSB repair (Bartrand et al., 2005; Brush et al., 2001). Furthermore, Rad24 interacts with the repair protein Rad57 specifically during meiosis, suggesting another link between checkpoint surveillance and repair (Hong and Roeder, 2002).

Apoptosis:

In multicellular organisms, programmed cell death frequently eliminates repair defective meiocytes, and the apoptotic machinery appears to be an important checkpoint target in both mouse and *C. elegans*. Reports differ as to whether *p53* is required for the induction of apoptosis in mouse repair mutants. The finding that inactivation of *p53* (or the CDK inhibitor $p21^{Cip1}$) allows $Atm^{-/-}$ mutant spermatocytes to partially overcome the G2/prophase arrest (Barlow et al., 1997) has been confirmed by some albeit not all subsequent reports (Ashley et al., 2004b; Scherthan et al., 2000). *p53*-independent apoptosis has been observed in spermatocytes harboring certain chromosomal translocations (Odorisio et al., 1998), while both p53-dependent and p53-independent apoptosis of spermatocytes occurs in several other mouse meiotic mutants (Salazar et al., 2005; Xu et al., 1996). These findings suggest that only a subset of the checkpoint

pathways that are active during mouse spermatogenesis trigger p53-dependent apoptosis.

In *C. elegans*, apoptosis of *rad-51* mutant oocytes is induced through the action of the p53-homolog *ced-1* (Alpi et al., 2003). Prior to pachytene stage of meiotic G2/prophase, translation of *ced-1/p53* mRNA is inhibited by the RNA-binding protein GLD-1 (Schumacher et al., 2005). GLD-1 levels drop during pachytene leading to an increase in CED-1 protein levels in pachytene oocytes, which in *rad-51* mutant oocytes allows the recombination checkpoint signal to be translated into a proapoptotic signal (Schumacher et al., 2005).

Development:

In budding yeast, checkpoint activation inhibits spore development concomitantly with cell cycle progression as a consequence of the inhibition of the transcription factor Ndt80, which controls the expression of genes required for both processes (Chu and Herskowitz, 1998; Hepworth et al., 1998). Curiously, the *Drosophila* recombination checkpoint affects the patterning of the embryo. The recombination defective *spn* mutants exhibit defects in karyosome formation and Gurken accumulation similar to that of mutants lacking the translation initiation factor *vasa*. However, unlike the *spn* mutants, the *vasa* mutant phenotype is not *mei-41* dependent, suggesting that *Vasa* acts downstream of the *Drosophila* recombination checkpoint. Consistent with this, Vasa is modified in a Chk2-

dependent manner in *spnB* mutants (Abdu et al., 2002; Ghabrial and Schupbach, 1999).

Adaptation

The meiotic checkpoints, at least in budding yeast, appear to be less responsive to DNA damage than the mitotic DNA damage checkpoint. In mitotic cells, a single irreparable DSB can trigger an extended checkpoint delay (Lee et al., 2000; Sandell and Zakian, 1993). Meiotic cells, on the other hand, are able to progress through meiosis and form spores even if a DSB remains unrepaired (Malkova et al., 1996). Moreover, despite the large number of DSBs typically introduced during meiosis – more than 200 recombination events are estimated to occur per meiosis – the cell cycle block of many repair mutants is transient. As indicated above, exogenous DNA damage may be sensed differently than recombination intermediates, which may partially explain the less dramatic response. An additional, non-exclusive possibility is that meiotic cells adapt more easily to damage than mitotic cells. Adaptation is known to occur in mitotic cells and allows cells with very limited DNA damage to overcome the checkpoint-dependent block and progress through the cell cycle (Lee et al., 2000; Lupardus and Cimprich, 2004).

Adaptation has also been demonstrated for the recombination checkpoint in budding yeast. A factor likely involved in this process is protein phosphatase 1 (PP1). Overexpression of the catalytic subunit of PP1, *GLC7* shortens the

G2/prophase delay of many meiotic repair mutants and can alleviate the arrest caused by constitutively active *MEK1* (Bailis and Roeder, 2000; Hochwagen et al., 2005a). Glc7, associates with a variety of targeting factors that provide substrate specificity. In one strain background, inactivation of *GIP1*, a meiosis-specific substrate-targeting factor of Glc7, causes a block in meiotic G2/prophase (Bailis and Roeder, 2000). However, *gip1* mutants do not arrest in other strain backgrounds (Tachikawa et al., 2001), suggesting that there may be other specificity factors acting redundantly to Gip1. Glc7 is inhibited by the FK506-binding protein Fpr3 (Hochwagen et al., 2005a). Fpr3 interacts with Glc7 through its proline isomerase (PPIase) domain and the PPIase domain of Fpr3, though not its catalytic activity, is required to prevent premature adaptation. Inactivation of Fpr3 using the small-molecule inhibitor rapamycin and Fpr3 PPIase mutants cause a reduced checkpoint delay in many repair mutants, similar to the overexpression of *GLC7*. Furthermore, co-overexpression of *GLC7* and *FPR3* re-establishes the checkpoint delay (Hochwagen et al., 2005a). Thus, adaptation to persistent recombination intermediates depends on the modulation of PP1 activity. Glc7 may allow adaptation by dephosphorylating Red1 or Red1-dependent targets. Indeed, Glc7 interacts with Red1, and Red1 can be dephosphorylated by Glc7 *in vitro* (Bailis and Roeder, 2000; Tu et al., 1996). Moreover, a mutant of Glc7 that fails to interact with Red1 (*glc7-T152K*) exhibits a DSB-dependent cell cycle arrest in meiotic G2/prophase that is bypassed by the inactivation of *RED1* (Bailis and Roeder, 2000).

In summary, meiotic recombination appears to be monitored by a battery of surveillance mechanisms. Moreover, many of the checkpoint factors involved in this surveillance are members of multiple checkpoint signaling cascades, and all pathways ultimately lead to cell cycle arrest in meiotic G2/prophase and/or apoptosis. Whether this means that the signals converge into one single pathway or whether the checkpoint components required for more than one checkpoint are assembled into distinct signaling modules by checkpoint specific factors remains to be determined.

The Meiosis I Spindle Checkpoint

Once cells have successfully completed meiotic recombination, the homologs need to be aligned correctly on the metaphase I spindle, such that (1) microtubules are attached to all (unmasked) kinetochores, and (2) the kinetochores of homologs face opposite poles of the spindle. In mitosis, correct bipolar attachment is sensed by the spindle checkpoint, which prevents anaphase onset as long as chromosomes are not properly attached to the spindle (Lew and Burke, 2003; Taylor et al., 2004). For anaphase to occur, a multi-subunit ubiquitin ligase, the anaphase promoting complex or cyclosome (APC/C) needs to target the separase inhibitor securin for degradation, thereby triggering the separase dependent destruction of sister chromatid cohesion (Peters, 2002). Kinetochores that are not bound to microtubules cause the sequestration of the APC/C adaptor protein Cdc20, a specificity factor required for securin destruction. Current models postulate that if a kinetochore is unattached, the kinetochore-associated

checkpoint factors Mad2 and Mad1 associate with that kinetochore. Once kinetochore-associated, they form a template that allows soluble Mad2 proteins to undergo a conformational switch, which binds and entraps Cdc20. Mad2-Cdc20 complexes may then act as templates themselves to further amplify the signal (Nasmyth, 2005). The checkpoint kinase Bub1 is required for the kinetochore association of Mad1 and Mad2 and also activates a second pathway of Cdc20 inhibition involving the checkpoint factor Bub3 and the Cdc20 binding factor Mad3 (Lew and Burke, 2003).

Both microtubule attachment and the tension that is created when the kinetochores of two connected chromosomes (sister chromatids in mitosis and meiosis II, homologs in meiosis I) are attached to opposite spindle poles appear to be monitored by the checkpoint. It is not clear, however, to what extent lack of microtubule attachment and lack of tension constitute separate signals (Lew and Burke, 2003). The Aurora kinase Ipl1 constitutes part of a tension sensitive microtubule severing activity required for the biorientation of kinetochores (Dewar et al., 2004; Tanaka et al., 2002). Thus, in the absence of tension, microtubules become detached from kinetochore, which may be enough to activate the spindle checkpoint.

The inactivation of spindle checkpoint components causes lethality in many higher eukaryotes but has little effect on the viability of proliferating yeast cells (Taylor et al., 2004). In contrast, deletion of *MAD1* or *MAD2* (but not *MAD3*)

significantly affects the fidelity of meiosis I chromosome segregation in budding yeast, suggesting that spindle checkpoint function is important for the correct alignment of homologs on the metaphase I spindle (Cheslock et al., 2005; Shonn et al., 2000; Shonn et al., 2003). Likewise, reduced Mad2 function also causes meiosis I chromosome missegregation during mouse oogenesis (Homer et al., 2005). It is thought that Mad1 and Mad2 delay the onset of anaphase I in response to incorrectly attached or unattached chromosomes, because artificial delay of anaphase I onset partially restores correct meiosis I chromosome segregation to *mad1* and *mad2* mutants (Homer et al., 2005; Shonn et al., 2000; Shonn et al., 2003).

Interestingly, even though loss of Mad3 does not affect the fidelity of meiotic chromosome segregation, both Mad2 and Mad3 are required to prevent securin destruction and thus anaphase I entry in *spo11* mutants, which cannot establish tension between homologs due to the absence of chiasmata (Shonn et al., 2003). The mechanism by which Mad2 delays anaphase entry likely differs from the role of Mad3, because unlike Mad2, Mad3 delays cells in already in meiotic G2/prophase irrespective of whether Spo11 is active (Cheslock et al., 2005; Shonn et al., 2003). Furthermore, Mad3 appears to be specifically required for chromosomes that have failed to recombine. In budding yeast wild-type cells, non-exchange chromosomes only missegregate in about 7% of cases, because a recombination-independent distributive system can non-specifically pair centromeres that have failed to align during meiotic recombination (Kemp et al.,

2004; Stewart and Dawson, 2004; Tsubouchi and Roeder, 2005). Inactivation of Mad1 and Mad2 causes missegregation of non-exchange chromosomes to increase to about 25%. Loss of Mad3, on the other hand causes completely randomized segregation (Cheslock et al., 2005). Thus, the spindle checkpoint components appear to act in several pathways to ensure correct meiosis I chromosome segregation.

In mitosis, the spindle checkpoint prevents anaphase initiation if microtubules are chemically destabilized using microtubule poisons, such as benomyl or nocodazole (Lew and Burke, 2003; Taylor et al., 2004). A similar Mad2-dependent metaphase I arrest in response to nocodazole treatment is also observed in mouse oocytes (Wassmann et al., 2003). On the other hand, mouse spermatocytes treated with colcemid, another microtubule poison, already arrest in meiotic G2/prophase (Tepperberg et al., 1997; Tepperberg et al., 1999). In budding yeast, it has been suggested that microtubule destabilization using benomyl causes an arrest in metaphase I (Shonn et al., 2000; Shonn et al., 2003). However, analysis of the exact arrest point of benomyl-treated yeast cells indicated that microtubule perturbation triggers a reversible arrest in meiotic G2/prophase rather than metaphase I. A similar arrest is also observed, if microtubules are destabilized by low temperatures (Hochwagen et al., 2005b). Unlike the spindle checkpoint response that occurs in mitosis, the meiotic G2/prophase arrest was caused by a dramatic downregulation of the meiotic transcriptional program and appears to be independent of Mad2 (Hochwagen et

al., 2005b). However, which factors are required for this response is at this point unclear.

Why delay in G2/prophase?

In principle, it would seem sufficient for meiotic cells with recombination defects or destabilized microtubules to arrest in metaphase I, because the damage would only become irreversible once the meiotic divisions are initiated. That delays are generally observed in G2/prophase indicates that preventing premature loss of cohesion and segregation of chromosomes fragment is not the only function of these checkpoints. In the case of recombination defects, an early block may allow for more time for recombination intermediates to be processed in the correct repair context. Importantly, however, meiotic G2/prophase may be the last stage to allow coordinate regulation of the meiotic cell cycle with the later meiotic development, because if cells are artificially arrested in metaphase I, developmental progression, i.e. spore formation, is already partially uncoupled from the cell cycle (Lee and Amon, 2003). Finally, G2/prophase is the last phase of the meiotic cell cycle from which repair deficient yeast cells can reenter vegetative growth before they become committed to the meiotic divisions (Esposito and Esposito, 1974; Shuster and Byers, 1989; Zenvirth et al., 1997). Thus, at least for budding yeast a G2/prophase delay also opens an alternative exit route that preserves viability when meiotic recombination fails.

Conclusion

Studies in a number of organisms have unveiled the existence of meiotic surveillance mechanisms or checkpoints that (1) ensure that meiotic program is correctly orchestrated and (2) allow cells to respond to damage or defects that would endanger genome integrity. Through their action, aberrant meiocytes are delayed in their meiotic progression to facilitate repair, or are culled through programmed cell death, thereby protecting the germline from aneuploidies that could lead to spontaneous abortions, birth defects and cancer predisposition in the offspring.

In the following chapters, experiments will be presented that investigate two aspects of meiotic surveillance, the control of recombination checkpoint silencing, and the meiotic response to microtubule perturbation.

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Chapter 2

The FK506 and Rapamycin Binding Protein Fpr3 Counteracts the Protein Phosphatase 1 to Maintain Recombination Checkpoint Activity.

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Hochwagen, A., Tham, W.-H., Brar, G., and Amon, A. (2005). The FK506 binding protein Fpr3 counteracts protein phosphatase 1 to maintain meiotic recombination checkpoint activity. Cell *122*, 861-873.

The screen for recombination checkpoint mutants was conducted in collaboration with Dr. Wai-Hong Tham. The construction and characterization of *cdc6-mn* was performed in collaboration with Gloria Brar.

Summary

The meiotic recombination checkpoint delays gamete precursors in G2/prophase until DNA breaks created during recombination are repaired and chromosome structure has been restored. Here we show that the FK506-binding protein Fpr3 prevents premature adaptation to damage and thus serves to maintain recombination checkpoint activity. Impaired checkpoint function is observed both in cells lacking *FPR3*, and in cells treated with rapamycin, a small molecule inhibitor that binds to the proline isomerase (PPIase) domain of Fpr3. *FPR3* functions in the checkpoint through controlling protein phosphatase 1 (PP1). Fpr3 interacts with PP1 through its PPIase domain, regulates PP1 localization and counteracts the activity of PP1 *in vivo*. Our findings define a branch of the recombination checkpoint involved in the adaptation to persistent chromosomal damage and a critical function for FK506-binding proteins during meiosis.

Introduction

FK506-binding proteins (FKBPs) share a common proline isomerase (PPIase) domain that catalyzes the interconversion between the *cis* and *trans* peptidyl proline bonds *in vitro*, and acts as a receptor for two clinically important drugs, FK506 and rapamycin. The best-studied aspect of FKBP biology is the ability of FKBP12 to bind and inhibit calcineurin or TOR kinase, when bound to FK506 or rapamycin, respectively, thereby mediating the immunosuppressive and antiproliferative effects of these drugs (Hamilton and Steiner, 1998; Heitman et al., 1992). Less is known about the physiological roles of FKBPs. Deletion of all four yeast FKBP does not affect cell proliferation under standard conditions (Dolinski et al., 1997). In humans, FKBP12 through its PPIase domain acts as a modulator of several different receptors (Breiman and Camus, 2002) and the yeast FKBP12-homologue *FPR1* is required for feed back control in aspartate homeostasis (Arevalo-Rodriguez et al., 2004). Interestingly, *Fkbp6*$^{-/-}$ mice and *as/as* (*Fkbp6*) rats exhibit a spermatogenesis defect (Crackower et al., 2003), and *shu* (Fkbp) mutant flies are defective in oogenesis (Munn and Steward, 2000). Although the basis of these defects is not understood, these observations raise the possibility that a role of FKBPs in gametogenesis is conserved across species.

Meiosis, a central event in gametogenesis is a specialized cell division, where two rounds of chromosome segregation, meiosis I and meiosis II, follow a single round of chromosome duplication, leading to the separation of homologous chromosomes and sister chromatids, respectively. Faithful segregation of

homologous chromosomes requires their physical connection through inter-homolog recombination**.** Recombination is initiated by the introduction of DNA double-strand breaks (DSBs) by the transesterase Spo11 (Keeney, 2001). DSBs are subsequently repaired using the homologous chromosome as a template, because repair off of the sister chromatid is blocked (Petes and Pukkila, 1995; Zickler and Kleckner, 1999). One factor important for meiotic DSB repair is Dmc1, a homolog of the bacterial DNA strand invasion factor RecA, which serves to direct nascent DSB toward the homologous chromosome (Bishop et al., 1992; Schwacha and Kleckner, 1997). Absence of Dmc1 leads to the accumulation of unrepaired DSBs and a checkpoint-dependent delay in meiotic G2/prophase (Bishop et al., 1992).

If broken chromosomes persist a conserved meiotic surveillance mechanism called the recombination or pachytene checkpoint delays cell cycle progression in meiotic G2/prophase (Lydall et al., 1996; Roeder and Bailis, 2000). In budding yeast, the G2/prophase delay is brought about by the inhibition of cyclin dependent kinase (CDK) activity (Roeder and Bailis, 2000) and by preventing activation of the transcription factor Ndt80, which induces the expression of factors necessary for meiotic chromosome segregation and spore formation (Pak and Segall, 2002; Tung et al., 2000). Factors implicated in the recombination checkpoint in yeast include components of the mitotic DNA damage signaling machinery (Mec1, Rad24, Rad17, Mec3, Ddc1), several meiosis-specific chromosomal proteins (Red1, Hop1, and Mek1) and a number of nucleolar

proteins (Roeder and Bailis, 2000). Yeast protein phosphatase 1 (PP1), Glc7, is not a checkpoint component *per se* but is thought to promote resumption of the cell cycle after recombination checkpoint-dependent delay by reversing phosphorylation events put in place by the checkpoint kinase Mek1 (Bailis and Roeder, 2000).

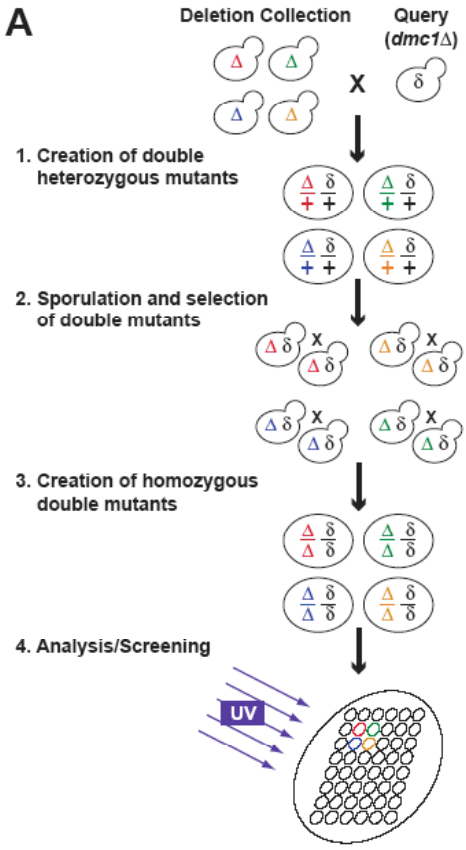
In a systematic search for novel recombination checkpoint components we identified the yeast FKBP Fpr3 as being required for continued cell cycle arrest. Using point mutants and rapamycin, we demonstrate that the proline isomerase domain but not its PPIase activity is required for the protein's checkpoint function. Our data also provide insight into the mechanism whereby Fpr3 functions in the recombination checkpoint. Fpr3 associates with protein phosphatase 1 through its proline isomerase domain and inhibits PP1 function *in vivo*. We propose that Fpr3 act as an inhibitor of PP1, thereby preventing premature adaptation to chromosomal damage.

Results

FPR3 is required for continued checkpoint arrest.

Loss of the strand invasion factor *DMC1* elicits a recombination checkpoint-dependent arrest in meiotic G2/prophase and hence a failure to form spores (Bishop et al., 1992; Lydall et al., 1996). In budding yeast, the formation of mature spores can be monitored through the accumulation of dityrosine, a spore wall component that renders sporulating colonies fluorescent under ultra-violet light (Briza et al., 2002; Briza et al., 1986). We used this assay to screen for mutations that allowed *dmc1Δ* mutants to progress through meiosis. The screen (outlined in Figure 1A) employed the *Saccharomyces cerevisiae* deletion collection (Giaever et al., 2002), which encompasses gene deletions of all non-essential yeast open reading frames (ORFs). We identified 15 deletions that allowed *dmc1Δ* cells to form fluorescent spores (Figure 1B). Fourteen deletions were previously known to alleviate the recombination checkpoint-mediated cell cycle delay (Figure 1B). Many of these eliminated DSBs, the primary signal eliciting the checkpoint response, by inactivating factors required for the formation of meiotic DSBs such as *SPO11, RAD50, MRE11* and *XRS2* (Figure 1B). Suppressor deletions were also identified in factors implicated in the recombination checkpoint response such as *HOP1, MEK1, RED1* and *DOT1*, although these mutations have also been shown to allow DSB repair from the sister chromatid, which could also account for the observed bypass suppression of the *dmc1Δ* mutation (San-Segundo and Roeder, 2000; Wan et al., 2004; Xu et al., 1997).

One suppressor deletion was novel and eliminated the gene encoding the FK506-binding protein *FPR3* (Benton et al., 1994; Manning-Krieg et al., 1994; Shan et al., 1994).



B

ORF	Gene name	Protein function
	DSB formation	
YER044C-A	*MEI4*	Required for meiotic DSB formation
YLR329W	*REC102*	Required for meiotic DSB formation
YHR157W	*REC104*	Required for meiotic DSB formation
YMR133W	*REC114*	Required for meiotic DSB formation
YGL213C	*SKI8/REC103*	Required for meiotic DSB formation
YGL214W		ORF overlaps with YGL213C
YHL022C	*SPO11*	Topoisomerase-like enzyme that catalyzes meiotic DSB formation
YMR224C	*MRE11*	Required for the formation and processing of meiotic DSBs
YNL250W	*RAD50*	Required for the formation and processing of meiotic DSBs
YDR369C	*XRS2*	Required for the formation and processing of meiotic DSBs
	Checkpoint proteins	
YDR440W	*DOT1*	Histone methylase
YIL072W	*HOP1*	DNA binding protein required for synapsis and chiasma formation
YOR531C	*MEK1*	S/T-kinase; meiotic Rad53 paralogue involved in recombination checkpoint
YLR263W	*RED1*	DNA binding protein required for synapsis and chiasma formation
	Novel	
YML074C	*FPR3*	Proline isomerase

Figure 1: A screen to identify components of the recombination checkpoint.
(A) Outline of the screen. Δ denotes a strain from the yeast deletion collection (different colors indicate different mutations), δ denotes the query mutation (*dmc1*Δ). In addition to the query mutation, the query strain (A5054) harbored the *pSTE5-URA3* construct that allowed respective selection for haploid (on medium lacking uracil) and diploid cells (on medium containing 5-fluoro orotic acid) at different steps of the mating scheme. The query mutation, a deletion of the entire ORF of *DMC1* (*dmc1*Δ*::HIS3*), was introduced into the deletion collection by

mass mating on plates and was subsequently homozygosed as described (Marston et al., 2004). Homozygous double mutants were sporulated for 3 days on a nitrocellulose filter that was placed onto a sporulation plate. The filter served to block out the agar auto-fluorescence and allowed screening by hand-held UV source directly on the plate. A caveat with our screening approach is the fact that a fraction of the deletion collection is known to be aneuploid (Hughes et al., 2000). Indeed, many initially UV-positive candidates were discovered to still harbor a wild-type copy of *DMC1* in addition to the *dmc1Δ::HIS3* query allele. We therefore isolated DNA of UV-positive mutants and performed a secondary screen, screening by PCR for the absence of a wild-type *DMC1* gene. Only isolates that did not show a wild-type size *DMC1* PCR fragment in this screen were kept for further analysis.

(B) Summary of genes identified in the screen.

We first compared the kinetics with which *dmc1Δ* and *dmc1Δ fpr3Δ* cells progressed through meiosis, using the timing of cyclin Clb3 protein accumulation and spindle pole body (SPB) separation as markers to assess progression out of meiotic G2/prophase into prometaphase I. Clb3 accumulation was strongly delayed in *dmc1Δ* cells, compared to wild-type cells, and was accelerated when *FPR3* was deleted (Figure 2A). We note, however, that compared to wild-type cells and *fpr3Δ* mutants, a three-hour delay in Clb3 accumulation persisted in *dmc1Δ fpr3Δ* cells (Figure 2A). The separation of SPBs occurred with similar kinetics as Clb3 accumulation. *dmc1Δ fpr3Δ* cells initiated SPB separation 7 hours after transfer into sporulation medium, whereas little separation occurred in *dmc1Δ* mutants (Figure 2B). Finally, sporulation efficiency increased dramatically

when *FPR3* was deleted in *dmc1Δ* cells (Figure 2C). Our data show that deletion of *FPR3* allows *dmc1Δ* cells to escape the checkpoint-dependent G2/prophase block and to complete the meiotic program.

The suppression of the checkpoint block in the absence of *FPR3* is not restricted to *dmc1Δ* mutants. Deletion of *FPR3* accelerated the timing of entry into meiosis I for *hop2Δ*, *rec8Δ*, and *mer3Δ* and *rad50S* cells (Alani et al., 1990; Klein et al., 1999; Leu et al., 1998; Nakagawa and Ogawa, 1999)(Figure 2D - G). However, as observed in *dmc1Δ* cells, deletion of *FPR3* did not eliminate the G2/prophase delay, suggesting that some aspects of the recombination checkpoint were still functional in the absence of *FPR3*. Interestingly, deletion of *FPR3* did not allow *zip1Δ* mutants (Sym et al., 1993) to exit the G2/prophase block more effectively, but instead appeared to slightly exaggerate it (Figure 2H). The reason why some but not all blocks are bypassed by deleting *FPR3* is at present unclear. The situation is likely more complex as suggested by the recent finding that *zip1Δ* and *mer3Δ* mutants have very similar phenotypes at low (23°C) and high (33°C) temperatures, but differ at the intermediate temperature (30°C) used in this study (Borner et al., 2004). Our findings nevertheless suggest that the prophase delay observed in *zip1Δ* cells (at 30°C) is qualitatively different from the delays caused by the deletions of *DMC1*, *HOP2*, *REC8*, or *MER3*.

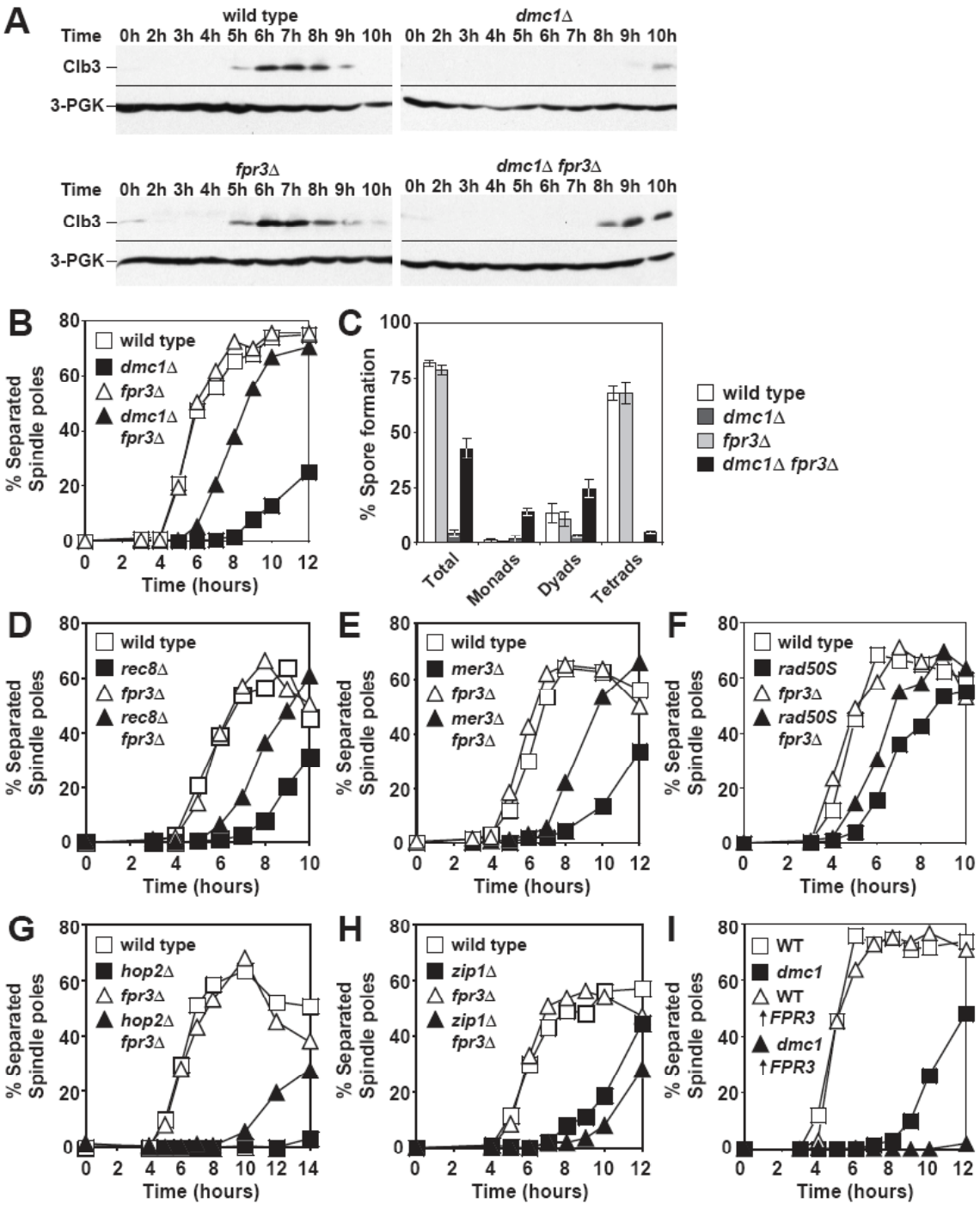


Figure 2: *FPR3* is required for maintenance of the recombination checkpoint block.

(A) Wild-type (A10125), *dmc1Δ* (A10122), *fpr3Δ* (A10124), and *dmc1Δ fpr3Δ* (A10123) cells were induced to undergo meiosis and samples were taken at the indicated times to analyze the amount of Clb3-HA protein by Western blot analysis. 3-Pgk served as a loading control in Western blots.
(B) Wild type (A9671), *dmc1Δ* (A9669), *fpr3Δ* (A9672), and *dmc1Δ fpr3Δ* (A9670) cells were induced to undergo meiosis and samples were taken at the indicated times to analyze the percentage of cells with separated SPBs.
(C) Sporulation efficiency of wild type (A6871), *dmc1Δ* (A6872), *fpr3Δ* (A6924), and *dmc1Δ fpr3Δ* (A6683) cells. Asci were classified as containing one (monads), two (dyads), or three/four spores (tetrads). Error bars indicate standard deviation in three independent experiments.
(D-I) Cells were induced to undergo meiosis and samples were taken at the indicated times to analyze the percentage of cells with separated SPBs. The following strains were used for this analysis: (D) Wild type (A9621), *rec8Δ* (A9619), *fpr3Δ* (A9620), and *rec8Δ fpr3Δ* (A9618). (E) Wild type (A11014), *mer3Δ* (A11012), *fpr3Δ* (A11015), and *mer3Δ fpr3Δ* (A11013). (F) Wild type (A9617), *rad50S* (A8990), *fpr3Δ* (A9615), and *rad50S fpr3Δ* (A8989). (G) Wild type (A8342), *hop2Δ* (A8339), *fpr3Δ* (A8345; this strain also harbored *fpr4Δ*), and *hop2Δ fpr3Δ* (A8360). (H) Wild type (A9697), *zip1Δ* (A9037), *fpr3Δ* (A9700), and *zip1Δ fpr3Δ* (A9119). (I) Wild type + *YEp352* (A13749), *dmc1Δ* + *YEp352* (A13751), wild type + *YEp352-FPR3* (A13750), *dmc1Δ* + *YEp352-FPR3* (A13752).

We next asked whether over-expression of *FPR3* would affect the recombination checkpoint. Wild-type cells expressing *FPR3* from a 2-micron plasmid progressed through meiosis with kinetics indistinguishable from cells carrying an empty control plasmid (Figure 2I). However, high levels of *FPR3* dramatically exaggerated the checkpoint-dependent cell cycle delay observed in *dmc1Δ* cells.

Our data indicate that high levels of *FPR3* lead to a maintained arrest in meiotic prophase in a DNA damage-dependent manner.

DSBs form normally and persist in *dmc1Δ fpr3Δ* cells.

Why are *dmc1Δ fpr3Δ* double mutants able to progress through meiosis? Two possibilities we considered were that (1) DSBs are not formed in the absence of *FPR3*, or that (2) the DNA damage caused by the absence of *DMC1* could be repaired once *FPR3* was eliminated. To test these hypotheses, we analyzed DSB formation and repair at the well-characterized *HIS4LEU2* hotspot (Storlazzi et al., 1995), as cells progressed through meiosis. DSBs appeared and were repaired in *fpr3Δ* cells with kinetics indistinguishable from that of wild-type cells (Figure 3A, C). Furthermore, DSBs formed and accumulated to the same extent in *dmc1Δ* and *dmc1Δfpr3Δ* mutants (Figure 3A, C), indicating that the lack of *FPR3* did not affect DSB formation. DSBs appeared to be resected with comparable kinetics in both strains, because the DSB band increased in heterogeneity at a similar rate in both strains (Figure 3A, C). This analysis did not allow us to determine whether the drop in DSB signal was solely due to hyperresection of the breaks or whether a subset of breaks were repaired from the sister chromatid. However, it was clear that no crossover repair products were formed in *dmc1Δ* and *dmc1Δfpr3Δ* mutants (Figure 3A, D), suggesting that deletion of *FPR3* did not re-activate cross-over repair in *dmc1Δ* mutants. The absence of cross-over products also indicates that deletion of *FPR3* does not cause over-activation of *RAD51* or

RAD54, which has previously been shown to bypass the requirement for *DMC1* in cross-over repair (Bishop et al., 1999; Tsubouchi and Roeder, 2003).

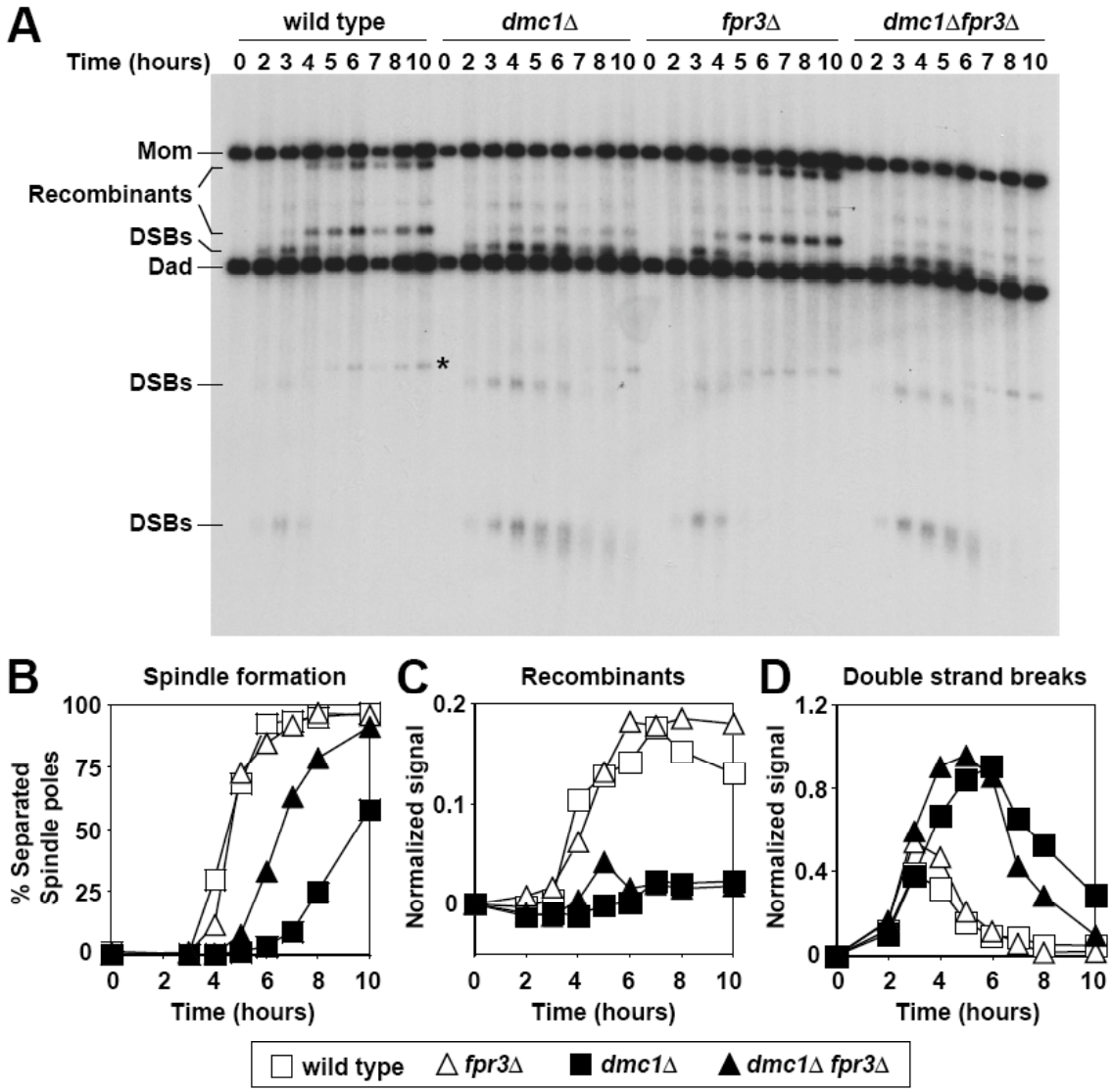


Figure 3: DSBs form normally in the absence of *FPR3*.

Wild-type (A7883), *dmc1*Δ (A7884), *fpr3*Δ (A7878), and *dmc1*Δ *fpr3*Δ (A7877) cells were induced to undergo meiosis and samples were taken at the indicated times to analyze recombination using the *HIS4LEU2* hotspot (Storlazzi et al.,

1995) by Southern blot analysis (A) and the percentage of cells with separated SPBs (B). The Southern blot was probed with probe A. * indicates a *RAD52*-dependent DNA fragment that is likely to be the result of ectopic recombination of the *HIS4LEU2* locus with *leu2::hisG* locus (Grushcow et al., 1999). Quantification of the slower migrating recombinant band and of the fastest migrating DSB band is shown in (C) and (D), respectively. To obtain the normalized signal the measured signal was divided by the signal of the parental band. The value of the 0 hour time point was then subtracted from all later time points to eliminate non-specific signal.

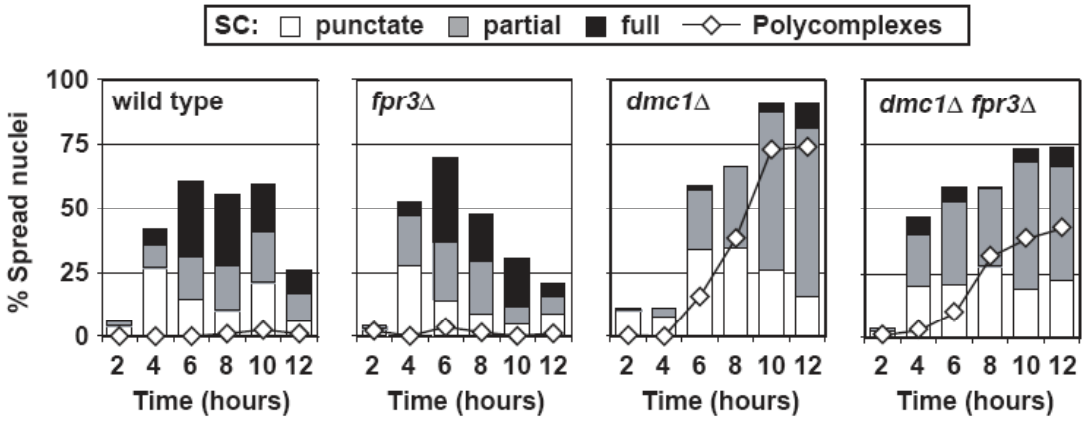


Figure 4: The defects in SC formation in *dmc1Δ* mutants are not rescued by inactivating *FPR3*.

Nuclei of wild-type (A7589), *dmc1Δ* (A7590), *fpr3Δ* (A7588), and *dmc1Δ fpr3Δ* (A7587) cells, all carrying a *ZIP1-GFP* fusion, were spread at the indicated time points, and SC structures were analyzed.

Despite the lack of crossover repair, *dmc1Δ fpr3Δ* cells entered meiosis I after only a two-hour delay compared to wild type and *fpr3Δ* single mutants, and three

to four hours before significant spindle formation could be observed in *dmc1Δ* mutants (Figure 3B). Consistent with the lack of DNA repair we found that deletion of *FPR3* also did not rescue the defect of *dmc1Δ* cells in synaptonemal complex formation (Figure 4). Our results indicate that deletion of *FPR3* neither eliminates DSB formation nor allows crossover repair of DSBs from the homolog in *dmc1Δ* cells, while still allowing progression through meiosis.

***FPR3* is a checkpoint factor.**

DSB repair using the sister chromatid as a template is largely inhibited during meiotic recombination making homologous chromosomes the preferred repair template (Petes and Pukkila, 1995; Schwacha and Kleckner, 1997). We took several approaches to test the possibility that deletion of *FPR3* allows repair of DSBs from the sister chromatid in *dmc1Δ* mutants. First, repair of DSBs is expected to improve the spore viability of *dmc1Δ* mutants. We performed this experiment in a *spo13Δ* background, because cells lacking *SPO13* undergo a single round of chromosome segregation, which partially alleviates the requirement for cross-over recombination and chiasma formation (Wagstaff et al., 1982). Thus, if repair of DSBs were to occur from the sister chromatid, *spo13Δ fpr3Δ dmc1Δ* spores ought to exhibit increased viability over *spo13Δ dmc1Δ* spores. This, however, was not the case (Table 1), indicating that deletion of *FPR3* does not allow significant repair off the sister chromatid in *dmc1Δ* mutants.

Table 1: Spore viabilities.

Strain	Genotype	Ploidy	Spore viability	N(spores)[2]
A6871	wild type	diploid	97.5 %	160
A6872	*dmc1Δ*	diploid	8 %	40
A8596	*fpr3Δ*	diploid	99 %	160
A6683	*dmc1Δ fpr3Δ*	diploid	10 %	40
A9035	*spo13Δ*	diploid	46 %	200
A9033	*spo13Δ dmc1Δ*	diploid	17 %	200
A9034	*spo13Δ fpr3Δ*	diploid	47 %	200
A9032	*spo13Δ dmc1Δ fpr3Δ*	diploid	12 %	200
A9802	*spo13Δ*	haploid[1]	20 %	40
A9799	*fpr3Δ*	haploid	23 %	40

[1] Both haploid strains harbor *MAT**a*** and *MATα* information to allow them to enter meiosis.

[2] Tetrads and dyads were dissected and viability of individual spores was determined.

Because *DMC1* is required for recombination, we also analyzed the effect of *fpr3Δ* on a prophase delay when the recombination machinery was intact. Haploid cells that harbor mating type information for both **a** and α can be induced to undergo meiosis and form viable offspring if *SPO13* is deleted and if the meiotic inhibition of sister chromatid repair is eliminated (De Massy et al., 1994; Wagstaff et al., 1982). If the inhibition of sister chromatid repair is maintained, *MAT**a**/α* haploids accumulate DSBs and delay in meiotic G2/prophase (De Massy et al., 1994). Consistent with *FPR3* having a checkpoint role, we observed that deletion of *FPR3* in *spo13Δ MAT**a**/α* haploids resulted in the bypass of the G2/prophase delay, but spore viability did not increase (Table 1, Figure 5). These

results indicate that a role of *FPR3* in preventing meiotic DSB repair off the sister chromatid is, if it exists at all, limited.

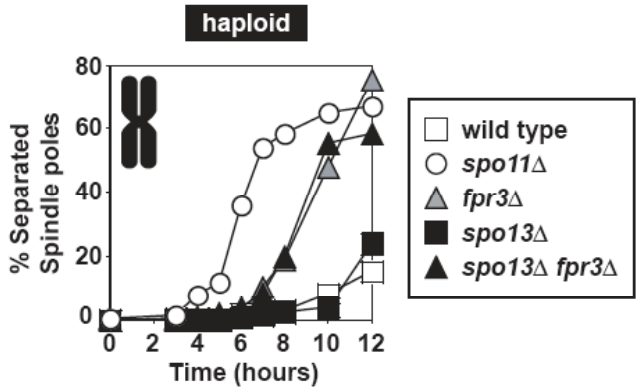


Figure 5: Deletion of *SPO13* does not bypass the delay of haploid *dmc1Δ fpr3Δ* mutants.

Cells were induced to undergo meiosis and samples were taken at the indicated times to analyze the percentage of cells with separated SPBs. The following strains were used: wild type (A8873), *spo11Δ* (A10272), *fpr3Δ* (A11288), *spo13Δ* (A9802), *spo13Δ fpr3Δ* (A9799).

To conclusively determine whether *FPR3* was indeed a *bona fide* checkpoint factor, we constructed a strain in which the homologous chromosome as well as the sister chromatid would be absent during meiotic G2/prophase, based on the premise that no homologous repair should be possible if all repair templates are removed. In this situation any observed bypass should be attributable to the checkpoint function of *FPR3*. To prevent cells from undergoing pre-meiotic DNA replication, we constructed a meiosis-specific knock-down allele of the pre-

replicative complex component *CDC6* (Cocker et al., 1996) by placing *CDC6* under the control of the mitosis-specific *SCC1* promoter (*cdc6*-meiotic-null; *cdc6-mn*). *cdc6-mn* cells duplicated their DNA normally during mitotic growth but underwent little pre-meiotic DNA replication (Figure 6C, Figure 7A). Nevertheless, diploid *cdc6-mn* mutants showed only a small delay in the progression through meiosis (Figure 6A, top right panel) and underwent DSB formation and meiotic recombination with almost wild-type kinetics and efficiency (Figure 7B). The observation that DSB formation occurs in cells depleted for *CDC6*, in which DNA replication is absent, but not in cells lacking the S phase cyclins *CLB5* and *CLB6*, in which DNA replication also does not occur (Borde et al., 2000; Smith et al., 2001) raises the interesting possibility that Clb5/6-CDK activity is required for DSB formation.

When both *MAT**a*** and *MAT*α information was provided, haploid *cdc6-mn* cells initiated meiotic recombination with only a single copy of their genome (Figure 6A). These cells possessed the full meiotic repair machinery but lacked a template to repair the DSBs and exhibited a cell cycle delay. The delay was DSB-dependent, because deletion of *SPO11*, the enzyme that catalyzes DSB formation (Keeney, 2001), allowed *MAT**a***/α *cdc6-mn* haploids to progress through meiosis with kinetics indistinguishable from cells with a full set of repair templates (Figure 6A).

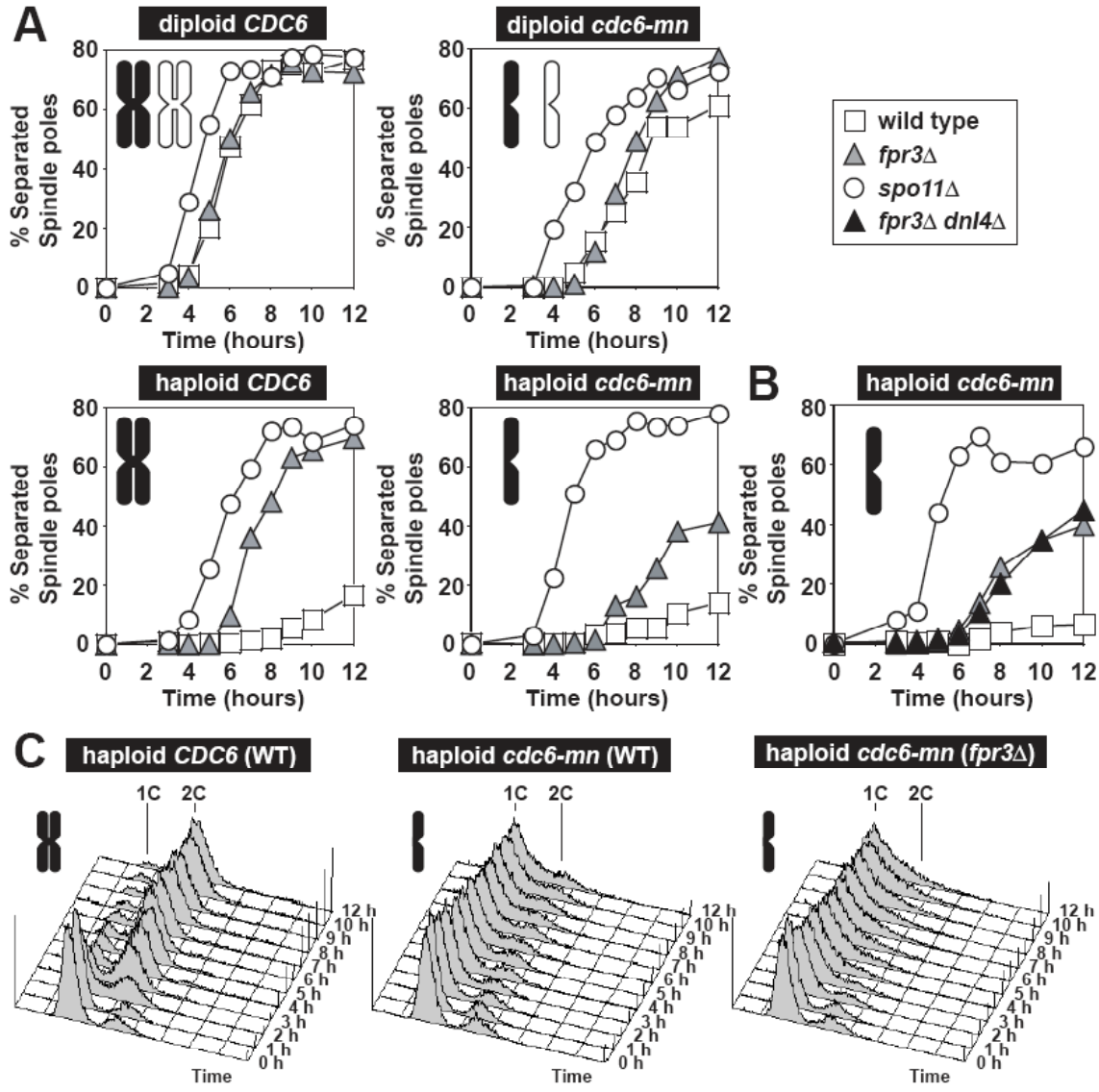


Figure 6: Homologous repair templates are not required for *FPR3*-mediated bypass of the recombination checkpoint block.

(A, B) Cells were induced to undergo meiosis and samples were taken at the indicated times to analyze the percentage of cells with separated SPBs. The following strains were used: diploid *CDC6* strains: wild type (A9671), *fpr3*Δ (A9672), *spo11*Δ (A12169); diploid *cdc6-mn* strains: wild type (A9603), *fpr3*Δ (A9602), *spo11*Δ (A12168); MAT**a**/α haploid *CDC6* strains: wild type (A8873),

*fpr3*Δ (A11288), *spo11*Δ (A10272); MAT**a**/α haploid *cdc6-mn* strains: wild type (A11550), *fpr3*Δ (A9723), *spo11*Δ (A12006), *fpr3*Δ *dnl4*Δ (A12007). Black and white chromosomes denote the C (complement) content of the strains.

(C) Flow cytometric analysis of DNA content. To improve the FACS profile quality, *LEU2* prototrophic versions of A8873, A11550 and A9723 were used.

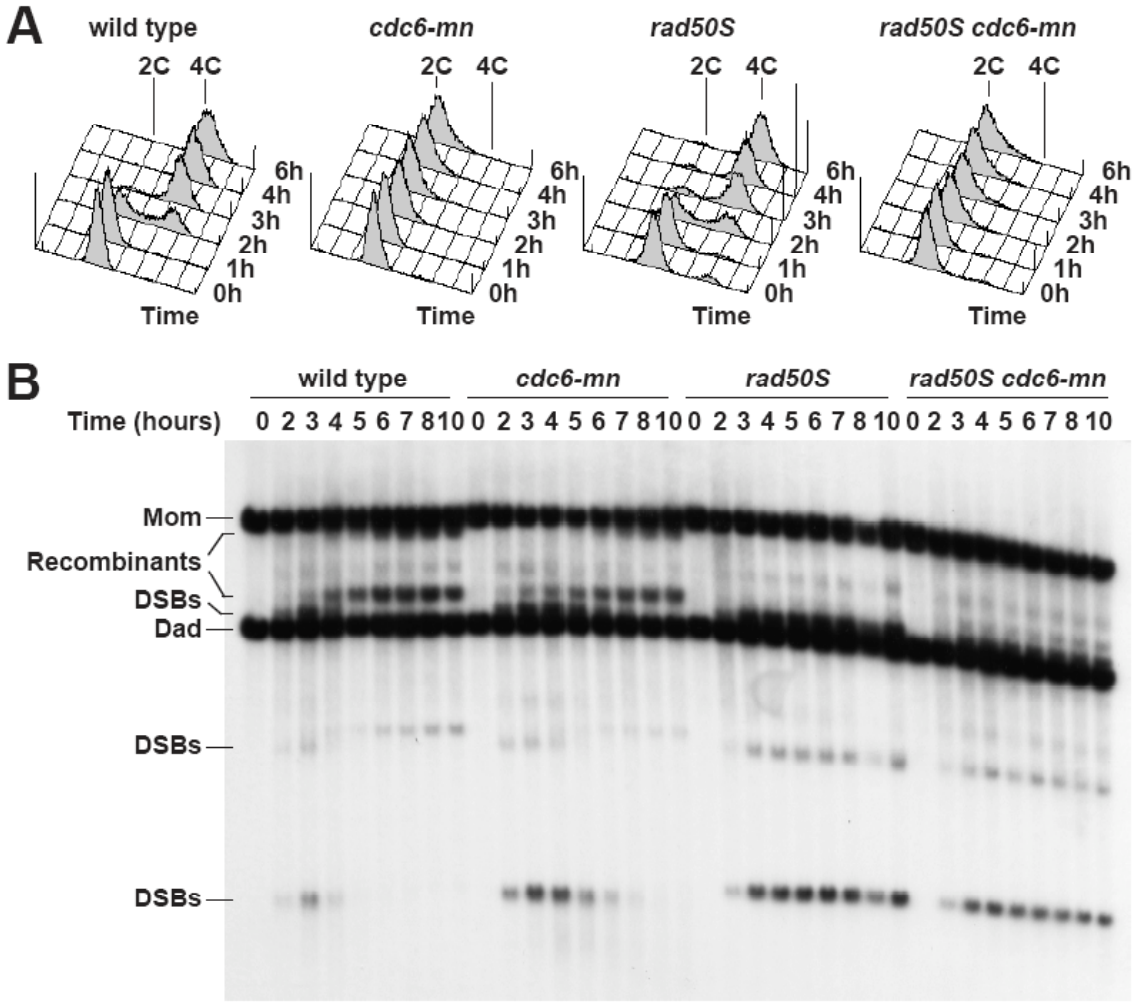


Figure 7: *cdc6-mn* mutants form DSBs and undergo recombination without pre-meiotic DNA replication.

Wild type (NKY1551), *cdc6-mn* (A10912), *rad50S* (A11675), and *rad50S cdc6-mn* (A11265) cells were induced to undergo meiosis and samples were taken at

the indicated times to analyze DNA content by flow cytometry (A), and recombination using the *HIS4LEU2* hotspot (Storlazzi et al., 1995) by Southern blot analysis (B). The Southern blot was probed with probe A.

If deletion of *FPR3* were to only activate repair from the sister chromatid it would be expected to have no effect on the cell cycle progression of *MAT**a**/α cdc6-mn* haploids. However, by 10 hours ~40% of haploid *cdc6-mn fpr3Δ* cells had entered meiosis I as judged by SPB separation even though little DNA replication had occurred by this time (Figure 6A lower right panel, 6C). The bypass of the cell cycle block was also not due to DSB repair mediated by the non-homologous end-joining pathway, because deletion of DNA ligase IV (Wilson et al., 1997b) did not affect the ability of *fpr3Δ* to bypass the delay of *MAT**a**/α cdc6-mn* haploids (Figure 6B). As observed in cells lacking *DMC1*, *REC8*, *MER3*, or *HOP2* (Figure 2), deletion of *FPR3* allowed only partial bypass of the delay, indicating that some aspect of the recombination checkpoint is still functional in the mutant. Furthermore, the finding that haploid *CDC6* cells lacking *FPR3* progress through meiosis more efficiently than *cdc6-mn fpr3Δ* cells may indicate that *FPR3* also has a role in preventing DSB repair off the sister chromatid. Thus, while the analysis of meiosis in haploid sister-less cells cannot exclude a role of *FPR3* in DSB repair, it clearly demonstrates a *bona fide* checkpoint role of *FPR3*.

Fpr3 spreads from the nucleolus into the nucleoplasm during meiosis.

Unlike the checkpoint factors *PCH2, MEK1* and *RED1*, *FPR3* did not appear to be developmentally regulated. Fpr3 protein levels remained constant during mitotic cell division and meiotic development (Figure 8 and data not shown).

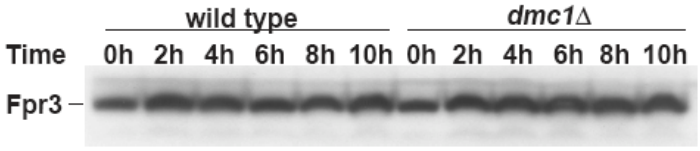


Figure 8: Fpr3 protein levels are constant throughout meiotic development. Wild-type (A6871) and *dmc1*Δ (A6872) cells were induced to undergo meiosis and the amount of Fpr3 protein was analyzed Western blot analysis at the indicated times.

Localization studies found Fpr3 enriched in the nucleolus during vegetative growth (Benton et al., 1994; Shan et al., 1994). In fact, Fpr3 was directly associated with nucleolar chromatin, because it remained localized to the low DAPI staining regions in spread nuclei (Figure 9A, B). Surprisingly, Fpr3 did not co-localize with core nucleolar markers such as Nop1 and Cdc14. Rather, it localized to a sub-compartment adjoining and frequently surrounding the Nop1- and Cdc14-positive nucleolar core structure (Figure 9B). In addition to the nucleolus, Fpr3 was also frequently localized to several foci on chromatin (Figure 9A, B and Figure 10), the nature of which is at present unclear.

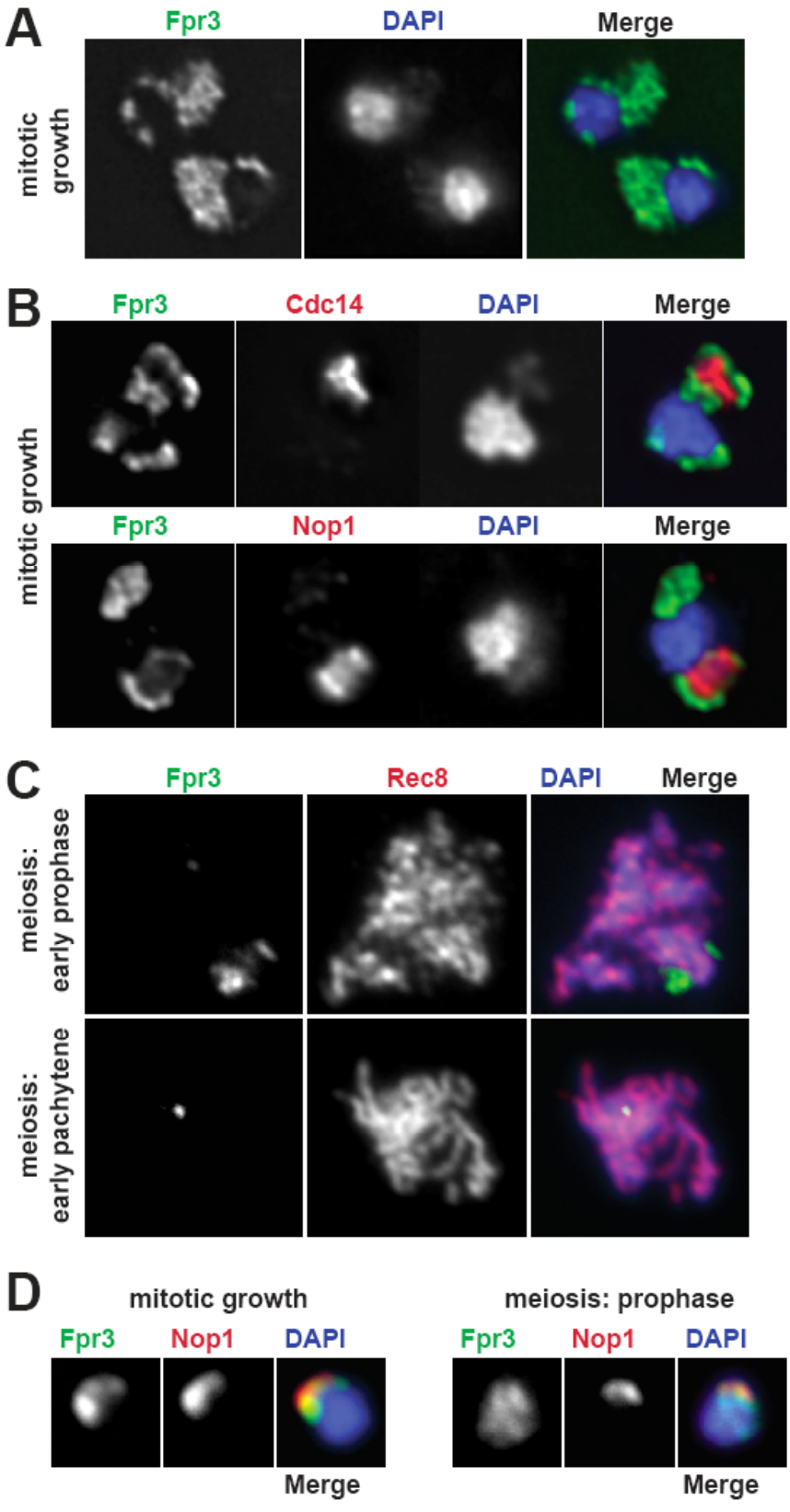

A
Fpr3
DAPI
Merge
mitotic growth
B
Fpr3
Cdc14
DAPI
Merge
mitotic growth
Fpr3
Nop1
DAPI
Merge
C
Fpr3
Rec8
DAPI
Merge
meiosis: early prophase
meiosis: early pachytene
D
mitotic growth
meiosis: prophase
Fpr3
Nop1
DAPI
Merge

Figure 9: Meiotic Fpr3 spreads from the nucleolus into the nucleus during meiosis.

(A) Deconvolved images of mitotic spreads of haploid wild-type (A10416) cells in late G1 (15 min after release from α-factor arrest). In the Merge, Fpr3 is shown in green, DNA in blue.

(B) Deconvolved images of mitotic spreads of cycling diploid wild-type cells (top: A7872, carrying *CDC14-HA*; bottom: A9671). In the Merge, Fpr3 is shown in green, Cdc14 in red and DNA in blue.

(C) Images of meiotic spreads of diploid wild-type cells carrying Rec8-HA (A1972). Top: early prophase cell, bottom: early pachytene cell. Rec8-HA is shown in red and Fpr3 in green.

(D) Whole cell immunofluorescence of wild-type (A6871) cells in exponential growth (left) or at 4h into meiosis (right). In the merge, Fpr3 is shown in green, Nop1 in red.

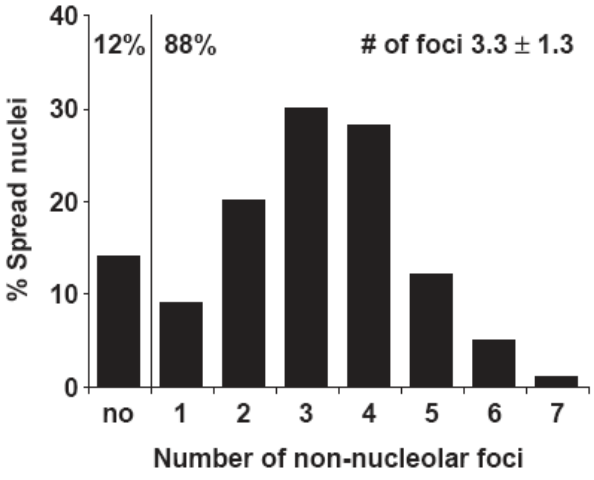


Figure 10: Distribution of non-nucleolar chromatin-associated Fpr3 foci present during meiosis.

Quantification of non-nucleolar Fpr3 foci from late G1 cells (haploid wild-type (A10416); 30 minutes after release from α-factor). Percentages on top indicate the

number of cells with no extra foci (12%) versus cells with extra foci (88%). Cells with extra Fpr3 foci contained on average 3.3±1.3(s.d.) foci.

When cells were starved to induce meiosis, the volume of both the Fpr3-positive and the Nop1-positive nucleolar compartment decreased dramatically, and a further loss in nucleolar volume was observed as cells progressed through the meiotic program (Figure 9C and data not shown). Concomitantly, Fpr3 lost its nucleolar chromatin association, such that by pachytene, when chromosomes were fully synapsed, Fpr3 staining was restricted to a single chromosome-associated focus (Figure 9C, bottom panels). We do not know the identity of the Fpr3 marked chromosomal region. As cells were induced to undergo meiosis, most of the cellular pool of Fpr3 became more diffusely distributed throughout the nucleus, as judged by whole-cell immunofluorescence of meiotic cells (Figure 9D). Our results indicate that at the time when the recombination checkpoint becomes active during meiosis, Fpr3 is present throughout the nucleus. The significance of the dynamic localization of Fpr3 is at present unclear.

***FPR3* and *PCH2* do not function together in the recombination checkpoint.**

Pch2, like Fpr3, is found in the nucleolus (San-Segundo and Roeder, 1999), which raised the possibility that the two proteins act in a common pathway. However, the effects of deleting *FPR3* and *PCH2* on the G2/prophase delays of recombination mutants were not identical. Deletion of *FPR3* alleviated the prophase delay of *dmc1Δ* but not *zip1Δ* mutants (Figure 2). Deletion of *PCH2*

allowed *zip1Δ* mutants to enter meiosis I (San-Segundo and Roeder, 1999), whereas it enhanced the prophase block of *dmc1Δ* cells (Figure 11A, B). These results indicate that, at least at 30°C, the G2/prophase delays of *zip1Δ* and *dmc1Δ* mutants are not caused by the same mechanism.

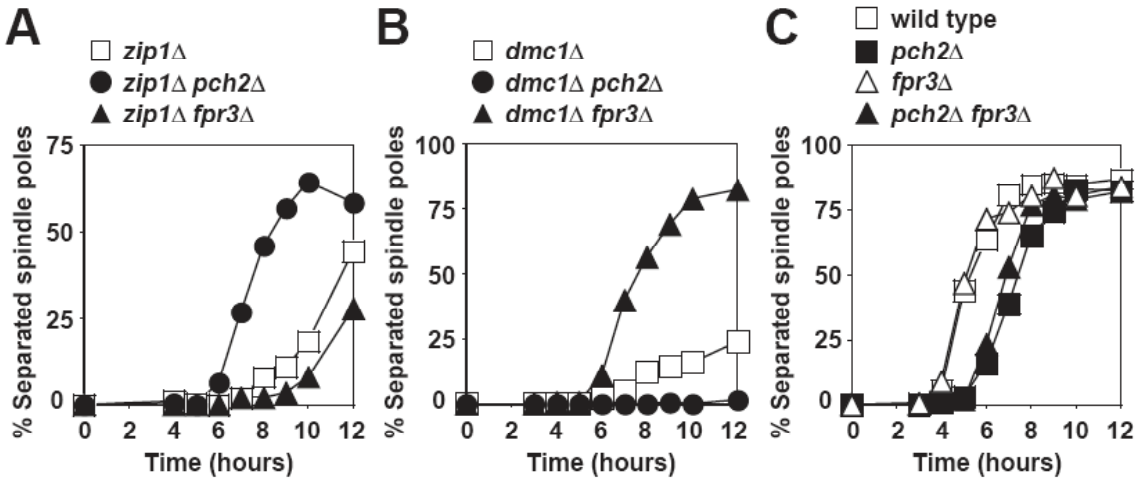


Figure 11: Distinct functions of *FPR3* and *PCH2* in the recombination checkpoint.

Cells were induced to undergo meiosis and samples were taken at the indicated times to analyze the percentage of cells with separated SPBs. The following strains were used:

(A) *zip1Δ* (A9037), *zip1Δ pch2Δ* (A9036), *zip1Δ fpr3Δ* (A9119).

(B) *dmc1Δ* (A7884), *dmc1Δ pch2Δ* (A10843), *dmc1Δ fpr3Δ pch2Δ* (A10843).

(C) Wild-type (A7883), *pch2Δ* (A11026), *fpr3Δ* (A7878), *fpr3Δ pch2Δ* (A10842).

PCH2 may be a component of a checkpoint pathway acting in parallel to the checkpoint response defined by *FPR3*. However, several lines of evidence argue against this possibility and instead support a role for *PCH2* in DSB repair. First,

pch2Δ cells themselves exhibited a two-hour delay in cell cycle progression (San-Segundo and Roeder, 1999) that could not be bypassed by the deletion of *FPR3* (Figure 11C). Second, while DSBs have largely disappeared by 4 hours in wild-type cells (Figure 3), they persisted at least until the 6-hour time point in *pch2Δ* mutants (Figure 12). Accordingly, crossover repair products were also observed with a two-hour delay. Moreover, the DSBs of *dmc1Δ pch2Δ* mutants did not get hyper-resected as rapidly as in *dmc1Δ* cells (compare Figure 12 with Figure 3A). Together these findings indicate that *PCH2* has a role in the processing of DSBs in both wild type and *dmc1Δ* mutants, which argues against a common role of *PCH2* and *FPR3* in the recombination checkpoint.

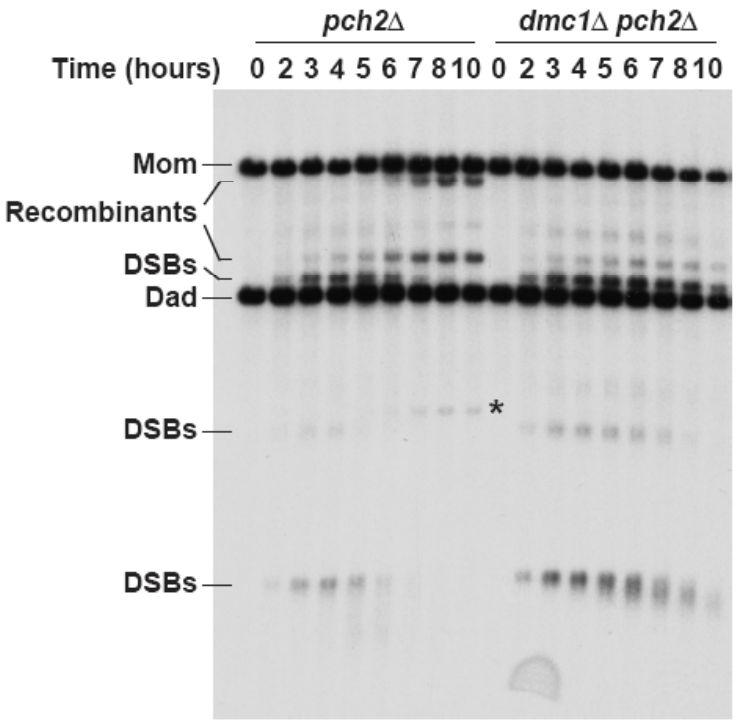


Figure 12: *PCH2* is required for DSB repair.

pch2Δ (A11026) and *dmc1Δ pch2Δ* (A10843) cells were induced to undergo meiosis and samples were taken at the indicated times to analyze recombination using the *HIS4LEU2* hotspot (Storlazzi et al., 1995) by Southern blot analysis. The Southern blot was probed with probe A. * indicates a *RAD52*-dependent DNA fragment that is likely to be the result of ectopic recombination of the *HIS4LEU2* locus with *leu2::hisG* locus.

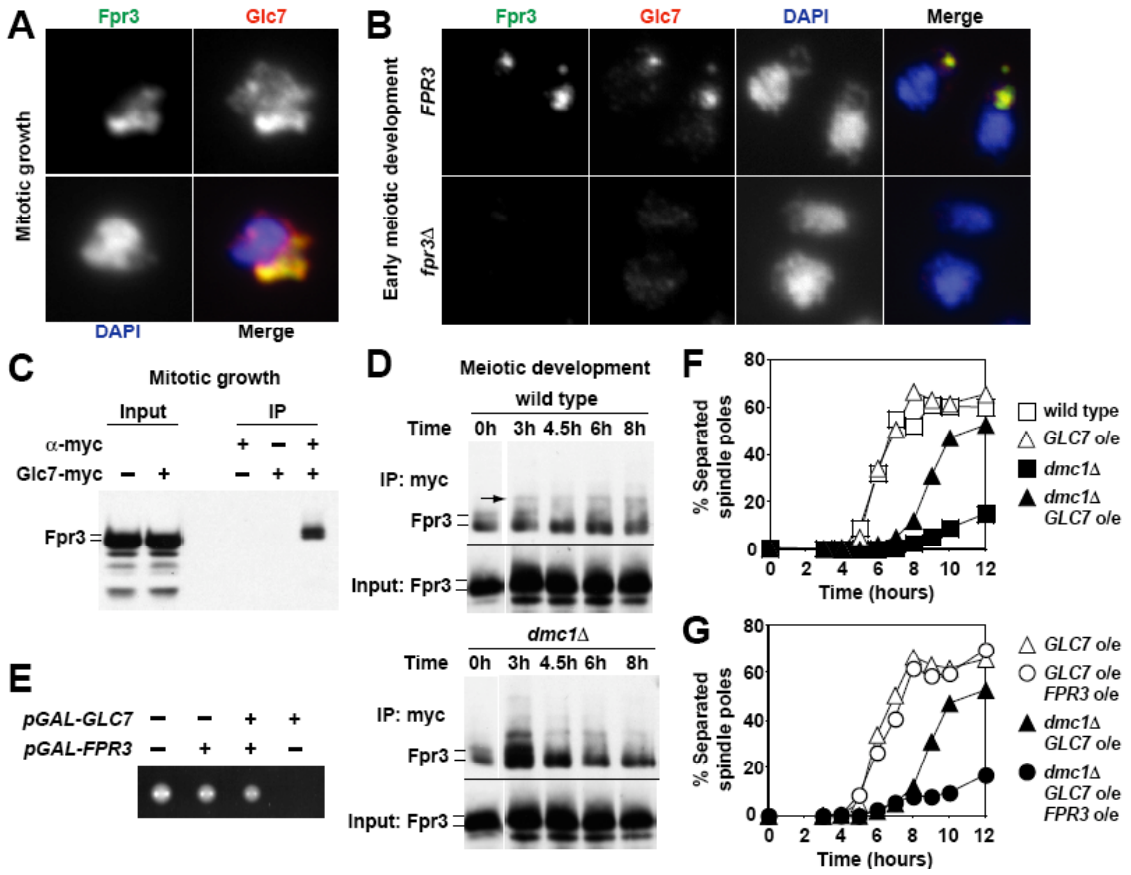


Figure 13: Fpr3 interacts with and counteracts Glc7.

(A) Images of spread mitotic wild-type cell carrying *Glc7-myc* (A6030; haploid W303). Glc7 is shown in red, Fpr3 in green and DNA in blue.

(B) Early meiotic (0h) *dmc1Δ* (A12445) and *dmc1Δfpr3Δ* (A12443) cells carrying *Glc7-myc*.

(C, D) Western blots detecting Fpr3 after immunoprecipitation of Glc7-myc from (C) cycling mitotic cells (A6030) or (D) cells progressing through meiosis; wild type (A12444), *dmc1Δ* (A12445). The arrow indicates a meiosis-specific cross-reacting band that suggests a meiosis-specific modification of Fpr3.

(E) Segregants of a cross between *pGAL-GLC7* cells with *pGAL-FPR3* cells (A1631 × A12368, W303). Tetrads were micro-manipulated on plates containing 2% galactose to induce overproduction of Glc7 and Fpr3.

(F, G) Cells were induced to undergo meiosis and samples were taken at the indicated times to analyze the percentage of cells with separated SPBs. The following strains were used for this analysis: (F) Wild type + *YEp352* (A13749), *dmc1Δ* + *YEp352* (A13751), *pHOP1-GLC7* + *YEp352* (A13753), *dmc1Δ pHOP1-GLC7* + *YEp352* (A13757). (G) *pHOP1-GLC7* + *YEp352* (A13753), *pHOP1-GLC7* + *YEp352-FPR3* (A13754), *dmc1Δ pHOP1-GLC7* + *YEp352* (A13757), *dmc1Δ pHOP1-GLC7* + *YEp352-FPR3* (A13758).

Fpr3 associates with and anchors Glc7/PP1 in the nucleolus.

PP1/Glc7 is a checkpoint factor (Bailis and Roeder, 2000) that like Fpr3 is expressed during both mitosis and meiosis and found enriched in the nucleolus during mitotic growth (Bloecher and Tatchell, 2000). Furthermore, a large-scale affinity purification study showed that Fpr3 co-purifies with a subset of nucleolar factors one of which is Glc7 (Ho et al., 2002). We therefore examined whether Fpr3 and Glc7 form a complex. Fpr3 forms a complex with Glc7 during mitosis (Figure 13C) and meiosis (Figure 13D) as evident from their ability to co-immunoprecipitate from both mitotic and meiotic extracts. Consistent with this, Glc7 co-localized with the nucleolar pool of Fpr3 on chromatin spreads of nuclei obtained from mitotically dividing and early meiotic cells (Figure 13A, B).

Furthermore, Fpr3 was required for Glc7 association with the nucleolus in both mitotic and early meiotic cells (Figure 13B, and data not shown). The loss of Glc7 from the nucleolus was not due to a general disorganization of the organelle, as Nop1 localization was not affected by deletion of *FPR3* (Figure 14).

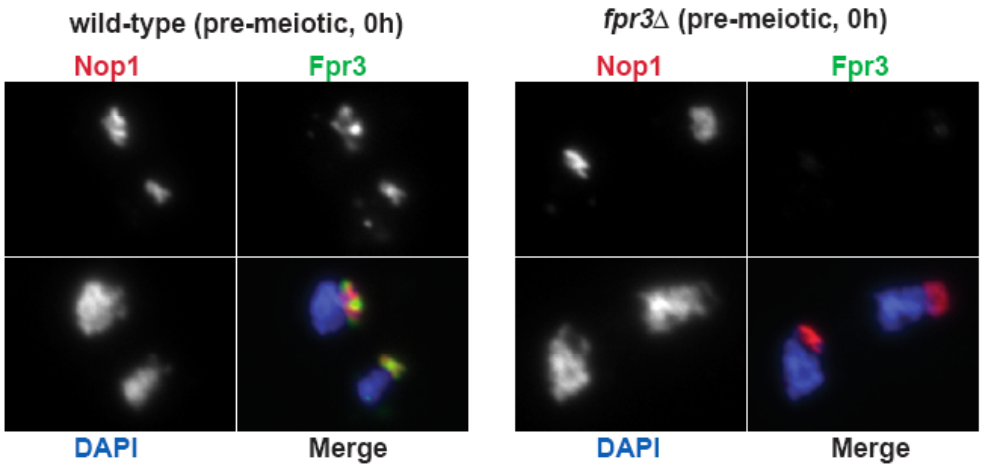


Figure 14: Nop1 localization is not affected by inactivation of *FPR3*.
Nop1 localization was analyzed on spread meiotic nuclei of wild type (A12444), *fpr3* (A12442).

As cell enter the meiotic program, both Fpr3 and Glc7 leave their nucleolar compartment and spread throughout the nucleus (Figures 9D and 13B), such that at later stages in meiosis, when the nucleolar signal of Fpr3 became restricted to a single dot, Glc7 could not be detected in the nucleolus anymore (Bailis and Roeder, 2000). Nevertheless, Glc7 and Fpr3 remained in a complex throughout meiosis (Figure 13D), indicating that they remained in the nucleoplasm as a complex. Interestingly, we observed a transient increase in co-

immunoprecipitation efficiency in extract obtained from *dmc1Δ* cells as compared to wild-type cells around the time of DSB formation (3h time point, Figure 13D), which may point to a functional connection between these two proteins within the recombination checkpoint. Our attempts to reproduce the interaction between Fpr3 and Glc7 using recombinant proteins were not successful indicating either that Fpr3 and Glc7 do not interact directly, or that Fpr3 and/or Glc7 need to be modified in order to interact. Consistent with the latter idea is the observation that Glc7 appeared to preferentially associate with a slower migrating form of Fpr3 (arrow, Figure 13D) in meiotic extracts. Fpr3 has been shown to be both phosphorylated and sumoylated (Wilson et al., 1997a; Wohlschlegel et al., 2004). Whether the slower migrating Fpr3 represents such a post-translationally modified form is at present unclear. Our data suggests that as cells undergo meiotic recombination, a complex consisting of Fpr3 and Glc7 leaves the nucleolus and spreads throughout the nucleus.

Fpr3 antagonizes Glc7 function.

FPR3 is required for maintained recombination checkpoint-induced cell cycle delay. In contrast, *GLC7* is required for the adaptation to DNA damage and overproduction of the phosphatase allows cells to bypass the recombination checkpoint delay (Bailis and Roeder, 2000). These findings together with our observation that the two proteins form a complex raise the possibility that Fpr3 functions as an inhibitor of Glc7. Consistent with this idea we found that over-expression of *FPR3* suppressed the lethality caused by high levels of *GLC7*

(Figure 13E). The suppression of the *GLC7*-induced lethality was not simply a result of lowering *GLC7* expression from the *GAL1-10* promoter, due to the presence of an additional copy of this promoter, because introduction a *GAL1-10* promoter alone did not suppress the lethality associated with overexpressing *GLC7*.

Overexpression of *FPR3* also counteracted Glc7 activity in the context of the recombination checkpoint. *GLC7* under the control of the strong meiotic *HOP1* promoter (*pHOP1-GLC7*) led to a partial bypass of the prophase delay in *dmc1Δ* (Figure 13F). This bypass was similar to that caused by the deletion of *FPR3* and only minimally accelerated by the deletion of *FPR3* (data not shown). Importantly, overexpression of *FPR3* prevented the *GLC7*-induced bypass of the cell cycle delay observed in *dmc1Δ* mutants (Figure 13G), indicating that Fpr3 counteracts Glc7 function in the recombination checkpoint. Interestingly, *GLC7* and/or *FPR3* overexpression only affected meiotic progression in *dmc1Δ* cells (when the recombination checkpoint is activated) but not in wild-type cells (Figures 2H and 13F), supporting a role for these two proteins in the cellular adaptation to persistent DNA damage. Our results indicate that Fpr3 associates with Glc7 to inhibit the phosphatase and maintain recombination checkpoint activity.

The PPIase domain of *FPR3* is necessary for complex formation with Glc7.

The carboxy-terminus of Fpr3 contains a proline isomerase domain that possess PPIase activity *in vitro* (Benton et al., 1994; Manning-Krieg et al., 1994; Shan et al., 1994). To investigate whether the PPIase domain is required for the interaction between Fpr3 and Glc7, we created a series of point mutations in the hydrophobic pocket of the PPIase domain (Figure 15A), based on mutations that have previously been demonstrated to decrease PPIase activity (DeCenzo et al., 1996; Koser et al., 1993; Timerman et al., 1995). We furthermore analyzed a spontaneous mutation (T345A) that changed a threonine to alanine at a position frequently occupied by polar or charged residues in other FKBPs. Two mutant forms of Fpr3, Y386D and F341Y/D342V, were stable during mitosis (Figure 16A), but displayed reduced stability during meiotic development (Figure 15B). The other point mutations did not affect protein stability (Figure 15A, B). Furthermore, all mutant proteins localized to the nucleolus normally in pre-meiotic cells (Figure 16B). We found that three different mutations of the PPIase domain (T345A, Y386D, F341Y/D342V) caused a loss of the interaction between Fpr3 and Glc7 in both pre-meiotic and meiotic cells, as judged by immunolocalization studies on meiotic spreads (Figure 15C, D) and coimmunoprecipitation analysis (Figure 15B). Two other mutations in the isomerase domain (W363L and F402Y) did not affect the binding between Fpr3 and Glc7 (Figure 15B, C, D). Analysis of the *in vitro* PPIase activity of recombinant Fpr3 point mutants showed that both W363L and F402Y mutants had lost PPIase activity (Figure 15E), consistent with observations in other

FKBPs (DeCenzo et al., 1996; Timerman et al., 1995). The T345A mutation reduced Fpr3 PPIase activity to about half of wild-type levels (Figure 15E). Our observations show that the PPIase domain of Fpr3 is required for the association between Fpr3 and Glc7. The disparity between the *in vitro* isomerase activities and *in vivo* binding activity of the T345A, W363L and F402Y mutants furthermore suggests that the proline isomerase activity itself is not required for the interaction between Fpr3 and Glc7.

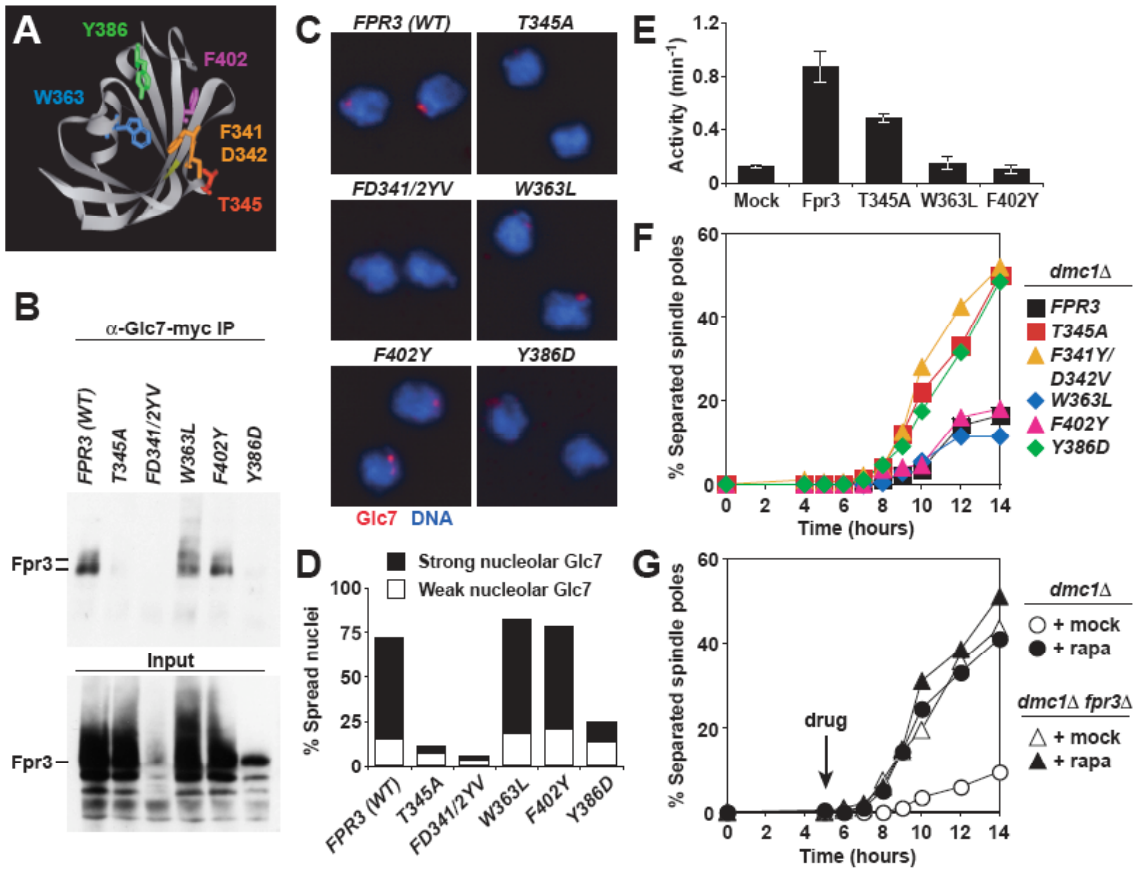


Figure 15: The PPIase domain of Fpr3 is required for checkpoint function.

(A) Predicted structure of the PPIase domain of Fpr3 (Guex and Peitsch, 1997; Peitsch, 1995; Peitsch, 1996) to illustrate positions of mutated residues. Wild-type residues are depicted. Colors correspond to color code in (E).

(B) Glc7 was immunoprecipitated from meiotic extracts at 3h and probed for the presence of Fpr3 from *dmc1*Δ and carry point mutations at the endogenous *FPR3* locus and harboring a *GLC7-myc* fusion. The following strains were used: wild type *FPR3* (A12658), *T345A* (A12659), *F341Y/D342V* (A12660), *W363L* (A12661), *F402Y* (A12662), *Y386D* (A12663).

(C, D) Spread early meiotic cells (at the time of transfer into sporulation-inducing medium) were analyzed for the presence of Glc7. Quantifications are shown in (D) and representative images shown in (C).

(E) Proline isomerase activity of recombinant Fpr3 (wild type), Fpr3 T345A, Fpr3 W363L, and Fpr3 F402Y was measured using a colorimetric assay as described in Materials and Methods. Activity describes the reaction rate (the change in OD_{395}) when the data was fit to a first order reaction. Error bars show standard deviations from three experiments.

(F) Cells were induced to undergo meiosis and samples were taken at the indicated times to analyze the percentage of cells with separated SPBs. The following strains were used: wild type *FPR3* (A9674), *T345A* (A9675), *F341Y/D342V* (A9676), *W363L* (A9677), *F402Y* (A9678), *Y386D* (A9679).

(G) 5 hours after meiotic induction, *dmc1*Δ (A7594) and *dmc1*Δ*fpr3*Δ (A7593) cells were treated with 10 μM rapamycin or 1% methanol (mock) and the percentage of cells with separated SPBs was determined.

The proline isomerase domain of *FPR3* is necessary for *FPR3's* checkpoint function.

Fpr3's PPIase domain is essential for the checkpoint role of Fpr3. A C-terminal truncation of Fpr3 that removed the entire PPIase domain (amino acids 300 – 411)

was unable to complement a deletion of *FPR3* (data not shown). Furthermore, the same point mutations that exhibited a loss of interaction between Fpr3 and Glc7 (T345A, Y386D, F341Y/D342V) also caused a loss of *FPR3* function *in vivo* as assayed by their inability to maintain a *dmc1Δ* arrest (Figure 15B, E). The two other mutations in the isomerase domain (W363L and F402Y) that did not affect Fpr3 binding to Glc7 also did not affect Fpr3 function *in vivo*. The strong correlation between the ability of Fpr3 to bind Glc7 and the checkpoint activity of Fpr3 suggests that the interaction between Fpr3 and Glc7 is important for Fpr3's checkpoint function.

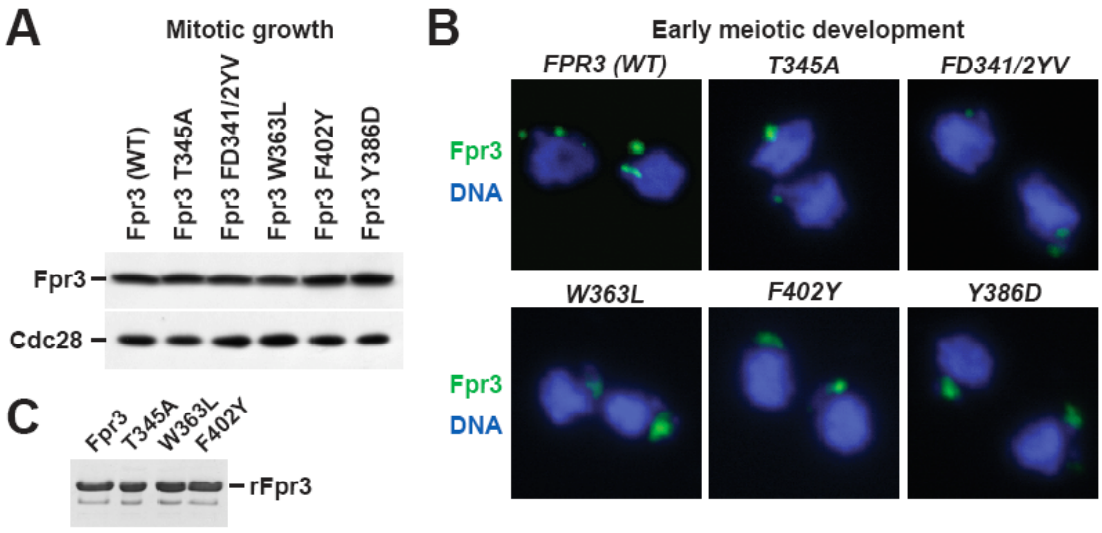


Figure 16: Effects of point mutations in the proline isomerase domain of Fpr3 on protein levels and protein localization during vegetative growth and meiotic development.

(A) Protein levels of Fpr3 point mutants in cycling cells were determined by Western blot. Cdc28 serves as loading control. Wild type *FPR3* (A9674), *T345A*

(A9675), *F341Y/D342V* (A9676), *W363L* (A9677), *F402Y* (A9678), *Y386D* (A9679).

(B) Localization of Fpr3 point mutants on spread nuclei of wild type *FPR3* (A12658), *T345A* (A12659), *F341Y/D342V* (A12660), *W363L* (A12661), *F402Y* (A12662), *Y386D* (A12663) at the time of transfer into sporulation medium.

(C) 1 g of each purified recombinant Fpr3 protein (wild type and point mutants) as used in the in vitro PPIase assay.

As a final test of the importance of Fpr3's isomerase domain in the recombination checkpoint, we examined the effects of two well-characterized small molecule inhibitors of Fpr3, FK506 and rapamycin, on the recombination checkpoint-induced G2/prophase delay. Treatment of *dmc1* cells with either FK506 or rapamycin allowed them to progress through the meiotic divisions (Figure 15F, Figure 17). Rapamycin exhibited its effect at substantially lower doses than FK506 (Figure 17). Selectivity for rapamycin has previously been observed for Fpr3 (Shan et al., 1994).

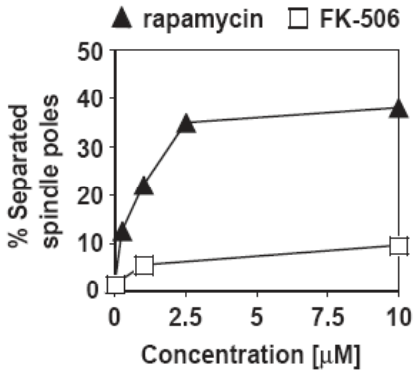


Figure 17: Effects of various concentrations of rapamycin and FK506 on the recombination checkpoint arrest.

*dmc1*Δ (A6872) cells were treated with 1% methanol or different concentrations of rapamycin or FK506 two hours after meiotic induction. The percentage of separated spindle poles 14 hours after meiotic induction was quantified.

Drug addition specifically affected Fpr3 and not other checkpoint factors because exposure to rapamycin bypassed the *dmc1*Δ arrest to levels similar to those observed when *FPR3* was deleted. Moreover, the effect of rapamycin was not enhanced by a deletion of *FPR3* indicating that rapamycin acted by inhibiting Fpr3 (Figure 15F). Taken together, the effects of Fpr3 point mutations and rapamycin on the recombination checkpoint indicate that the proline isomerase domain of Fpr3 but not its isomerase activity is required for the protein's checkpoint function.

Discussion

Fpr3 is a novel component of the recombination checkpoint.

The recombination checkpoint is a conserved meiosis-specific surveillance mechanism (Roeder and Bailis, 2000). In the present study we identified the FK506 and rapamycin binding protein Fpr3 as being required for maintained checkpoint arrest. Many meiotic checkpoint factors, in particular the components of the canonical mitotic DNA damage checkpoint machinery, Rad24, Rad17, Mec3, Ddc1, and Mec1, while being important sensors and transducers of the DNA damage signal in mitotic cells, have a poorly understood second role during meiosis in preventing DSB repair from the sister chromatid (Grushcow et al., 1999; Thompson and Stahl, 1999). Separating checkpoint and repair functions for these factors during meiosis has generally not been trivial. Here, we developed a tool to analyze the checkpoint contribution of any putative recombination checkpoint factor independently of its repair function. By constructing haploid cells that do not replicate their genome but still enter the meiotic program, we eliminated all homologous repair templates for meiotic recombination – the sister chromatid as well as the homologous chromosomes. This allowed us to unambiguously classify *FPR3* as a checkpoint factor. The same assay will be very helpful in evaluating the checkpoint roles of factors that also function to promote DSB repair.

What is the function of Fpr3 in the recombination checkpoint?

A role for PP1 in the exit from meiotic prophase has been observed in both budding yeast and *Xenopus*. In budding yeast PP1 appears to counteract the activity of the checkpoint kinase Mek1 (Bailis and Roeder, 2000), while in *Xenopus* it activates the cell cycle phosphatase Cdc25 (Margolis et al., 2003). Several lines of evidence suggest that Fpr3 functions at least in part through PP1. First, PP1 and Fpr3 influence the checkpoint arrest in opposing ways. PP1 is required for the exit from meiotic prophase (Bailis and Roeder, 2000; Margolis et al., 2003), whereas *FPR3* is necessary to inhibit premature exit from the checkpoint arrest. Second, Fpr3 and Glc7 share a similar nucleolar localization pattern and associate with each other in both mitotic and meiotic cells. This association can be abrogated by introducing point mutations into the proline isomerase domain of Fpr3. The same point mutations also cause loss of Fpr3's checkpoint activity. Finally, *FPR3* antagonizes *GLC7* function *in vivo*. In mitotic cells, the lethality associated with overexpression of *GLC7* was efficiently suppressed by high levels of *FPR3*. In meiotic cells, overexpression of *FPR3* prevented the bypass of the recombination checkpoint caused by high levels of *GLC7*.

Together, our data suggest a model in which *FPR3* maintains the checkpoint arrest by antagonizing *GLC7* function. This idea is consistent with our observation that the partial alleviation of the checkpoint delay in *dmc1Δ* cells by overexpression of *GLC7* is only insignificantly enhanced by the additional

deletion of *FPR3*. The fact that inactivation of *FPR3* only bypasses the arrests of *dmc1Δ*, *hop2Δ*, *rec8Δ*, and *mer3Δ* mutants after an initial delay is also consistent with the above model. Because *FPR3* does not affect the checkpoint pathway itself, the checkpoint signal remains active in these mutants and could be responsible for the initial delay. Unrestrained Glc7 activity would however eventually override the checkpoint arrest and promote entry into the meiotic divisions. It has not escaped our attention that this model of Glc7 regulation is reminiscent of the regulation of the protein phosphatase Cdc14, which is kept inactive in the nucleolus by an inhibitory subunit Cfi1/Net1 (Stegmeier and Amon, 2004). The finding that the nucleolar structure occupied by Fpr3 and Glc7 differs from that occupied by Cdc14, furthermore raises the interesting possibility that distinct domains of the nucleolus may serve different signaling functions.

Based on our observations, we propose *FPR3* and *GLC7* function in the adaptation to persistent DNA damage. Adaptation, that is, continued cell cycle progression after an initial arrest even if the DNA damage remains, is a phenomenon has been studied in yeast and vertebrates (Toczyski et al., 1997), and involves the inactivation of the checkpoint kinases Rad53 (the mitotic homologue of Mek1) and Chk1, respectively (Pellicioli et al., 2001; Yoo et al., 2004). In the absence of *FPR3* or upon overexpression of *GLC7*, adaptation may be accelerated. Indeed, our observation that some aspects of the checkpoint remain active in the absence of *FPR3*, as well as the fact that the effects of changing *FPR3* and *GLC7* levels can only be observed when the recombination checkpoint

has been activated by persistent chromosomal damage are consistent with a role of the two proteins in adaptation. Intriguingly, one factor required for checkpoint adaptation is casein kinase II (Toczyski et al., 1997). Fpr3 has been identified as a physiological substrate of CKII (Wilson et al., 1997a). It will therefore be of interest to investigate the role of CKII phosphorylation of Fpr3 in the context of the recombination checkpoint. Conversely, the presence of both Fpr3 and Glc7 during the mitotic cell cycle raises the possibility that these two factor are also involved in the adaptation response outside of meiosis.

The PPIase domain of Fpr3 is required for its checkpoint function.

Fpr3 is one of four FKBPs in yeast. FKBPs are a highly conserved protein family, but the cellular roles of many FKBPs remain poorly understood (Hamilton and Steiner, 1998). This is particularly true of the proline isomerase domain. The PPIase domain of FKBPs is of interest not only because it acts as the receptor for rapamycin and FK506, two drugs of considerable clinical importance, but also because of a PPIase activity associated with this domain that has thus far remained an *in vitro* phenomenon (Hamilton and Steiner, 1998). Part of the problem to define an *in vivo* function for the FKBP PPIase activity is the lack of a suitable *in vivo* assay, and the generally transient nature of the isomerization event. However, even when targeted point mutations were analyzed that exhibited varying defects in PPIase activity *in vitro*, these variations often did not correlate with the functionality of the domain *in vivo* (Timerman et al., 1995). This has led to the speculation that the PPIase domain may function in some cases as a protein

interaction domain rather than as an enzyme (Hamilton and Steiner, 1998). Fpr3, like other FKBPs, possesses PPIase activity *in vitro* (Benton et al., 1994; Manning-Krieg et al., 1994; Shan et al., 1994), and our analysis shows that Fpr3 checkpoint activity is lost when several residues in the PPIase domain are mutated. However, some point mutations that cause a complete loss of PPIase activity still exhibited wild-type function in the cell, whereas another point mutation that exhibits only a partial reduction in PPIase activity caused a complete loss of checkpoint function *in vivo*. It therefore appears that the PPIase activity of Fpr3 is not required for the protein's checkpoint function. It is however clear that the PPIase domain of *FPR3* is essential for its checkpoint function. Both point mutations in the PPIase domain and treatment of *dmc1*Δ cells with rapamycin led to a phenotype similar if not identical to that of deleting *FPR3*. The observation that FKBP12, which consists only of a PPIase domain can inhibit the phosphatase activity of calcineurin (when bound to FK-506) by blocking substrate access to the catalytic site (Harrar et al., 2001), may suggest a similar activity of Fpr3 toward Glc7.

Is the checkpoint function of Fpr3 shared by other FKBPs?

The yeast genome contains a close homologue of Fpr3 called Fpr4 that has a role in rDNA silencing (Kuzuhara and Horikoshi, 2004). *FPR3* and *FPR4* appear to share some common function since over-expression of either factor rescues the temperature sensitivity of a *tom1* mutant (Davey et al., 2000), and since double deletion of both genes causes a slight inhibition of cell proliferation in our strain

background (unpublished observations). However, even though *FPR4* is expressed at low levels in meiosis, inactivation of *FPR4* did not allow *dmc1Δ* or *hop2Δ* mutants to enter meiosis I, and the *fpr3Δ fpr4Δ* double mutant did not bypass the arrest significantly better than the *fpr3Δ* single mutant (unpublished observations). Thus, if *FPR4* has a role in the recombination checkpoint it is likely to be a very minor one.

Mouse *Fkbp6* is distantly related to *FPR3* and so far the only mammalian FKBP with a known role in meiotic progression. Male *Fkbp6*$^{-/-}$ mice show severe defects during meiotic G2/prophase, leading to an arrest prior to pachytene and to apoptosis (Crackower et al., 2003). Interestingly, disruptions and truncations of other mammalian checkpoint factors, such as *Atm*, *Brca1*, and *Brca2*, also cause infertility in mice (Baarends et al., 2001). Thus, although the *Fkbp6*$^{-/-}$ phenotype is quite different from the phenotype caused by the inactivation of *FPR3*, its similarity to the phenotypes of other checkpoint mutants in mouse raises the possibility of a role of FKBPs in mammalian recombination checkpoint signaling. If this were the case, the risks of defective gamete formation would have to be considered when using the immunosuppressive and anti-proliferative drugs rapamycin and FK506.

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Materials and Methods

Yeast strains and plasmids

Strains used in this work are listed in Table 2. Unless noted otherwise, the genetic background of all strains is SK1 (Kane and Roth, 1974). Gene deletions were constructed by one-step gene replacement using either the method of (Longtine et al., 1998) or transferring existing deletions from the deletion collection (Giaever et al., 2002) into the SK1 background. *CLB3-3HA* and *GLC7-13MYC* were constructed by one-step gene replacement. *ZIP1-GFP*, *CDC14-3HA* and *REC8-3HA* have been described previously (Klein et al., 1999; Visintin et al., 1999; White et al., 2004). The version of the *HIS4LEU2* hotspot used in this study was described by (Storlazzi et al., 1995). *MAT**a**/α* strains were created by integrating a plasmid coding for α mating type information (kindly provided by S. Chu and I. Herskowitz) into the *URA3* locus of *MAT**a*** strains. A8873, A11550, and A9723 were rendered leucine prototroph by integrating YIplac128 (Gietz and Sugino, 1988) at the *leu2* locus. To construct *FPR3* point mutants, the genomic *Eco*RI fragment coding for the COOH-terminus and the *3'UTR* of *FPR3* was cloned into pIC19R (Marsh et al., 1984). The genomic *Hind*III fragment of *URA3* was then inserted into the *3'UTR* of *FPR3*, yielding p807. p808 (*FPR3-TA*) was a spontaneous mutation resulting from PCR amplification of the genomic *FPR3* sequence. p809 (*FPR3-FDYV*), p810 (*FPR3-WL*), p811 (*FPR3-FY*), and p812 (*FPR3-YD*) were created by site-directed mutagenesis (QuickChange XL, Stratagene) using p807 as a substrate. Point mutations were integrated at the genomic *FPR3* locus by transforming the *Eco*RI fragments of the plasmids into

A7458 (*FPR3Δ C-3HA::TRP1*). The GST-Fpr3 expression vector was constructed by amplifying the *FPR3* sequence from genomic DNA introducing a BamHI site upstream of the ATG (primer: 5'-CGC **GGA TCC** GCG ATG TCT GAT TTG TTA CCA CTA GCT ACC-3') and subcloning the BamHI/HinP1I digested fragment into pGBDU-C1 (James et al., 1996). The BamHI/SalI fragment from the resulting plasmid was cloned into pGEX4T-1 (GE Healthcare) to yield p1201. Expression plasmids for the Fpr3 point mutants were constructed by replacing the EcoRI/SpeI fragment of p1201 with the corresponding fragment of p808, p810, or p811 to yield p1247, p1248, and p1249, respectively.

Table 2: Strains.

Strain	Relevant genotype
A1631	*MATa, pGAL-GLC7::LEU2 (W303)*
A1972	*MATa/alpha, REC8-3HA::URA3/REC8-3HA::URA3*
A5054	*MATa, pSTE5-URA3::TRP1, dmc1Δ::HIS3*
A6030	*MATa, GLC7-13Myc::KanMX6 (W303)*
A6683	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/fpr3Δ::KanMX*
A6871	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::KanMX/+*
A6872	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/+*
A6924	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::KanMX/fpr3Δ::KanMX*
A7587	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/fpr3Δ::KanMX, ZIP1-GFP::URA3/+*
A7588	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::KanMX/fpr3Δ::KanMX, ZIP1-GFP::URA3/+*
A7589	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::KanMX/+, ZIP1-GFP::URA3/+*
A7590	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/+, ZIP1-GFP::URA3/+*
A7593	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/fpr3Δ::TRP1*
A7594	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+*
A7872	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::KanMX/+, CDC14-3HA/+*
A7877	*MATa/alpha, his4X::LEU2-(Bam)-URA3/his4B::LEU2, arg4-Nsp/arg4-Bgl II, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/fpr3Δ::KanMX*
A7878	*MATa/alpha, his4X::LEU2-(Bam)-URA3/his4B::LEU2, arg4-Nsp/arg4-Bgl II, dmc1Δ::HIS3/+, fpr3Δ::KanMX/fpr3Δ::KanMX*
A7883	*MATa/alpha, his4X::LEU2-(Bam)-URA3/his4B::LEU2, arg4-Nsp/arg4-Bgl II,*

	dmc1Δ::HIS3/+, fpr3Δ::KanMX/+
A7884	*MATa/alpha, his4X::LEU2-(Bam)-URA3/his4B::LEU2, arg4-Nsp/arg4-Bgl II, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/+*
A8339	*MATa/alpha, hop2Δ::HIS3/hop2Δ::HIS3, fpr3Δ::TRP1/+*
A8342	*MATa/alpha, hop2Δ::HIS3/+, fpr3Δ::TRP1/+*
A8345	*MATa/alpha, hop2Δ::HIS3/+, fpr3Δ::TRP1/fpr3Δ::TRP1, fpr4Δ::KanMX/fpr4Δ::KanMX*
A8360	*MATa/alpha, hop2Δ::HIS3/hop2Δ::HIS3, fpr3Δ::TRP1/fpr3Δ::TRP1*
A8596	*MATa/alpha, fpr3Δ::TRP1/fpr3Δ::TRP1*
A8873	*MATa, ura3::MATalpha::URA3*
A8989	*MATa/alpha, rad50S::URA3/rad50S::URA3, fpr3Δ::KanMX/fpr3Δ::KanMX*
A8990	*MATa/alpha, rad50S::URA3/rad50S::URA3, fpr3Δ::KanMX/+*
A9032	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3Δ::TRP1, spo13Δ::KanMX/spo13Δ::KanMX*
A9033	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+, spo13Δ::KanMX/spo13Δ::KanMX*
A9034	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/fpr3Δ::TRP1, spo13Δ::KanMX/spo13Δ::KanMX*
A9035	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+, spo13Δ::KanMX/spo13Δ::KanMX*
A9036	*MATa/alpha, zip1Δ::LYS2/zip1Δ::LYS2, pch2Δ::KanMX/pch2Δ::KanMX, fpr3Δ::KanMX/+*
A9037	*MATa/alpha, zip1Δ::LYS2/zip1Δ::LYS2, pch2Δ::KanMX/+, fpr3Δ::KanMX/+*
A9038	*MATa/alpha, zip1Δ::LYS2/zip1Δ::LYS2, pch2Δ::KanMX/pch2Δ::KanMX, fpr3Δ::KanMX/fpr3Δ::KanMX*
A9119	*MATa/alpha, zip1Δ::LYS2/zip1Δ::LYS2, pch2Δ::KanMX/+, fpr3Δ::KanMX/fpr3Δ::KanMX*
A9602	*MATa/alpha, cdc6::pSCC1-3HA-CDC6::KanMX6/cdc6::pSCC1-3HA-CDC6::KanMX6, fpr3Δ::TRP1/fpr3Δ::TRP1*
A9603	*MATa/alpha, cdc6::pSCC1-3HA-CDC6::KanMX6/cdc6::pSCC1-3HA-CDC6::KanMX6, fpr3Δ::TRP1/+*
A9615	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/fpr3Δ::TRP1, spo11-Y135F-HA::URA3/+*
A9617	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+, spo11-Y135F-HA::URA3/+*
A9618	*MATa/alpha, rec8Δ::KanMX/rec8Δ::KanMX, fpr3Δ::TRP1/fpr3Δ::TRP1*
A9619	*MATa/alpha, rec8Δ::KanMX/rec8Δ::KanMX, fpr3Δ::TRP1/+*
A9620	*MATa/alpha, rec8Δ::KanMX/+, fpr3Δ::TRP1/fpr3Δ::TRP1*
A9621	*MATa/alpha, rec8Δ::KanMX/+, fpr3Δ::TRP1/+*
A9669	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+*
A9670	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3Δ::TRP1*
A9671	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+*
A9672	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/fpr3Δ::TRP1*
A9674	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/FPR3(WT)::URA3*
A9675	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(T345A)::URA3*
A9676	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(F341Y/D342V)::URA3*
A9677	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(W363L)::URA3*

A9678	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(F402Y)::URA3*
A9679	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(Y386D)::URA3*
A9697	*MATa/alpha, zip1Δ::LYS2/+, pch2Δ::KanMX/+, fpr3Δ::KanMX/+*
A9700	*MATa/alpha, zip1Δ::LYS2/+, pch2Δ::KanMX/+, fpr3Δ::KanMX/fpr3Δ::KanMX*
A9723	*MATa, cdc6::pSCC1-3HA-CDC6::KanMX6, fpr3Δ::TRP1, ura3::MATalpha::URA3*
A9799	*MATa, spo13Δ::KanMX, fpr3Δ::TRP1, ura3::MATalpha::URA3*
A9802	*MATa, spo13Δ::KanMX, ura3::MATalpha::URA3*
A10122	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+, CLB3-3HA:KanMX*
A10123	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3Δ::TRP1, CLB3-3HA:KanMX*
A10124	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/fpr3Δ::TRP1, CLB3-3HA:KanMX*
A10125	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+, CLB3-3HA:KanMX*
A10272	*MATa, spo11Δ::TRP1, ura3::MATalpha::URA3*
A10416	*MATa, RAD52-GFP::TRP1*
A10842	*MATa/alpha, his4X::LEU2-(Bam)-URA3/his4B::LEU2, arg4-Nsp/arg4-Bgl II, dmc1Δ::HIS3/+, fpr3Δ::KanMX/fpr3Δ::KanMX, pch2Δ::KanMX/pch2Δ::KanMX*
A10843	*MATa/alpha, his4X::LEU2-(Bam)-URA3/his4B::LEU2, arg4-Nsp/arg4-Bgl II, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/+, pch2Δ::KanMX/pch2Δ::KanMX*
A10844	*MATa/alpha, his4X::LEU2-(Bam)-URA3/his4B::LEU2, arg4-Nsp/arg4-Bgl II, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::KanMX/fpr3Δ::KanMX, pch2Δ::KanMX/pch2Δ::KanMX*
A10912	*MATa/alpha, his4B::LEU2/his4X::LEU2(Bam)-URA3, arg4-BglII/arg4-Nsp, cdc6::pSCC1-3HA-CDC6::KanMX6/cdc6::pSCC1-3HA-CDC6::KanMX6*
A11012	*MATa/alpha, mer3Δ::KanMX/mer3Δ::KanMX, fpr3Δ::TRP1/+*
A11013	*MATa/alpha, mer3Δ::KanMX/mer3Δ::KanMX, fpr3Δ::TRP1/fpr3Δ::TRP1*
A11014	*MATa/alpha, mer3Δ::KanMX/+, fpr3Δ::TRP1/+*
A11015	*MATa/alpha, mer3Δ::KanMX/+, fpr3Δ::TRP1/fpr3Δ::TRP1*
A11026	*MATa/alpha, his4X::LEU2-(Bam)-URA3/his4B::LEU2, arg4-Nsp/arg4-Bgl II, dmc1Δ::HIS3/+, fpr3Δ::KanMX/+, pch2Δ::KanMX/pch2Δ::KanMX*
A11265	*MATa/alpha, his4B::LEU2/his4X::LEU2(Bam)-URA3, arg4-BglII/arg4-Nsp, rad50S::URA3/rad50S::URA3, cdc6::pSCC1-3HA-CDC6::KanMX6/cdc6::pSCC1-3HA-CDC6::KanMX6*
A11288	*MATa, fpr3Δ::TRP1, ura3::MATalpha::URA3*
A11550	*MATa, cdc6::pSCC1-3HA-CDC6::KanMX6, ura3::MATalpha::URA3*
A11675	*MATa/alpha, his4B::LEU2/his4X::LEU2(Bam)-URA3, arg4-BglII/arg4-Nsp, rad50S::URA3/rad50S::URA3*
A12006	*MATa, cdc6::pSCC1-3HA-CDC6::KanMX6, spo11Δ::TRP1 ura3::MATalpha::URA3*
A12007	*MATa, cdc6::pSCC1-3HA-CDC6::KanMX6, dnl4Δ::TRP1, fpr3Δ::TRP1, ura3::MATalpha::URA3*
A12168	*MATa/alpha, cdc6::pSCC1-3HA-CDC6::KanMX6/cdc6::pSCC1-3HA-CDC6::KanMX6, spo11Δ::TRP1/spo11Δ::TRP1*
A12169	*MATa/alpha, spo11Δ::TRP1/spo11Δ::TRP1*
A12368	*MATalpha, pGAL-FPR3::TRP1 (W303)*
A12442	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/fpr3Δ::TRP1,*

	GLC7-13Myc::KanMX/GLC7-13Myc::KanMX
A12443	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3Δ::TRP1, GLC7-13Myc::KanMX/GLC7-13Myc::KanMX*
A12444	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+, GLC7-13Myc::KanMX/GLC7-13Myc::KanMX*
A12445	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+, GLC7-13Myc::KanMX/GLC7-13Myc::KanMX*
A12658	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/FPR3(WT)::URA3, GLC7-13Myc::KanMX/+*
A12659	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(T345A)::URA3, GLC7-13Myc::KanMX/+*
A12660	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(F341Y/D342V)::URA3, GLC7-13Myc::KanMX/+*
A12661	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(W363L)::URA3, GLC7-13Myc::KanMX/+*
A12662	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(F402Y)::URA3, GLC7-13Myc::KanMX/+*
A12663	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/fpr3(Y386D)::URA3, GLC7-13Myc::KanMX/+*
A13749	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+, leu2::YIPlac128::LEU2, [YEP352::URA3]*
A13750	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+, leu2::YIPlac128::LEU2, [YEP352-FPR3::URA3]*
A13751	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+, YIPlac128::LEU2, [YEP352::URA3]*
A13752	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+, YIPlac128::LEU2, [YEP352-FPR3::URA3]*
A13753	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+, pHOP1-GLC7::LEU2, [YEP352::URA3]*
A13754	*MATa/alpha, dmc1Δ::HIS3/+, fpr3Δ::TRP1/+, pHOP1-GLC7::LEU2, [YEP352-FPR3::URA3]*
A13757	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+, pHOP1-GLC7::LEU2, [YEP352::URA3]*
A13758	*MATa/alpha, dmc1Δ::HIS3/dmc1Δ::HIS3, fpr3Δ::TRP1/+, pHOP1-GLC7::LEU2, [YEP352-FPR3::URA3]*
NKY1551	*MATa/alpha, his4B::LEU2/his4X::LEU2(Bam)-URA3, arg4-BglII/arg4-Nsp* (Storlazzi et al., 1995)

Growth conditions and drug treatment

For mitotic spreads and immunoprecipitation, cells were grown to OD_{600} = 0.8 – 1.0 in YPDA. Conditions for α-factor release were as described (Visintin et al.,

1999). To induce meiosis, cells were seeded at OD_{600} = 0.3 and pre-grown in YP + 2% potassium acetate at 30°C for approximately 16 hours. To initiate meiosis (corresponding to the 0h time point in the meiotic time courses), cells were washed once with sterile water, resuspended at OD_{600} = 1.9 in 0.3% potassium acetate and cultured at 30°C. Rapamycin (Sigma) and FK506 (tacrolimus; AG Scientific) were dissolved in methanol to yield a 1mM stock solutions (100X) that were stored at –80°C. Rapamycin or FK506 stock solution or methanol was added to the sporulating cultures at the indicated time points.

Southerns

Southern blot analysis was conducted following the protocol of Hunter et al. (Hunter and Kleckner, 2001). Briefly, genomic DNA was isolated by spheroplasting and subsequent phenol extraction. Per lane, 1 μg total DNA was digested with XhoI and separated on a 0.6% agarose gel. Gels were blotted by alkaline transfer onto Hybond-N+ membranes (Amersham Biosciences). ^{32}P-labeled probe A was prepared using the Megaprime DNA labeling system (Amersham Biosciences) and was hybridized to the blot in dextran sulfate buffer. Blots were exposed on a BAS-MS Imaging plate (Fuji), analyzed in a BAS-2500 image reader, and quantified using ImageQuant software (Amersham Biosciences).

Immunofluorescence and Spreads

Indirect immunofluorescence on whole cells was carried out as described in (Visintin et al., 1999). Rat α-tubulin antibody (Oxford Biotechnology) and FITC-conjugated α-rat antibody (Jackson ImmunoResearch) were used at 1:200 and 1:100 dilutions, respectively. For each time-point 200 cells were scored. Meiotic spreads were performed as described by (Nairz and Klein, 1997). For Glc7, monoclonal 4A6 α-myc antibody (Upstate Cell Signaling) or rabbit α-myc (Gramsch) were used at 1:150 and FITC-conjugated α-mouse or α-rabbit antibody (Jackson ImmunoResearch) were used at dilution and 1:100 dilution respectively (images were colored differently for consistency). Antibodies raised against the N- and C-terminal fragments of Fpr3 were kindly provided by J. Thorner (Benton et al., 1994). To detect Fpr3 on nuclear spreads, N-terminal α-Fpr3 was used at 1:2500 and FITC- or CY3-conjugated α-rabbit antibody (Jackson ImmunoResearch) was used at 1:2000. Nop1 was visualized using mouse monoclonal 28F2 antibody (EnCor Biotechnology) at 1:2000 and CY3-conjugated α-mouse antibody (Jackson ImmunoResearch) at 1:2000. For whole-cell immunofluorescence, α-Fpr3 was used at 1:150 and FITC-conjugated α-rabbit antibody at 1:100, 28F2 antibody was used at 1:250 and CY3-conjugated α-mouse antibody at 1:150. Conditions for visualizing Cdc14-HA and Rec8-HA have been described previously (Klein et al., 1999; Marston et al., 2003). α-Zip1 antibody (kindly provided by N. Kleckner) and FITC-conjugated α-rabbit antibody were diluted at 1:200 and 1:50, respectively. Immunofluorescence samples were analyzed using a Zeiss Axioplan 2 microscope and a Hamamatsu

ORCA-ER camera. Where indicated, images were deconvolved from 0.2 μm z-stacks using the 3D restoration software of Openlab 3.1.5 (Improvision) typically employing 12 to 17 iterations.

Immunoprecipitation

Cells pellets were broken with glass beads in an equal volume of breakage buffer (50 mM potassium phosphate (pH = 7.4), 10 mM KCl, 10% glycerol, 0.01% NP40, 2.75 mM DTT, 2x complete protease inhibitors – EDTA (Roche)). Glc7 was immunoprecipitated from ~5 mg of total protein in the presence of 150 mM NaCl using monoclonal mouse α-myc 9E10 antibody (Covance) and Protein G sepharose (Pierce). Extracts were washed extensively with breakage buffer containing 150 mM NaCl before bound proteins were eluted with SDS loading buffer.

Recombinant Fpr3 and proline isomerase measurements

Recombinant wild-type and mutant Fpr3 were expressed as GST-fusion proteins in BL-21 CodonPlus (DE3)-RIL (Stratagene). Expression was induced in log phase cells with 1 mM isopropyl-β-thiogalactopyranoside for 3 hours at 30°C. Cells were lysed by sonication in 50 mM Tris pH 7.4, 0.5 mM dithiotreitol, supplemented with Complete protease inhibitors (Roche). Recombinant GST-Fpr3 was purified by FPLC (ÄKTA) on a Q sepharose column using a 100 mM - 640mM NaCl gradient. The peak fraction was applied to glutathione sepharose 4B (Amersham) and recombinant Fpr3 was released from the beads by thrombin

cleavage at room temperature using the Thrombin Cleavage Capture Kit (Novagen). Proline isomerase activity of recombinant Fpr3 was assayed following the procedure of Shan et al. (1994). 50 μg of the respective recombinant protein were diluted to a final volume of 900 μl in reaction buffer (50 mM HEPES pH7.8, 100 mM NaCl). Immediately before the start of the assay 100 μl chymotrypsin (5 mg/ml in reaction buffer) was added. The assay was initiated by adding 10 μl peptide substrate (5 mM Suc-Ala-Leu-Pro-Phe-pNA [BACHEM] dissolved in trifluoroethanol containing 470 mM LiCl). PPIase activity was observed at 4°C in a CARY 50 Bio UV-Vis Spectrophotometer (Varian) at 395 nm. The resulting data points were fit to first order kinetics.

Other Techniques

The structure of Fpr3 was modeled on the crystal structures of homologous FKBPs using SwissModel (Guex and Peitsch, 1997; Peitsch, 1995; Peitsch, 1996), and visualized using DS Viewer Pro software (accelrys). Flow cytometric analysis of total cellular DNA content and immunoblot analysis were performed as described in (Visintin et al., 1998). For immunoblot analysis, C-terminal α-Fpr3 antibody (Benton et al., 1994) was used at a dilution of 1:2500 and α-Cdc28 was used at 1:1000.

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Chapter 3

A Novel Response to Microtubule Perturbation in Meiosis.

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The microarray analysis of the experiments shown in Figures 6, 7, and 11 was conducted by Dr. Gunnar Wrobel, Marie Cartron, Dr. Pierre Demougin, and Dr. Christa Niederhauser-Wiederkehr in the laboratory of Dr. Michael Primig, Basel, Switzerland. The experiments shown in Figure 6, 7, 10, and 11 were conducted in collaboration with Monica G. Boselli.

Summary

During the mitotic cell cycle, microtubule depolymerization leads to a cell cycle arrest in metaphase due to activation of the spindle checkpoint. Here we show that under microtubule-destabilizing conditions, such as low temperature or the presence of the spindle-depolymerizing drug benomyl, meiotic budding yeast cells arrest in G1 or G2/prophase, instead of metaphase. Cells arrest in G1 if microtubule perturbation occurs as they enter the meiotic cell cycle, and in G2/prophase if cells are already undergoing pre-meiotic S-phase. Concomitantly, cells down-regulate genes required for cell cycle progression, meiotic differentiation and spore formation in a highly coordinated manner. Decreased expression of these genes is likely to be responsible for halting both cell cycle progression and meiotic development. Our results point towards the existence of a novel surveillance mechanism of microtubule integrity that may be particularly important during specialized cell cycles when coordination of cell cycle progression with a developmental program is necessary.

Introduction

In the course of gamete production, a specialized cell division called meiosis creates four haploid cells from one diploid progenitor. Many aspects of cell cycle regulation are similar during proliferative mitotic growth and meiosis, but the different division pattern of meiosis requires modification of the mitotic cell cycle machinery to fit the needs of the meiotic differentiation program. During the meiotic cell cycle DNA is replicated once and then separated twice during meiosis I and meiosis II without an intervening S-phase. In addition, a prolonged G2/prophase (meiotic prophase) separates pre-meiotic DNA replication from the first meiotic division. During meiotic prophase homologous chromosomes align and meiotic recombination creates the links between homologs that are necessary for proper meiosis I chromosome segregation. After completion of prophase, homologous chromosomes are segregated first during meiosis I, followed by the segregation of sister chromatids during meiosis II.

Concurrent with the chromosomal events, cells progress through an intricate developmental program that culminates in the production of highly specialized cell types, such as sperm and egg, or spores in budding yeast. For gamete formation to occur successfully, it is essential that the meiotic cell cycle and the developmental program are tightly coupled via molecular interactions that we are only beginning to understand. Mutations that uncouple meiotic cell cycle progression from spore formation emphasize how important these interactions are. For example, cells that fail to decrease cyclin-dependent kinase (CDK) activity

cannot disassemble the meiosis I spindle but other aspects of the meiotic cell cycle and the developmental program (spore production) continue, leading to the formation of defective gametes (Buonomo et al., 2003; Marston et al., 2003).

The complex transcriptional program that underlies gametogenesis appears to be one key level of control that couples the meiotic cell cycle to gamete development. Stage-specific expression of crucial meiotic regulators controls most meiotic processes, including meiotic recombination, formation of the synaptonemal complex (SC), meiosis I chromosome segregation, and spore wall formation. Factors required for the respective cell cycle stage and the corresponding developmental genes are coordinately up-regulated within characteristic transcriptional waves, creating a link between cell cycle and development (Chu et al., 1998; Chu and Herskowitz, 1998; Primig et al., 2000). For example, nutrient limitation that provides the signal for entry into the meiotic cell cycle is relayed through the transcription factor Ime1 (Honigberg and Purnapatre, 2003), which is not only essential for initiating cell cycle entry, but is also responsible for inducing the transcription of genes required for the early metabolic program of gametogenesis. It does so by interacting with the transcriptional modulator Ume6 that coordinates expression of at least 80 loci involved in metabolic as well as early and middle meiotic gene functions (Williams et al., 2002). Similarly, at the end of meiotic prophase the transcription factor Ndt80 not only induces expression of genes essential for entry into meiosis

I but also that of genes important for pro-spore wall assembly (Chu et al., 1998; Chu and Herskowitz, 1998; Primig et al., 2000).

Several meiotic events are monitored by surveillance mechanisms known as checkpoints. Checkpoints halt cell cycle progression until the event that is surveyed has been completed, thereby ensuring that the cell cycle, as well as developmental events occur in an ordered manner. Typically, the transcription factors required to initiate the next phase of the meiotic cell cycle are under direct checkpoint control. For example, the transcription factor *NDT80* is a direct target of the recombination (or pachytene) checkpoint (Roeder and Bailis, 2000; Tung et al., 2000), which prevents entry into meiosis I and pro-spore wall assembly until the DNA damage created during meiotic recombination is repaired. Thus, both the initiation of pro-spore wall assembly and entry into meiosis I are coupled to the completion of meiotic prophase.

The integrity of the microtubule cytoskeleton is also monitored by surveillance mechanisms. In mitotic cells, perturbations of the microtubule cytoskeleton lead to unattached kinetochores, which causes activation of the spindle assembly checkpoint (Lew and Burke, 2003; Shonn et al., 2000; Shonn et al., 2003). The spindle checkpoint component Mad2 binds to and inhibits the Anaphase Promoting Complex or Cyclosome (APC/C), a crucial activator of chromosome segregation (Hwang et al., 1998). This inhibition causes a cell cycle arrest in metaphase until the spindle defects have been repaired. Disruption of the

microtubule cytoskeleton during the meiotic cell cycle by exposure to the microtubule depolymerizing drug benomyl, has also been reported to cause a transient delay in metaphase I that, much like in the mitotic cell cycle, is dependent upon the checkpoint component Mad2 (Shonn et al., 2000).

Here we report that severe disruption of the yeast microtubule cytoskeleton after premeiotic DNA replication leads to a G2/prophase rather than a metaphase I arrest, with low protein levels of cyclin Clb3 and incompletely synapsed chromosomes. Whole-genome expression profiling revealed that this arrest is accompanied by a substantial change in the meiotic gene expression program. In particular, genes essential for meiotic recombination, cell cycle progression or spore formation/maturation are not expressed at wild-type levels. Down-regulation of meiotic transcripts also occurs when microtubules are destabilized by culturing cells at 10°C. Finally, we demonstrate that the G2/prophase arrest caused by microtubule depolymerization is independent of the checkpoints controlling spindle assembly and meiotic recombination. Our results indicate that the transcriptional response to microtubule perturbations serves to bring both the meiotic developmental program and the cell cycle to a halt. Our data also point towards the existence of a novel mechanism of microtubule integrity surveillance that coordinates the meiotic cell cycle with spore development.

Results

Benomyl reversibly arrests cells during meiosis.

To study the consequences of microtubule perturbation on meiotic cell cycle progression and spore formation we treated cells with the microtubule-depolymerizing drug benomyl. Previous reports indicated that a benomyl concentration of 60 μg/ml elicits a metaphase I delay in meiotic cells (Shonn et al., 2000). We also observe a small delay in nuclear divisions at this concentration compared to a mock treated culture (0.4% dimethylsulfoxide/DMSO; Figure 1A). However, at 60 μg/ml, benomyl caused only incomplete microtubule depolymerization and occasional short spindles were observed (data not shown). To more completely depolymerize microtubules we incubated cells with 90 μg/ml or 120 μg/ml benomyl. The delay in nuclear division appeared more pronounced as the benomyl concentration was increased (Figure 1B), with, as observed previously (Sora et al., 1988), no cells undergoing nuclear divisions at the highest concentration of the spindle toxin. In the presence of benomyl at 120 μg/ml, microtubules disappeared within minutes and only the spindle pole bodies (SPBs) exhibited weak reactivity with anti-tubulin antibodies (Figures 1C and D).

The benomyl concentration used in this analysis was 3 to 8 times higher than the concentration typically used to arrest mitotically growing cells (Machin et al., 1995; Saunders et al., 1997). It was therefore important to determine that such high benomyl concentrations were not toxic to the cells. To investigate this, cells were incubated for 5 hours with the compound, washed and released into

sporulation medium. Cells rapidly resumed the meiotic program, initiated meiotic spindle formation as judged by the separation of SPBs (Figure 1E) and underwent the nuclear divisions with kinetics indistinguishable from mock treated cells (Figure 1F). Furthermore, the severe sporulation defect was reversed when benomyl was removed from the medium (Figure 1G), and spore viability was indistinguishable from that of mock-treated cells (mock-treated: 93%; benomyl-treated: 96%; n = 240). Our results indicate that high levels of benomyl effectively inhibit progression through meiosis in a reversible manner without apparent adverse effects on meiotic cell cycle progression and spore formation.

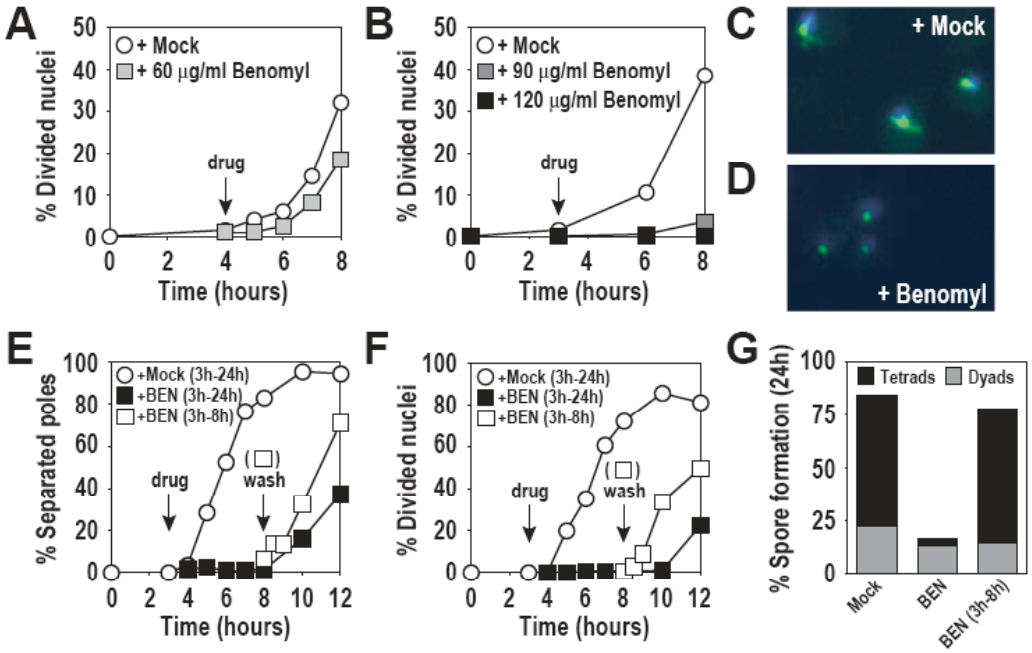

Figure 1: High levels of benomyl reversibly arrest meiotic cells.

(A) Wild-type (A727) cells. 4 hours after meiotic induction at room temperature (black arrow, designated 'drug') cells were re-suspended in medium containing 60 □g/ml benomyl (grey squares) or 0.4% DMSO (Mock; white circles).

(B) Wild-type (A727) cells. 3 hours after meiotic induction at 30°C (black arrow) cells were re-suspended in medium containing 90 μg/ml benomyl (dark grey squares), 120 μg/ml benomyl (black squares), or 0.4% DMSO (Mock, white circles). The percentage of cells in (A) and (B) having undergone at least one nuclear division was determined by 4',6-diamidino-2-phenylindole (DAPI) staining at the indicated time points.

(C, D) Wild-type (A727) cells from cultures treated with 0.4% DMSO (C) or 120 μg/ml benomyl (D) fixed one hour after treatment. DAPI-stained nuclear masses in blue, tubulin in green.

(E, F, G) Wild-type (A727) cells. 3 hours after meiotic induction at 30°C (arrow designated 'drug') cells were re-suspended in medium containing 120 μg/ml benomyl (black squares) or 0.4% DMSO (Mock, white circles). 5 hours after drug addition (arrow designated 'wash') the benomyl culture was split and half the culture was washed and released into fresh sporulation medium containing 0.4% DMSO (white squares). (E) Percentage of cells containing more than one focus of tubulin staining. (F) Percentage of cells having undergone nuclear divisions (DAPI). (G) Spore formation 24 hours after induction of meiosis. Asci were classified as containing two (dyads) or three/four (tetrads) spores (n = 500).

Benomyl treatment prevents Clb-CDK accumulation in meiotic cells.

Treatment of mitotically growing cells with 15 μg/ml benomyl causes cells to arrest in metaphase, with unseparated sister chromatids and high levels of mitotic (Clb) CDK activity (Lew and Burke, 2003). We obtained similar results when we increased this concentration to 120 μg/ml benomyl (Figure 2A, B). To determine the effects of high levels of benomyl on meiotic cell cycle progression we characterized the cell cycle arrest caused by addition of the drug. Exposure to 120

g/ml benomyl caused cells to arrest with unseparated SPBs (Figures 1D and 2C) as has been previously observed in mitotically growing cells treated with this drug (Jacobs et al., 1988). However, in contrast to mitotically growing cells, meiotic cells treated with benomyl during or after completion of S phase exhibited a dramatic delay in the accumulation of Clb3 protein and associated kinase activity (Figure 2). Furthermore, Cdc28, the catalytic subunit of CDKs, was phosphorylated on tyrosine 19, which reflects a cell cycle arrest prior to entry into the chromosome segregation phase (Morgan, 1997). Thus, whereas mitotic cells treated with high levels of benomyl arrest in metaphase, meiotic cells cultured under similar conditions during S phase and prophase arrest prior to the accumulation of Clb-CDKs that is necessary for entry into meiosis I.

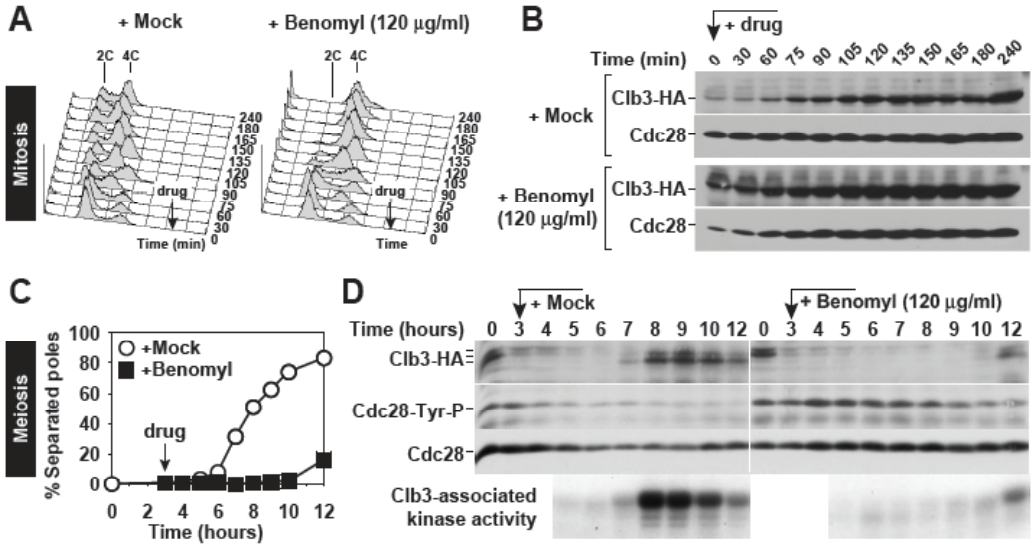

Figure 2: Meiotic cells arrest with low levels of Clb3 protein and low Clb3-associated kinase activity.

(A, B) Wild-type cells carrying a *CLB3-3HA* fusion (A4563) were enriched in G1 by acetate starvation and released into YPD medium containing 0.4% DMSO (left panel) or 120 μg/ml benomyl (right panel). (C) Total cellular DNA content determined flow cytometry. Black arrows indicate time of drug addition. (D) Western blot analysis of Clb3.

(C, D) Wild-type cells carrying a *CLB3-3HA* fusion (A4563) at room temperature. 3 hours after meiotic induction (black arrow) cells were re-suspended in medium containing 120 μg/ml benomyl (black squares) or 0.4% DMSO (Mock, white circles). (A) Percentage of cells containing more than one focus of tubulin staining. (B) Western blot analysis of Clb3 (top panel), Cdc28 tyrosine 19 phosphorylation (2nd panel), and Cdc28 (loading control, 3rd panel), and autoradiogram of H1-kinase activity of Clb3-3HA immunoprecipitated from crude extracts (bottom panel).

Chromosome pairing is defective in the presence of high levels of benomyl.

To further characterize the arrest induced by high levels of benomyl we examined chromosome pairing and synapsis. Homologous chromosomes align during prophase and this process is completed when a multi-layered structure, the synaptonemal complex (SC), has formed between homologs (Roeder, 1997; Zickler and Kleckner, 1999). We spread meiotic nuclei at various time points before and after benomyl addition and stained them with an antibody against the SC component Zip1 (Sym et al., 1993). The appearance of the first Zip1 foci was noticeably delayed in benomyl-treated cells as compared to mock-treated cells, and SC formation along the entire length of chromosomes was even further delayed (Figure 3A).

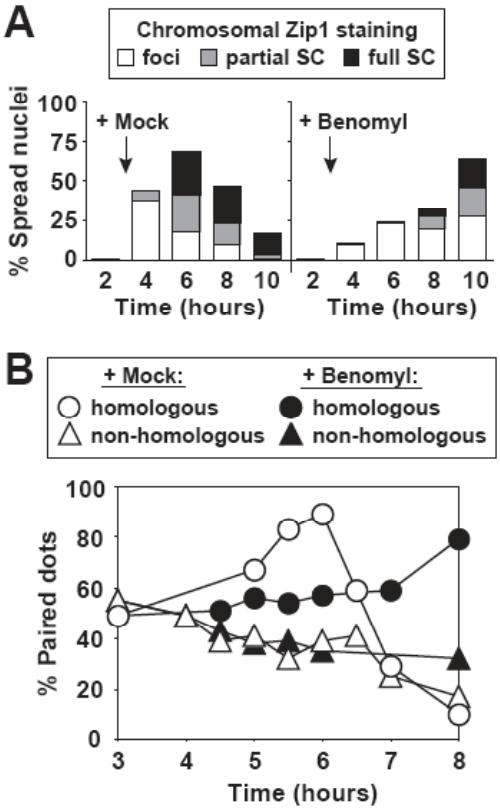

Figure 3: Benomyl exposure causes delayed homolog pairing and SC formation.

(A) Cells from experiment in Figures 2C, D were surface-spread and stained for Zip1. Spread nuclei were classified based on the presence of no staining, dispersed Zip1 foci (white), elongated Zip1 foci (partial SC; grey), or Zip1 fully covering chromosomes (full SC; black). Black arrows indicate time of drug addition. 200 nuclei were counted for each time point.

(B) Wild-type cells carrying *TetO* arrays at homologous (A5009, circles) or non-homologous (A4967, triangles) chromosomal positions. 3 hours after meiotic induction at room temperature (black arrow) cells were re-suspended in medium containing 120 □g/ml benomyl (black symbols) or 0.4% DMSO (Mock, white symbols). Pairing was determined in 100 live cells at the indicated time points.

Chromosomes were considered unpaired if two clearly separated GFP dots were visible, and paired if the two GFP dots were partially or fully overlapping.

To examine the effects of benomyl on homolog pairing we integrated tandem arrays of the Tet operator sequence (*TetO*) at the *LEU2* locus on chromosome III (*LEU2* arrays) or at the *URA3* locus on chromosome V (*URA3* arrays). The arrays were visualized by expressing a Tet repressor (TetR)-GFP fusion protein, which binds to the *TetO* arrays (Michaelis et al., 1997). To assay pairing we created diploid cells with *LEU2* arrays on both copies of chromosome III, which allowed us to analyze the behavior of a homologous locus. We assessed pairing by determining whether one or two GFP dots were visible within the cell. In this assay, only one GFP dot is visible if the arrays are paired or closely associated. As a control for non-specific array clustering, we also examined diploid strains with one *LEU2* array and one *URA3* array, i.e. the two arrays were at non-homologous positions. Around or shortly after pre-meiotic S-phase (3 hour time point) the homologous loci appeared more or less randomly arranged with respect to each other, since co-localization of *LEU2/LEU2* (homologous) occurs at similar frequency as co-localization of *LEU2/URA3* (non-homologous; Figure 3B). In the mock-treated culture the homologous *LEU2* loci increasingly co-localized during prophase reaching maximal pairing six hours after transfer into sporulation medium. Pairing was then lost as cells completed the first meiotic division. The non-homologous *LEU2/URA3* combination on the other hand exhibits a slight drop in co-localization, presumably because the ongoing homolog alignment

restricts random co-localization of non-homologous sequences. In the benomyl-treated culture, association of homologous *LEU2* loci was very much delayed (Figure 3B). Thus, this delay in homolog pairing correlates well with the effect of benomyl on SC formation (Figure 3A). Our results indicate that benomyl interferes with the pairing and synapsis of homologous sequences during meiosis.

Benomyl treatment causes cells to arrest in G1 or G2/prophase.

Our results suggest addition of benomyl during or shortly after S phase triggers a cell cycle arrest in G2/prophase. We next analyzed in more detail, whether benomyl affected the progression through S phase. To examine the effects of benomyl on pre-meiotic S phase, we determined the DNA content of benomyl-treated cells. Cells were induced to sporulate and after 4 hours benomyl was added. Progression through pre-meiotic S phase was not affected by benomyl. The 4C peak continued to increase for at least one hour after benomyl addition (Figures 4 A-C). Quantification of the extent of DNA replication occurring within an hour of mock or benomyl treatment showed that the extent of DNA replication was similar in the two cultures (Figure 4A).

We did, however, notice that an unusually high fraction of cells with a 2C DNA content persisted in the culture at later time-points after benomyl addition (Figures 4B and C). This finding raised the possibility that the initiation of DNA replication was inhibited by benomyl. Thus, due to the partial asynchrony of sporulating cultures, some cells had not yet started pre-meiotic S phase at the

point of benomyl addition and these lagging cells were prevented from entering pre-meiotic S phase by benomyl treatment.

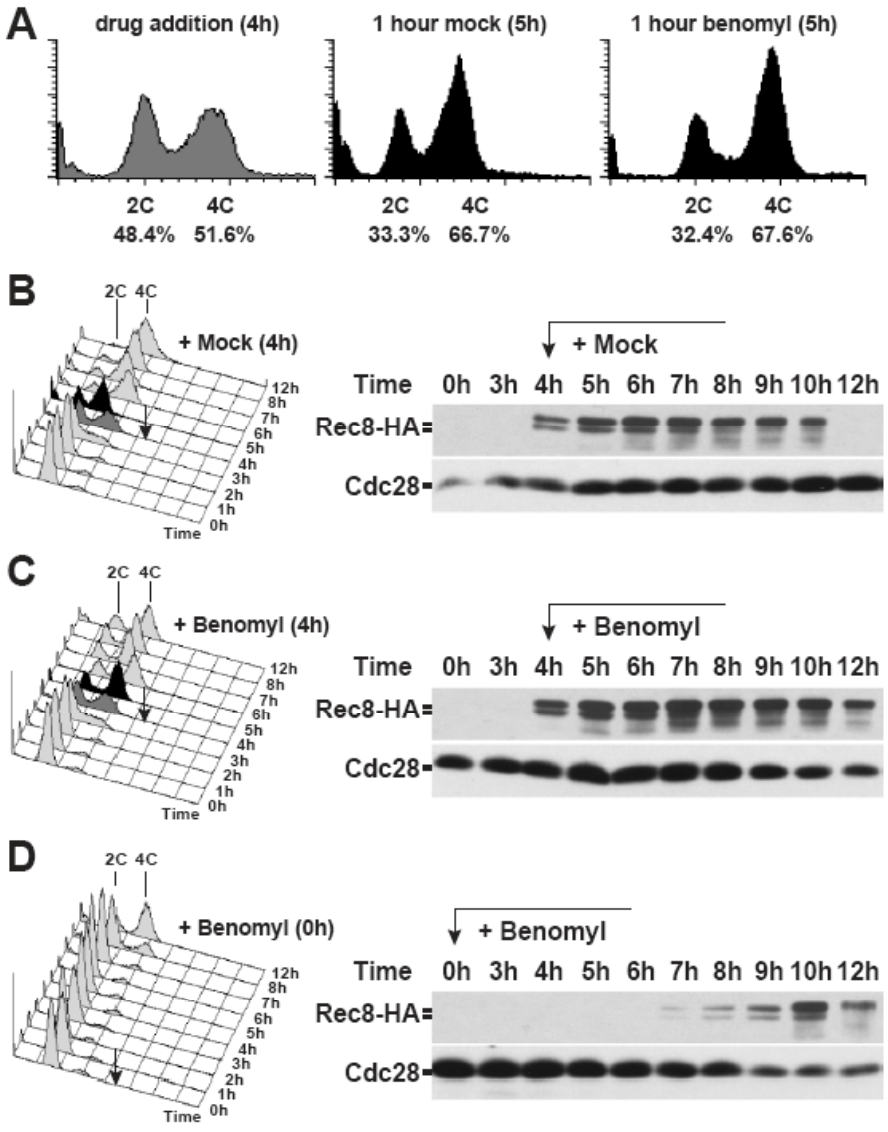

Figure 4: Benomyl treatment does not affect pre-meiotic S-phase but prevents entry into meiosis.

Wild-type cells carrying a *REC8-3HA* fusion (A1972). 4 hours after meiotic induction at room temperature (black arrow) cells were re-suspended in medium

containing 0.4% DMSO (A; Mock) or 120 μg/ml benomyl (B). Total cellular DNA content determined by flow cytometry (left panels). Dark grey cytometry profiles indicate time of drug addition (4h), black profiles are from one hour after drug addition (5h). Western blot samples were used to monitor Rec8 levels during the same time course. (C) Percentages of cells with 2C and 4C DNA content at 4h and 5h were determined by quantifying the area beneath the respective peaks ± 1/2 the respective inter-peak distance. (D) Cells were treated as in (B) except cells were re-suspended directly into sporulation medium containing 120 μg/ml benomyl at the start of the meiotic time course.

To test this possibility we analyzed the consequences on meiotic progression when benomyl was added to meiotic culture at the time of meiotic induction (Time = 0 hours). Analysis of this culture revealed that exposure to benomyl markedly delayed premeiotic DNA synthesis. This delay was due to a defect in entry into the meiotic program because accumulation of an early meiosis-specific protein, Rec8 (Klein et al., 1999), was significantly delayed (Figure 4D).

The failure of benomyl-treated to enter into the meiotic cell cycle was not due to a general toxicity of the drug but was mediated by the absence of microtubules. Cells harboring the benomyl-dependent allele of β-tubulin *tub2-150* form overly stable microtubules that need to be destabilized by supplementing the growth medium with at least 40 μg/ml benomyl (Machin et al., 1995; Thomas et al., 1985). Despite this need for benomyl, cells harboring the *tub2-150* allele are still sensitive to high doses of benomyl and grow very slowly in medium containing

120 μg/ml benomyl (data not shown). We reasoned, however, that this allele would allow us to shift the sensitivity curve compared wild-type cells making the response to benomyl less severe. As described below, cells carrying the *tub2-150* allele entered S-phase in the presence of the drug (Figure 5C). Thus, the arrest prior to pre-meiotic S phase is dependent on microtubule depolymerization. Our results indicate that benomyl causes two cell cycle arrests. It prevents entry into the first meiotic division if the drug is administered during or after premeiotic S phase, and entry into the meiotic cell cycle if benomyl is administered during G1.

High levels of benomyl interfere with gene expression during meiosis.

The observation that benomyl, added before meiotic induction, caused a significant delay in the production of the meiosis-specific protein, Rec8, raised the possibility that the drug interfered with transcription and/or translation of meiotic factors. To address this possibility, we analyzed RNA levels of the meiosis specific gene *IME2* (Guttmann-Raviv et al., 2002). Consistent with the delayed accumulation of Rec8 protein, we also observed delayed accumulation of *IME2* mRNA, when benomyl was added at the time of meiotic induction (Figure 5A). This result suggests that benomyl causes an arrest in G1 by interfering with the accumulation of these two and perhaps other early meiotic cell cycle regulators.

To determine whether the benomyl-induced G2/prophase arrest was accompanied by transcriptional down-regulation we analyzed the consequences of adding

benomyl four hours after transfer into meiosis-inducing conditions. mRNA levels of *IME2* and the cell cycle regulator *CLB5* dropped sharply upon benomyl addition (Figure 5B). Surprisingly, mRNA levels of two constitutively expressed genes, *CDC28* and *RPL3*, showed a similar response, while expression of the large ribosomal RNA and the small nuclear RNA *SNR6* remained unchanged upon benomyl addition (Figure 5B). Our results suggest that benomyl treatment during G1 or S phase/G2/prophase leads to changes in meiotic gene expression, with a number of important cell cycle genes being drastically down-regulated.

The down-regulation of meiotic transcripts depends at least in part on microtubule depolymerization.

To determine whether the decline in mRNA levels brought about by benomyl was due to the microtubule depolymerizing effect of the drug, we examined the consequences of benomyl treatment on *IME2* mRNA levels in *tub2-150* cells (Machin et al., 1995; Thomas et al., 1985). Cells carrying the *tub2-150* allele were grown in the presence of 50 μg/ml benomyl and upon transfer into meiosis-inducing medium were incubated with 120 μg/ml benomyl. *tub2-150* cells entered the meiotic cell cycle normally, and *IME2* mRNA accumulated to normal levels (Figure 5C). When benomyl was removed after 4 hours and cells were transferred into medium lacking the drug, *IME2* mRNA levels did not further increase indicating that transcription was fully induced in the presence of high levels of benomyl (Figure 5C). When benomyl was added again at a concentration of 120 μg/ml (during or after completion of DNA replication; Time = 4 hours), *IME2*

expression decreased somewhat though not nearly as dramatically as in wild-type cells (compare Figures 5B and C). These results suggest that the decline in *IME2* RNA levels (and presumably that of other transcripts) brought about by benomyl is at least in part due to the microtubule-depolymerizing effect of the drug.

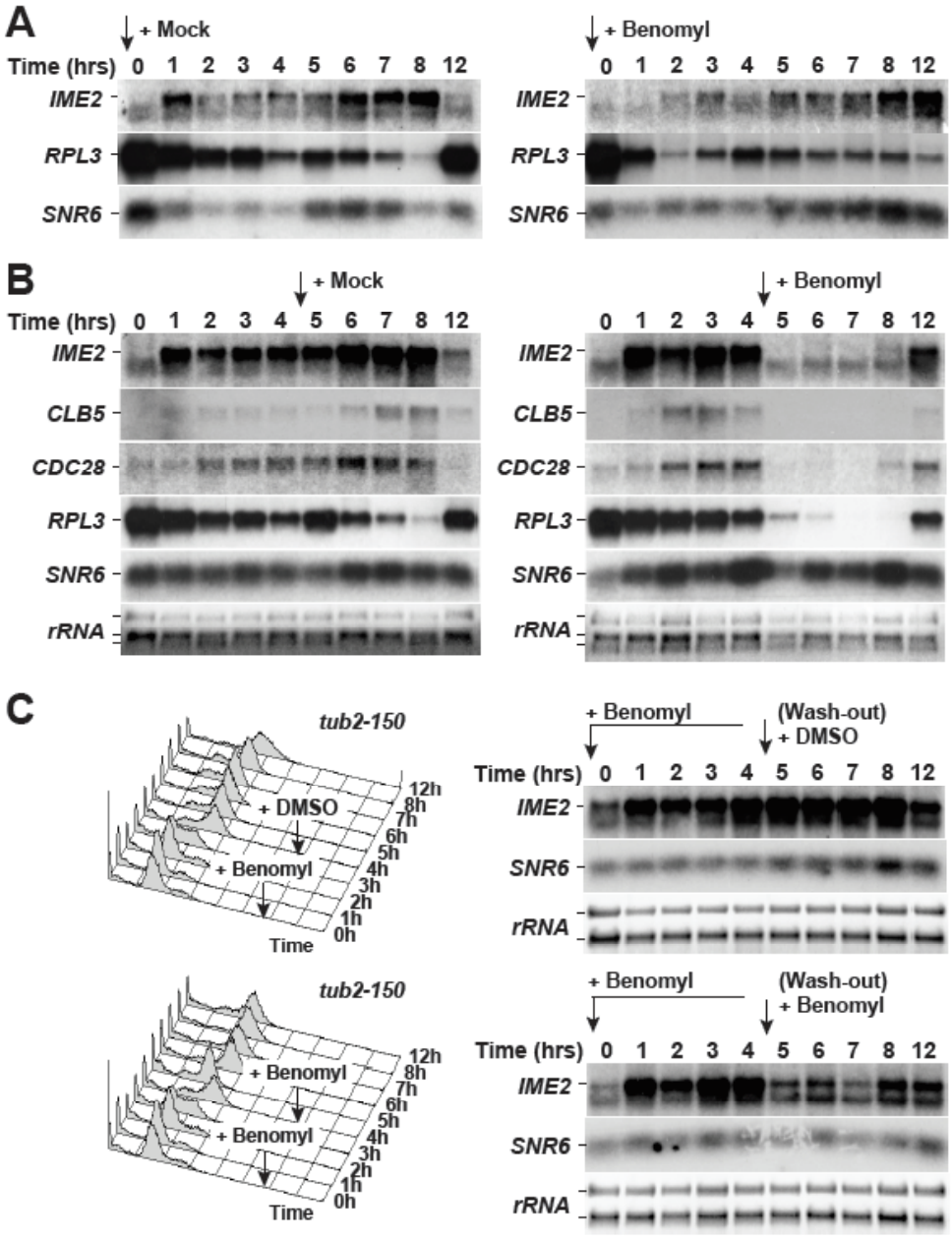

Figure 5: Benomyl treatment triggers changes in meiotic gene expression.
(A) Northern blot analysis of *IME2, RPL3* and *SNR6* RNA levels. Wild-type cells (A1972) induced to undergo meiosis at room temperature in medium containing 0.4% DMSO (Mock, left panels) or 120 μg/ml benomyl (right panels).
(B) The same strain as in (A) was allowed to progress through meiosis for 4 hours. After 4 hours (black arrow) cells were re-suspended in medium containing 0.4% DMSO (Mock, left panels) or 120 μg/ml benomyl (right panels). Northern blot analysis of *IME2, CLB5, CDC28, RPL3* and *SNR6* RNA levels. rRNA used as a loading control. rRNA levels were determined by staining with ethidium bromide, all other RNAs were detected by autoradiography.
(C) *tub2-150* cells (A5779) were pre-grown at 30°C in media containing 50 μg/ml benomyl and then sporulated at room temperature in medium containing 120 μg/ml benomyl. After four hours cells were re-suspended in fresh medium containing 0.4% DMSO (Mock, top panels) or 120 μg/ml benomyl (bottom panels). Total RNA samples were analyzed by Northern blotting. Mock and benomyl-treated samples were run in the same gel, but are separated in these figures for clarity. Total DNA content was analyzed by flow cytometry.

Benomyl causes a global change in meiotic gene expression.

A large number of genes, including loci that are also involved in mitotic growth, are differentially regulated as cell progress through meiosis (Schlecht and Primig, 2003). Previous work has identified at least seven broad meiotic expression profiles, six of which involve transient up-regulation of transcription during the process (Chu et al., 1998; Primig et al., 2000). To determine how general the effects of benomyl on gene expression were, we examined the effect of benomyl on meiotic gene expression at a genome-wide level as cells progressed through

meiosis in the presence or absence of benomyl. The experimental protocol is outlined in Figure 6A. Duplicate samples were harvested from cultures at several stages in pre-meiotic and meiotic development: after saturation in rich medium (YPD), after acetate starvation (YPA), and 4 hours after induction of meiosis (SP4). At this point cells were filtered and resuspended in normal SPO medium, or medium containing DMSO or benomyl. Duplicate samples were then harvested one hour later from the SPO culture (SF5), DMSO culture (SD5), or benomyl culture (SB5), and four hours later from the DMSO culture (SD8), or benomyl culture (SB8).

Total RNA samples from these time points were hybridized to Affimetrix S98 GeneChips covering approximately 6,400 yeast transcripts. 1189 transcripts displayed a strong variation of signals between different samples. These loci were grouped using a hierarchical clustering algorithm and sorted by signal strength during mitotic growth, such that genes strongly expressed in rich medium and pre-sporulation medium were preferentially placed on top of the cluster (Figure 6). To get an overview of the gene functions dominating the different subparts of the hierarchical clustering we used the *goCluster* tool to identify non-overlapping branches of the hierarchical tree that showed the strongest enrichment of genes annotated with a common GeneOntology (GO) term (Harris et al., 2004). The effects of benomyl were most obvious one hour after drug addition, comparing DMSO-treated (SD5) with benomyl treated (SB5) cells (Figure 6, Figure 7). A substantial number of genes was up-regulated by benomyl treatment in a highly

coordinated manner, notably genes encoding factors involved in drug response and transport (GO:0015893: drug transport; GO:0050896: response to stimulus), as well as subunits of the proteasome and genes required for ubiquitin-dependent proteolysis (GO:0006511: ubiquitin-dependent protein catabolism). Among the genes whose expression decreased in the presence of benomyl we identified a large number of genes involved in protein translation (GO:0006412: protein biosynthesis), and factors implicated in meiotic development (GO:0030437: sporulation [sensu fungi], GO:0030476: spore wall assembly [sensu fungi], GO:0030154: cell differentiation). This suggests that benomyl treatment up-regulates genes involved in protein turnover and the response to stress, while negatively affecting many factors involved in cell growth and the cell cycle. Analysis of the transcriptional effects of benomyl on mitosis also showed an upregulation of stress response genes and transporters, but, in contrast to the meiotic cell cycle, no transcripts were down-regulated (Sue Biggins, personal communication).

We then analyzed the transcriptional effects of benomyl on individual genes within those functional groups. In addition to causing an up-regulation of genes involved in protein turnover, the presence of benomyl led to a detoxification response. In particular, we observed increased expression of genes involved in transport (*SNQ2, YOR1*), multi-drug and chemical stress resistance (*CIN5, PDR5, PDR16, SNG1*) (Figure 7B) and stress response, including *FLR1*, a gene encoding a benomyl-inducible multi-drug resistance permease (see Figure 9).

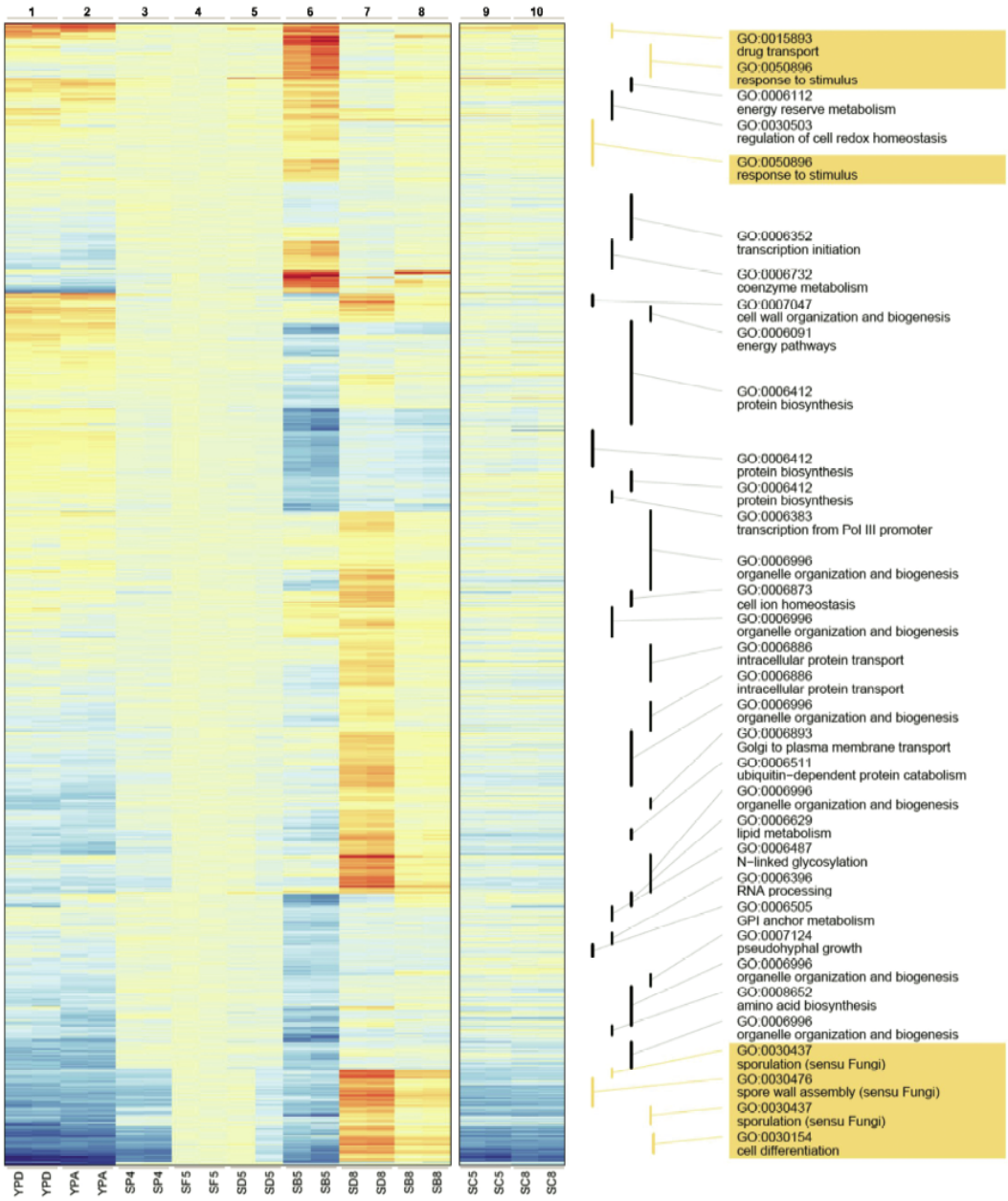

Figure 6: Expression profiling the meiotic transcriptome in the presence of benomyl.

1189 genes showing the strongest variation between the different samples were selected by those for which the ratio between the sample and duplicate standard deviations was greater than 5. These transcripts were subsequently grouped by hierarchical clustering. Genes strongly expressed in rich medium (YPD) and pre-

sporulation medium (YPA) were preferentially placed on top of the cluster. All data were normalized to the values obtained with sporulating and filtered cells that were considered the optimal reference sample (SF5). Each column represents a sample as shown at the bottom, each row is a transcript. Blue and red indicate low and high expression values, respectively, according to the scale given below the heatmap. The *goCluster* tool identified enriched GO terms from the category "Biological Process" in the branches of the clustering tree by comparing the frequency of occurrence among the transcripts comprising a branch and the total number of genes on the array, respectively. *goCluster* subsequently identified the GO categories with the strongest enrichment (lowest p-values) over all non-overlapping branches of the hierarchical tree. The region covered by each of these branches is marked at the right side of the figure in association with the corresponding GO term. Branches with less than 7 transcripts were omitted for readability. Duplicate samples were cultured in rich medium with glucose (YPD) or acetate (YPA), in sporulation medium for 4 hours (SP4). After 4 hours cells were transferred to new medium and cultured the following medium: SPO for 1 hour (SF5), SPO + DMSO for 1 and 4 hours (SD5, SD8), SPO + benomyl for 1 and 4 hours (SB5, SB8). Additional samples are from cells shifted to 10°C after four hours of sporulation and harvested 1 and 4 hours later (SC5, SC8).

At the same time, benomyl treatment caused a widespread down-regulation meiosis-specific genes and general cell cycle factors (Figure 7C). These include genes essential for meiotic transcriptional control (*IME2, IME4, NDT80*) and early meiotic functions, such as recombination (*EXO1*, *MSH4, MSH5, REC102, REC107, REC114, SAE3, SPO11*), SC formation (*HOP1, HOP2, MND1, MER1, RED1, ZIP1, ZIP2*), sister chromatid cohesion and chromosome segregation (*CSM2, IRR1/SCC3, REC8, SCC2, SGO1*), spindle pole body formation (*SPO1*), the recombination checkpoint (*PCH2, MEK1, DDC1*) and control of M-phase

(*CDC5, MND2, SPO13*). As a consequence of the decreased expression of early meiotic genes, factors involved in post-meiotic functions like spore wall formation and maturation (e.g. *ADY4, DON1, SMA1, SMK1, SPO74, SPR3, SPR6, SSP2*) were also expressed later and to a lower level. Among factors important for both mitotic and meiotic cell cycle progression we observed coordinated transcriptional down-regulation of the protein kinase *CDC28* and five of its six B-type cyclin regulatory subunits (*CLB1, CLB3, CLB4, CLB5, CLB6* – note that *CLB2* is not expressed during the meiotic cell cycle; Figures 7C and D) and components of the anaphase promoting complex/Cyclosome (APC/C; *APC4, APC5, APC11, CDC20, CDC23, CDC26*).

Microtubule depolymerization may affect the expression of this large group of genes by influencing the expression of a smaller set of transcription factors that regulate the former. To test this, we directly examined a number of transcription factors involved in stress response, cell cycle regulation and meiotic expression. Indeed, we found that mRNA levels of several transcription factors, *SWI4*, *NDD1* (G1 and G2/M specific induction), *ABF1* (general regulator of mitotic and meiotic genes) as well as *IME1*, *UME6* and *NDT80* (required for meiotic activation and re-repression) were down-regulated at the transcriptional level in the presence of benomyl (Figure 7E). This was not a general effect since other transcription factors involved in stress-response (Gcn4) and cell cycle control (Swi6, Mpb1, Mcm1, Fkh1) did not show a significant decrease of their expression levels (see the *S. cerevisiae* section of GermOnline at http://www.germonline.org). These

results suggest that the wide-spread transcriptional changes that occur after benomyl-induced microtubule destabilization could be correlated with the down-regulation of a set of transcription factors that regulate these genes.

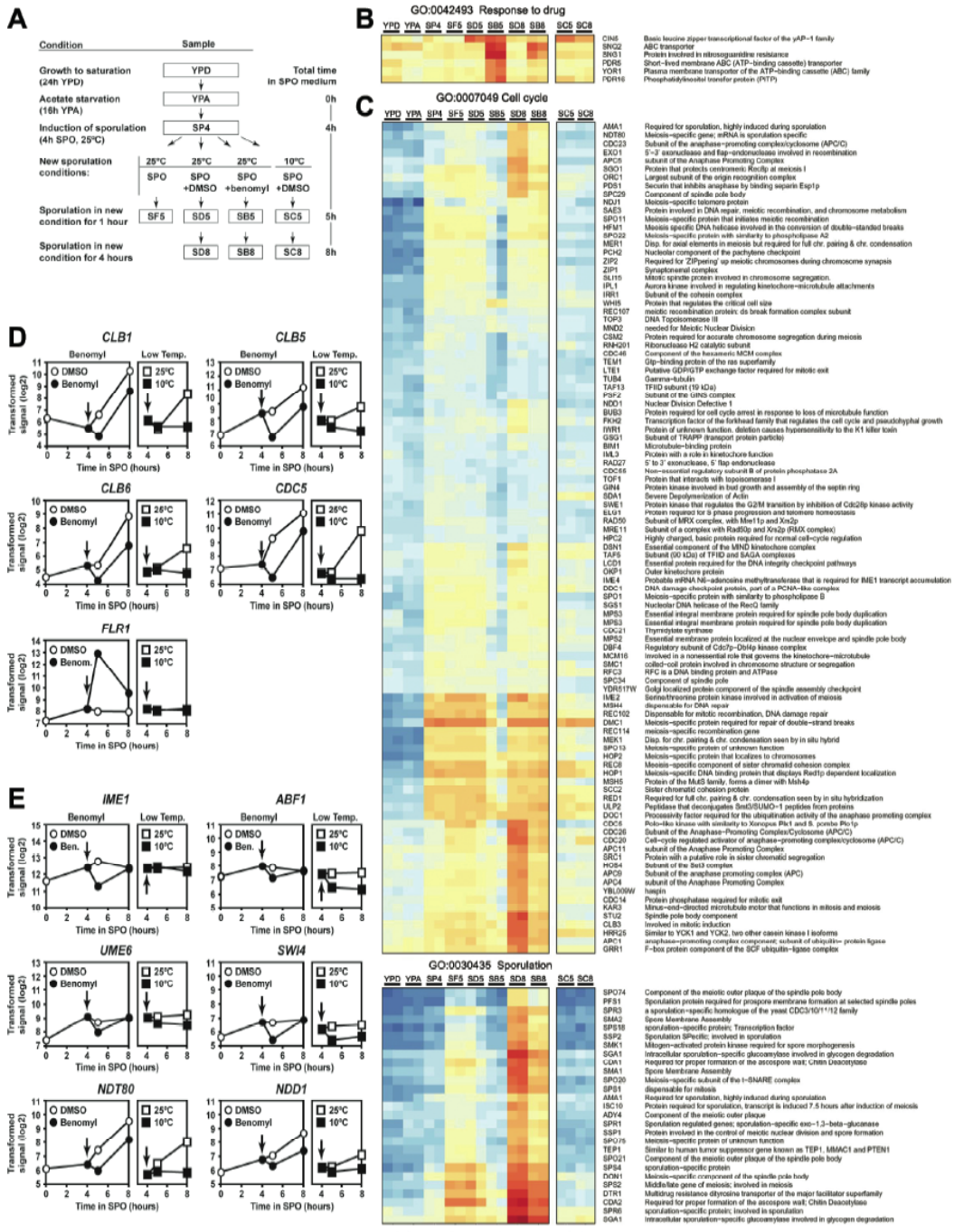

Figure 7: Benomyl treatment triggers a transcriptional response from genes involved in detoxification, cell cycle control and the meiotic program.

(A) Flow-chart of the experimental protocol. Boxes indicate the samples analyzed in Figure 6 and 7. Box labels correspond to column labels in B, C. Flow chart also indicates the total time cultures spent in sporulation (SPO) medium. The time spent in SPO medium corresponds to the x axis of the graphs in D and E.

(B, C) Loci involved in drug response and mitotic or meiotic cell cycles as well as sporulation. The complete dataset was analyzed using *goCluster* and split into 100 subgroups with similar expression using partitioning around medoids (Kaufman and Rousseeuw, 1987). The GO categories associated with the genes in each of the resulting groups were analyzed for statistically significant over-representation by comparing the frequencies of occurrence of the same GO term within the group and all transcripts represented on the microarray, respectively. The hypergeometric distribution was employed to determine the resulting p-values (Hosack et al., 2003). To correct for the multiple testing procedure we used data from 100 randomized datasets to determine a p-value cutoff that would result in a false discovery rate of 10%. A total of 586 different GO terms were identified as being enriched in one or several of the 100 clusters. Blue and red indicate low and high expression values, respectively.

(D, E) Expression patterns of manually selected genes involved in drug response, cell cycle control and the regulation of mitotic and meiotic gene expression. Signals are given as log2 transformed values on the y-axis, and are graphed versus the total time the cultures spent in SPO medium. Black arrows indicate the time of drug treatment or shift to low temperature. Note that low abundance mRNAs that are also cell cycle regulated (e.g. *SWI4*) are often not or only barely detectable in non-synchronized heterogeneously growing cell populations.

Cold shock causes similar effects as benomyl.

Low temperatures are also known to destabilize microtubules (Gupta et al., 2001). We furthermore observed that meiotic cells exposed to temperatures below 12°C arrested as mononucleate cells with unseparated spindle poles (Figure 8).

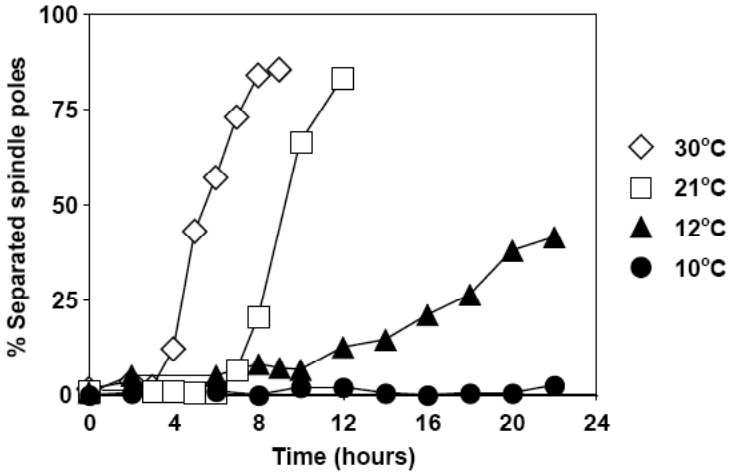

Figure 8: Temperature effect on meiotic progression.
Wild-type cells (A3973) were cultured in SPO medium at room temperature (21°C) for two hours before cells were shifted to the respective temperatures (10°C, 12°C, 21°C, and 30°C). Spindle morphology was determined at the indicated time points by immunofluorescence.

To determine whether cold temperatures affect transcription in a manner similar to that of benomyl, we analyzed the transcriptional changes in sporulating cultures that, instead of being exposed to benomyl, were shifted to low temperature (10°C) for 1 hour (SC5) and 4 hours (SC8). Low temperature stress did not lead to induction of genes involved in detoxification and drug transport but did cause,

among other effects, a general shut-down of the meiotic transcriptional program very similar to that observed in benomyl treated cells (Figure 7B, C). One notable exception is the transcription factor *IME1*, whose expression while down-regulated by benomyl does not appear to be markedly affected by low-temperature stress (Figure 7E). Taken together, these results suggest that a wide array of genes involved in cell proliferation and meiotic progression, including the three major early meiotic kinases *IME2*, *CDC28*, and *CDC5*, are down-regulated to halt cell cycle progression in the presence of damaged microtubules.

The down-regulation of meiotic genes by low temperatures or benomyl treatment was not caused by a general meiotic stress response pathway, because cells lacking the major stress kinase *HOG1* still arrested in G2/prophase after benomyl treatment (data not shown), and exposure of meiotic cells to hypertonic stress (0.4 M potassium chloride) did not elicit a change in gene expression comparable to benomyl or cold treatment. mRNA levels of *IME2, HOP1* and *RPL3* declined after treatment with benomyl or exposure to cold but remained at normal levels after exposure to hypertonic stress (Figure 9). Furthermore, cell cycle arrest occurred in either G1 or G2/prophase after exposure to benomyl or low temperature, but not if cells were exposed to hypertonic stress (Figure 9, top row). On the other hand, expression analysis suggested that the detoxification response was benomyl-specific. We confirmed this by analyzing expression of the multi-drug resistance permease, *FLR1*, by Northern blot analysis (Figure 9). Thus, microtubule perturbation (chemically or by low temperature) but not other stress

causes a specific transcriptional response that leads to meiotic cell cycle arrest in G1 or G2/prophase.

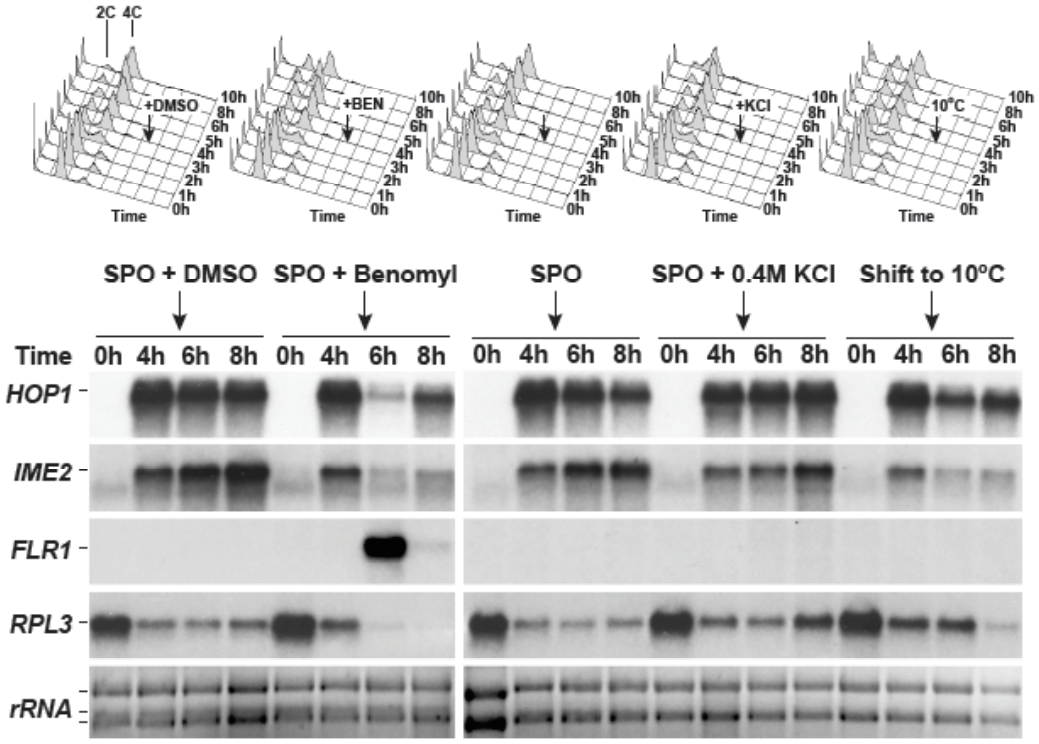

Figure 9: Benomyl-induced changes in meiotic gene expression are not a general stress response but resemble the response to low temperatures.
Wild-type cells (A1972). 4 hours after induction of meiosis at room temperature (black arrows) cells were either cultured at room temperature in SPO medium containing various additives: 0.4% DMSO; 0.4% DMSO + 120 □g/ml benomyl; no additive; 0.4M potassium chloride; or cultured at 10°C. Cellular DNA content was determined by flow cytometry (top panels). Total RNA samples were analyzed by Northern blotting (bottom panels). All samples were run in the same gel, but are separated in this figure for clarity.

The G2/prophase arrest caused by benomyl treatment is independent of known meiotic checkpoints.

Several checkpoints have been characterized to date that cause a cell cycle arrest prior to meiotic chromosome segregation. We therefore determined whether any of the known meiotic checkpoints were responsible for the G2/prophase arrest brought about by benomyl treatment. Since microtubule destabilization appeared to be the cause for the arrest, we first tested whether the G2/prophase arrest we observed was dependent on the spindle assembly checkpoint. Deletion of the spindle assembly checkpoint component *MAD2* did not bypass the G2/prophase arrest. *mad2Δ* cells treated with benomyl exhibited the same delay in Clb3 accumulation as wild-type cells (Figure 10A). Thus, the G2/prophase arrest caused by benomyl treatment is not due to activation of the spindle checkpoint.

The recombination checkpoint (pachytene checkpoint) arrests cells in G2/prophase by the down-regulating Clb-CDK activity and by inhibiting the transcription factor Ndt80 responsible for the transcriptional activation of genes necessary for entry into meiosis I and spore formation (Roeder and Bailis, 2000). Clb-CDK activity is kept low by at least two mechanisms. The protein kinase Swe1 phosphorylates Cdc28 on tyrosine 19, thereby inhibiting its activity (Leu and Roeder, 1999). At the same time *CLB* cyclin transcription is repressed through the inhibition of Ndt80 (Tung et al., 2000). To determine whether the recombination checkpoint is required for the G2/prophase arrest caused by benomyl treatment we analyzed the response of *mek1Δ* and *swe1Δ* mutants to

benomyl. Mek1 is the meiotic homolog of the mitotic DNA-damage checkpoint kinase Rad53 and a central player in the recombination checkpoint (Bailis and Roeder, 2000; Xu et al., 1997). Cells lacking *MEK1* arrested with low levels of Clb3 protein after exposure to benomyl (Figure 10B). Similar results were obtained when Swe1 was inactivated (Figure 10A). We furthermore excluded the possibility that the recombination checkpoint and the spindle assembly checkpoint act together to cause the arrest by examining the response of *mek1Δ mad2Δ* double mutants to benomyl treatment (Figure 10C). Our results indicate that inactivation of both the recombination checkpoint and the spindle checkpoint does not allow benomyl-treated cells to enter meiosis I.

Finally, we tested whether the presence of unprocessed meiotic double-strand breaks contributed to the G2/prophase arrest caused by benomyl treatment. Cells lacking *RAD50* do not form meiotic double strand breaks (Cao et al., 1990), yet they still arrested in G2/prophase with low levels of Clb3 protein upon exposure to benomyl (Figure 10B). We conclude that the benomyl-triggered G2/prophase arrest is independent of double strand break formation and is not due to activation of either the recombination or the spindle checkpoint.

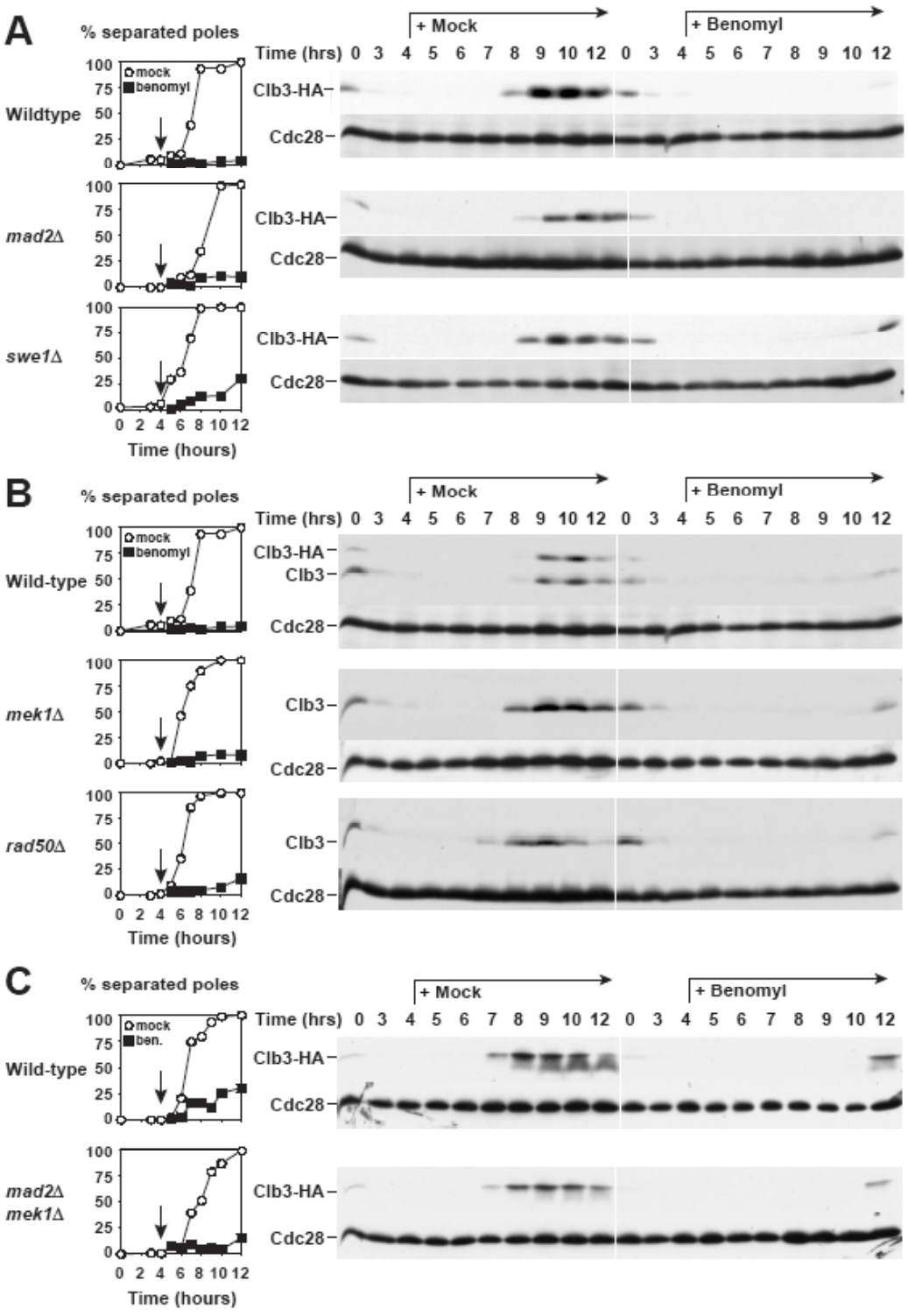

Figure 10: The benomyl-induced G2/prophase arrest does not require the spindle checkpoint or the recombination checkpoint.

Wild-type (4563), *mad2Δ* (A4843), *swe1Δ* (A4704), *mek1Δ* (A4838), *rad50Δ* (A1771), and *mad2Δ mek1Δ* (A12927). 4 hours after induction of meiosis at room temperature (black arrow) cells were re-suspended in medium containing 0.4% DMSO (Mock; white circles and left panels) or 120 μg/ml benomyl (black squares and right panels). Graphs on the left indicate percentage of cells containing more than one focus of tubulin staining. Western blot analysis was used to monitor Clb3 and Cdc28 (loading control) levels during the same time course. (A, C) A4563, A4843, A4704, A12927 carry a *CLB3-3HA* fusion. Polyclonal α-Clb3 antibody was used to determine Clb3 levels in (B).

Discussion

Our analyses reveal a profound effect of microtubule destabilization on meiotic progression in budding yeast. In the presence of the microtubule depolymerizing drug benomyl, cells fail to enter the meiotic program and arrest in G1. When benomyl is added during or after pre-meiotic S-phase, it causes cells to arrest in G2/prophase with low levels of Clb-CDK activity and incompletely paired chromosomes. Upon microtubule depolymerization, cells respond with a complex change in the pattern of meiotic gene expression that affects both meiosis-specific genes as well as loci important for both mitotic and meiotic cell cycle progression. In particular, treatment with benomyl or low-temperature stress causes a shut-down of the meiotic transcriptional cascade. Our data further indicate that this transcriptional change is not a general stress response but specific to perturbation of the microtubule cytoskeleton and is likely to be responsible for the cell cycle and developmental arrest. Finally, our results show that the effects of benomyl on meiotic cell cycle progression are not mediated by any known checkpoint pathways pointing to the existence of a novel mechanism responsible for monitoring microtubule integrity and responding to perturbations.

A novel response to microtubule perturbation.

It has been reported that treatment of cells with only 60 □g/ml benomyl causes a delay in metaphase I (Shonn et al., 2000). However, at this concentration, cells were still able to segregate their chromosomes indicating that a transient meiotic spindle could still form (Shonn et al., 2000). When benomyl is added at a

concentration of 120 μg/ml, microtubules completely depolymerize. We find that treatment of meiotic cells with such a high dose of benomyl causes a G1 arrest when it is added during induction of meiosis, or a G2/prophase arrest when cells are treated during S-phase or G2/prophase. Furthermore, the G2/prophase arrest is accompanied by a dramatic drop in mRNA levels of meiosis-specific genes and meiotically expressed cell cycle regulators. A key question is whether these events are the result of a general stress response or of a specific response mediated by microtubule perturbations. Several lines of evidence indicate that the latter is the case. First, benomyl is a well-characterized microtubule depolymerizing agent and the concentration of benomyl used in this study elicits the characteristic metaphase arrest during the mitotic divisions. Second, at least the G2/prophase arrest caused by benomyl is fully reversible. When the drug is removed, cells progress through meiosis and form viable spores with normal efficiency. Third, cells carrying the *tub2-150* allele, in which microtubules are stabilized and less vulnerable to microtubule depolymerizing agents (Machin et al., 1995; Thomas et al., 1985) are able to enter meiosis and progress through pre-meiotic S phase in the presence of 120μg/ml benomyl, indicating that the G1 arrest caused by benomyl is mediated by the drug's microtubule depolymerizing function. The transcriptional response observed when benomyl is added 4 hours after induction of meiosis is also at least in part mediated by benomyl-induced microtubule perturbations. In *tub2-150* cells renewed exposure to benomyl four hours after transfer into meiosis-inducing conditions did cause a drop in RNA levels, but this drop was significantly less dramatic than that observed when wild-

type cells are treated in this way. Given that *tub2-150* microtubules still respond to benomyl, some microtubule de-polymerization is likely to occur when the cell are re-exposed to the drug, which may explain the partial drop in RNA levels that is observed.

A fourth line of evidence indicating that the cell cycle response to benomyl is not a general stress response is that hypertonic stress, a condition not known to affect microtubule structures, fails to cause a cell cycle arrest in G2/prophase or to down-regulate mRNAs. In contrast, low temperature (Gupta et al., 2001) affects cell cycle progression and genome-wide transcript levels in a manner qualitatively very similar to that of benomyl. We were not able to examine the effects of other fungal microtubule drugs such as thiabendazole, MBC/carbendazim or nocodazole on meiotic cell cycle progression because these drugs are not sufficiently soluble in sporulation medium. However, it is interesting to note that microtubule de-polymerizing drugs such as colchicine and vinblastine that are structurally quite different from benomyl (Downing, 2000) perturb meiotic prophase in other organisms, such as mice, ciliates and plants (Allen et al., 1988; Kaczanowski et al., 1985; Loidl, 1988; Shepard et al., 1974; Zickler and Kleckner, 1998). Taken together, these findings indicate that the response of meiotic cells to high levels of benomyl or low temperatures is specific and due to microtubule perturbations.

Benomyl treatment causes as cell cycle arrest in G1 and G2/prophase.

The response of cells to benomyl during entry into the meiotic cell cycle (G1) and the response during S phase/G2/prophase appear similar. The G2/prophase arrest is accompanied by changes in expression levels of several hundred genes. Likewise, transcripts that are affected by benomyl during S phase/G2/prophase, such as *IME2* and *RPL3* are also down-regulated by benomyl treatment during G1. We do not know how benomyl causes a cell cycle arrest in G1 or G2/prophase but our data indicate that the arrest is a consequence of the fact that it causes microtubules to depolymerize. Cells carrying the *tub2-150* allele enter and progress through pre-meiotic S phase efficiently even when benomyl is added to the medium. In this context it is interesting to note that disruption of microtubule dynamics by inactivating the microtubule motor *KAR3* or its associated factor *CIK1* also causes defects in meiotic entry and a meiotic cell cycle arrest in G2/prophase (Bascom-Slack and Dawson, 1997; Shanks et al., 2004; Shanks et al., 2001). It is possible that a similar transcriptional response underlies the meiotic defects of these mutants.

The nature of the cell cycle arrest is also unclear. The G2/prophase arrest elicited by benomyl is neither triggered by the activation of the spindle assembly nor the recombination checkpoint, nor their combined activation. The arrest is also independent of the stress kinase Hog1, since *hog1Δ* cells still arrest in G2/prophase after benomyl treatment. This suggests that a novel, as yet

uncharacterized pathway mediates cell cycle arrest in G2/prophase in response to microtubule perturbations caused by benomyl and low temperature stress. We do not know much about this response, the factors involved, or the nature of the signal triggering it - possible candidates for signals would be unattached kinetochores or the level of free tubulin dimers. It is clear, however, that one of the consequences of this response is a dramatic change in gene expression. How are these changes in transcription mediated? Obvious targets of the response mechanism to microtubule depolymerization would be transcription factors involved in stress response or mitotic and meiotic gene expression. Meiosis is controlled by a complex transcriptional cascade (Kassir et al., 2003). Induction of early meiotic genes is necessary for the correct expression of the subsequent middle and mid-late meiotic genes. Thus, observed delays in induction of later meiotic genes are likely a consequence of a failure early in the expression cascade. However, the transcriptional decrease also coordinately affects general cell cycle factors including most components of the APC/C and other genes involved in chromosome segregation and cell cycle progression. We therefore favor the idea that the expression and/or activity of a number of mitotic and meiotic transcriptional regulators might be coordinately decreased in response to microtubule depolymerization. Indeed the meiotic transcription factors *IME1, UME6, ABF1*, and *NDT80*, as well as the cell cycle regulators *SWI4* and *NDD1* were expressed to lower levels in response to benomyl, while their transcriptional levels were re-established when cells began to escape from the arrest. Note that the *IME1* promoter does not appear to respond to temperature stress during early

meiosis (see Figure 9D) so the inactivation of the meiotic cascade by cold-shock may involve, if Ime1 is affected at all, a post-translational mechanism. A possible explanation for this orchestrated response of transcription factors to microtubule instability could be auto- and cross-regulation of the factors, which renders them inter-dependent. It has been suggested that Abf1 and Ndt80 are involved in their own regulation (Hepworth et al., 1998; Kassir et al., 2003; Miyake et al., 2004). Moreover, Ume6 is required for correct *IME1* and *NDT80* expression (Williams et al., 2002). Finally, Abf1 may be required for normal *IME1* and *UME6* expression (Prinz et al., 1995). Irrespective of the mechanism eliciting this response, it is clear that that the cell cycle arrest caused by benomyl is a consequence of the transcriptional response, because mRNA levels of factors essential for entry into meiosis I, such as B-type cyclins and the CDK Cdc28 are down-regulated.

Why does microtubule perturbation cause a G1 or G2/prophase arrest during the meiotic cell cycle?

In mitotically dividing cells, the sole arrest elicited by microtubule de-polymerization is a metaphase arrest (Jacobs et al., 1988) with no known effects on gene expression (Sue Biggins, personal communication). In cells undergoing the meiotic cell cycle, complete microtubule depolymerization causes cells to arrest in G1 or G2/prophase. Why is the response to benomyl different between these two types of cell cycles? The decision not to enter the meiotic cell cycle when microtubules become unstable may be related to the fact that the meiotic cell cycle occurs under conditions where nutrients are limited. Delays in cell cycle

progression caused by perturbations of the microtubule cytoskeleton or any other stresses could thus lead to cell death. Microtubule-depolymerization and other stress sensing mechanisms may therefore be in place during G1 that prevent entry into the cell cycle when conditions are not favorable for completion of this cell cycle. In this regard it is interesting to note that treatment of cells with the DNA replication inhibitor hydroxyurea also inhibits entry into the meiotic cell cycle (Davis et al., 2001).

The reason why meiotic cells whose microtubules have disassembled arrest in G2/prophase rather than metaphase I may lie in the way in which the meiotic cell cycle is organized. Once cells have entered meiosis I, cell cycle events are no longer coupled with the developmental program. For example, the monopolin complex, a kinetochore-bound protein complex that promotes the attachment of sister chromatids to microtubules emanating from the same spindle pole (Rabitsch et al., 2003), dissociates from kinetochores in a manner uncoordinated with other cell cycle events. When cells are arrested in metaphase I through the inactivation of the APC/C activator Cdc20, the developmental program continues. Cdc20-depleted cells initiate spore formation despite arresting with metaphase I spindles (Lee and Amon, 2003). Furthermore, the monopolin complex dissociates from kinetochores at the time when it dissociates from wild-type cells despite being arrested in metaphase I (Lee and Amon, 2003). Thus, if microtubule depolymerization were to cause cell cycle arrest in metaphase, as it does during the mitotic divisions, the results for meiotic development would be disastrous.

During the metaphase I arrest, the monopolin complex would dissociate from kinetochores and upon repolymerization of microtubules, attachment of microtubules to sister kinetochores in a co-oriented manner would not occur and hence meiosis I segregation would fail. In contrast, halting of cells in a cell cycle stage in which the cell cycle is still coordinated with the developmental program would allow cells to resume the meiotic cell cycle upon repolymerization of microtubules, and therefore permit the successful completion of the meiotic cell cycle. This is achieved by the arrest in G2/prophase, prior to the commitment to meiosis I. At this stage, both the developmental program and the cell cycle program still rely on the same transcription factor, Ndt80, for their initiation.

Transcriptional changes in response to microtubule depolymerization are not a phenomenon restricted to budding yeast. Changes in RNA levels upon colchicine treatment have been observed in a variety of tissue culture cells (Cleveland et al., 1981) and a microtubule-associated transcription factor that activates transcription following microtubule instability has been reported (Ziegelbauer et al., 2001). Furthermore, like we observed in budding yeast, several widely divergent organisms including mouse, *Allium*, and lily, exhibit a meiotic prophase arrest or delay upon exposure to microtubule poisons (Loidl, 1988; Shepard et al., 1974; Tepperberg et al., 1997). Indeed, the existence of a prophase "colchicine checkpoint" in mouse spermatocytes has been suggested previously (Tepperberg et al., 1997; Tepperberg et al., 1999). Interestingly, mammalian tissue culture cells show an arrest response to the microtubule drugs colchicine and nocodazole

even in mitotic prophase (Mikhailov and Rieder, 2002; Rieder and Cole, 2000). It will be interesting to determine whether these arrests are caused by a transcriptional response similar to that observed in budding yeast.

Acknowledgements

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Materials and Methods

Strains

Strains used are described in Table 1 and are derivatives of SK1 (Kane and Roth, 1974). *CLB3-3HA* and *mad2Δ::KanMX6* were constructed by a one step gene replacement method (Longtine et al., 1998). *mek1::KanMX4* and *swe1::KanMX4* were PCR-amplified from the corresponding knock-out strains in the *Saccharomyces* deletion collection (Giaever et al., 2002) and introduced into SK1. *REC8-3HA* and *URA3::TetO* were described in (Klein et al., 1999) and *LEU2::pURA3-TetR-GFP::TetO::HIS3* was described in (Marston et al., 2004). *rad50::URA3* was described in (Cao et al., 1990).

Table 1: Strains.

Strain	Relevant Genotype
A727	*MATa/α, ho::LYS2/ho::LYS2, lys2/lys2, his4X/his4X, leu2::hisG/leu2::hisG, ura3/ura3*
A1771	*MATa/α, rad50::URA3/rad50::URA3*
A1972	*MATa/α, REC8-3HA::URA3/REC8-3HA::URA3*
A4563	*MATa/α, CLB3-3HA::KanMX6/+*
A4704	*MATa/α, swe1Δ::KanMX4/swe1Δ::KanMX4, CLB3-3HA:KanMX6/+*
A4838	*MATa/α, mek1Δ::KanMX4/mek1Δ::KanMX4*
A4843	*MATa/α, mad2::KanMX/mad2::KanMX, CLB3-3HA:KanMX6/+*
A4967	*MATa/α, URA3::TetOx224 (Chr.V)/+ LEU2::pURA3-TetR-GFP/LEU2::pURA3-TetR-GFP::TetO::HIS3 (Chr.III)*
A5009	*MATa/α, URA3::TetOx224 (Chr.V) /URA3::TetOx224 (Chr.V), LEU2::pURA3::TetR-GFP/LEU2::pURA3::TetR-GFP*
A5779	*MATa/α, tub2-150/tub2-150, CLB3-3HA:KanMX6/+*
A12927	*MATa/α, mad2::KanMX/mad2::KanMX, mek1Δ::KanMX4/mek1Δ::KanMX4, CLB3-3HA:KanMX6/+*

Sporulation conditions

Cells were grown to saturation in YPD (YEP + 2% glucose) for 24 hours, diluted into YPA (YEP + 2% potassium acetate) at OD_{600}= 0.3 and grown over night (16 hours) at 30°C. Cells were washed with sterilized water the next day and resuspended in SPO medium (0.3% potassium acetate, pH = 7.0) at OD_{600}= 1.9 to induce sporulation. Cells were sporulated at 25°C or 10°C as indicated. Sporulation medium containing benomyl was always prepared freshly on the day of the experiment following the directions in (Shonn et al., 2000). Briefly, DMSO (dimethyl sulfoxide, Sigma-Aldrich) or benomyl (Methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate, Sigma-Aldrich; 30 mg/ml stock in DMSO) was dissolved in near-boiling SPO medium to avoid precipitation of benomyl. The medium was then allowed to slowly cool to room temperature. At the time of drug treatment, cells were filtered and immediately re-suspended in the medium containing benomyl or DMSO.

Immunofluorescence and Meiotic Spreads

Unless noted otherwise, 200 cells were scored for each time-point. Indirect immunofluorescence on whole cells was carried out as described in (Visintin et al., 1999). Rat α-tubulin antibody (Oxford Biotechnology) and FITC-conjugated α-rat antibody (Jackson ImmunoResearch) were diluted at 1:200 and 1:100, respectively. Meiotic spreads were performed as described by (Nairz and Klein, 1997). α-Zip1 antibody (kindly provided by N. Kleckner) and FITC-conjugated α-rabbit antibody (Jackson ImmunoResearch) were diluted at 1:150 and 1:50,

respectively. Immunofluorescence samples were analyzed using a Zeiss Axioplan 2 microscope.

Immunoprecipitation and Kinase Assays

H1 kinase assays were performed as described previously (Amon et al., 1993). Briefly, Clb3-3HA protein was immunoprecipitated from 50 μl crude extract (250 μg total protein) using α-HA antibody (Babco) and IgG Sepharose (Amersham Pharmacia). Sepharose beads were washed extensively with NP40 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40) and 25 mM MOPS (pH 7.0). Beads were pre-incubated with 6 μl buffer HBII (25 mM MOPS, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, supplemented with protease and phosphatase inhibitors) for 15 min, before adding 10 μl kinase reaction mix (25 mM MOPS, 2 mg/ml Histone H1, 0.2 mM ATP) containing 50 nCi [γ-^{32}P]ATP. Kinase reactions were allowed to proceed for 15 min at 25°C before they were stopped by addition of 10 μl 3X SDS loading buffer. Samples were separated on a 15% SDS acrylamide gel, fixed in 10% methanol/10% acetic acid, dried and analyzed by autoradiography.

Northern blot analysis

Northern blot analysis was performed as described in (Amon et al., 1993). Total RNA was purified by phenol extraction and ethanol precipitation. 10 μg RNA was loaded per lane and separated on 1.1% agarose gel containing 6% formaldehyde and 40 mM MOPS (pH = 7.0). Gels were blotted in 10X SSC (1.5M NaCl, 0.15M sodium citrate, pH = 7.0) onto Hybond-XL membranes (Amersham Biosciences).

Blots were probed over-night with randomly ^{32}P-labeled DNA probes typically spanning ~ 1 kb of the respective open reading frame.

cRNA target synthesis and microarray hybridization

Frozen yeast cell pellets stored at -80°C were quickly thawed and processed using the hot-phenol method (Kohrer and Domdey, 1991). Approximately 190 µg of total RNA was isolated from 0.7 x 10^8 cells. Subsequently, 80 µg of total RNA was mixed with 350 µl RLT buffer and 250 µl of 70 % ethanol. The mix was loaded onto an RNeasy Mini-Spin column (Qiagen) and RNA was eluted in 50 µl of double-distilled water. Total RNA quality was monitored by loading approximately 200 ng onto an RNA Nano 6000 Chip processed with the 2100 Bioanalyzer (Agilent) (Figure 11, top). Biotin labeling of RNA was performed as described in the Expression Analysis Technical Manual (Affymetrix) with minor modifications as previously published (Schlecht et al., 2004). Approximately 80 µg of labeled cRNA from each reaction was purified using RNeasy Mini-Spin columns and roughly 300 ng was analyzed on RNA Nano 6000 Chips (Figure 11, bottom). 220 µl of the hybridization mix containing target cRNA at a final concentration of 0.05 µg/µl was transferred into S98 GeneChips (Affymetrix) and incubated at 45°C on a rotator in a Hybridization Oven 640 (Affymetrix) for 16 hours at 60 rpm. The arrays were washed, stained and scanned as published (Schlecht et al., 2004).

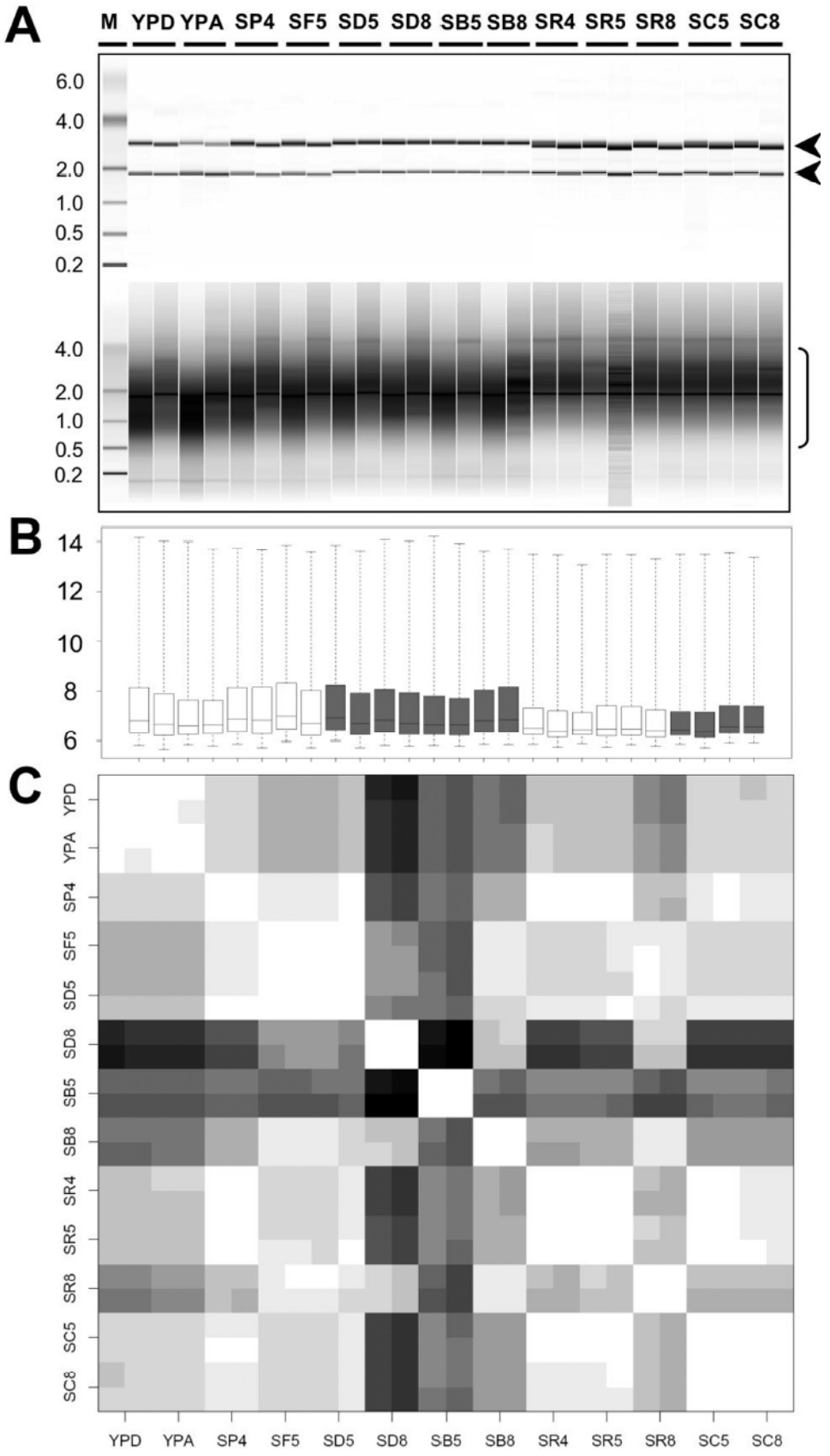
A
M YPD YPA SP4 SF5 SD5 SD8 SB5 SB8 SR4 SR5 SR8 SC5 SC8
6.0
4.0
2.0
1.0
0.5
0.2
4.0
2.0
1.0
0.5
0.2
B
14
12
10
8
6
C
YPD
YPA
SP4
SF5
SD5
SD8
SB5
SB8
SR4
SR5
SR8
SC5
SC8

Figure 11: RNA and data quality control.

(A) Sample replicates as shown on top were analyzed as published (51) using RNA Nano 6000 Chips and the 2100 Bioanalyzer and virtual gels were reconstituted using software provided by the manufacturer (Agilent). 18S and 28S ribosomal RNA bands (top) and cRNAs (bottom) are indicated by arrowheads and a bracket, respectively, and the sizes of RNA molecular weight markers are given in kbp. M indicates the marker lane. The lane showing sample 2 of SR5 was manually enhanced to compensate for a running artifact. (B) The box plot displays the distribution of expression values after normalization. The central dividing line of each box indicates the median of all measurements while the boxes above and below the line mark the central quartiles of the distribution. The signal intensities are given as log2 transformed values. (C) A grey scale representation of Pearson correlation coefficients for each possible pair of arrays is shown in a distance matrix. Black and white indicates low and high correlation between the expression values of two filtered data sets, respectively. The white squares on the central diagonal show that expression signals from replicate samples are highly reproducible.

DAT (image), CEL (feature or oligonucleotide probe level) and TXT (gene or probe set level) files of the GeneChips were generated using an Agilent GeneArray scanner (low intensity settings) and Microarray Analysis Suite 5.0 (Affymetrix).

Microarray data analysis

Raw data at the feature (oligonucleotide probe) level (CEL files) computed using the algorithm implemented in MAS 5.0 (Affymetrix) were imported into software

packages available via the BioConductor project (http://www.bioconductor.org/). Data from CEL files were normalized, analyzed and clustered using the R language for statistical computing (http://www.r-project.org) and the variance stabilization (VSN) algorithm (Huber et al., 2002) as implemented in the BioConductor package (http://bioconductor.org) following basic procedures as previously described (Schlecht et al., 2004). The Perfect Match values for all probes of a probe set were averaged to yield a single expression value for each gene represented on the array by employing the *median polish* algorithm as published (Irizarry et al., 2003). Oligonucleotide probes yielding questionable signals were marked during the image analysis and the features (probes) at the 3'-most position of each probe set were disregarded during signal computation to decrease the effect of signal artifacts and RNA degradation on data quality (since cRNA synthesis proceeds in a 5' to 3' direction). A detailed documentation of all processing and analysis steps (Leisch, 2002) is provided as supplemental material on our web portal at http://www.bioz.unibas.ch/primig/benomyl/ that provides access to web-specific figures, hyper-linked tables, supporting information and raw data files. The file provides a code in the R-programming language combined with comments in LATEX format. It can be downloaded together with the raw data as a single R package. Within R the code can be extracted or a PDF document providing an overview of the analysis can be generated. A graphical display of the expression data for each locus is accessible via the GermOnline knowledgebase at http://www.germonline.org (Primig et al., 2003; Wiederkehr et al., 2004a; Wiederkehr et al., 2004b). 1189 transcripts that displayed a strong

variation of signals between different samples as compared to (theoretically) identical replicates were grouped using a hierarchical clustering algorithm (Euclidian distance) and sorted over signal strength during mitotic growth. To search for functional GO annotations that are correlated with a particular expression pattern in a statistically significant manner we employed the *goCluster* tool. Briefly, all loci for which reliable data are available (without prior filtration) are first grouped via their expression patterns using k-means clustering and subsequently the genes bearing related functional annotation within each cluster are identified using a statistical test (G. Wrobel and M. Primig, manuscript in preparation). In addition, we directly identified a number of functionally related loci through other GO categories.

MIAME compliance

The TXT and CEL data files corresponding to cells in rich and pre-sporulation medium (YPD, YPA), sporulation medium (SP4, SF5), treated samples (SD5, SD8, SB5, and SB8) and cold-shocked samples as well as the appropriate controls (SR4, SR5, SR8, SC5, SC8) were uploaded to the GEO (NCBI) public data repository at http://www.ncbi.nlm.nih.gov/geo/ (Edgar et al., 2002). Files can be retrieved using the accession number GSE1693. CEL and TXT data files of all samples are also available at our web portal.

Other Techniques

Spore viability was analyzed by dissection of tetrads. Flow cytometric analysis of total cellular DNA content and immunoblot analysis was performed as in (Visintin et al., 1998). For immunoblotting, α-HA antibody (HA.11, Babco) was used at a 1:500 dilution, α-CLB3 (rabbit, Santa Cruz Biotechnology) was used at 1:200, α-Cdc28 antibody was used at 1:1000, and α-Cdc2-Tyr15-P antibody (Cell Signaling Technology) was used at 1:1000. Pairing behavior of GFP-marked chromosomes was analyzed *in vivo* on a Zeiss Axioplan 2 microscope.

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Chapter 4

Conclusions and Future Prospects.

Conclusion

A number of checkpoints monitor crucial meiotic processes and orchestrate meiotic progression to ensure that chromosome integrity is maintained during gamete production. In this work, two processes involved in meiotic surveillance were analyzed in detail. First, we identified the FK506 binding protein (FKBP) Fpr3 as a negative regulator of protein phosphatase 1 (PP1) activity. Through its proline isomerase domain Fpr3 associates with PP1 and prevents PP1 from precociously silencing the recombination checkpoint. Second, we identified and characterized an unexpected response of meiotic cells to microtubule depolymerization. By a mechanism that is independent of the spindle checkpoint, microtubule perturbation (using drugs or low temperatures) causes widespread downregulation of the meiotic transcriptional program leading to a cell cycle arrest in meiotic G2/prophase. Below, some further implications and possible future directions of this work are discussed.

Searching for Recombination Checkpoint Components.

It is unlikely that most major meiotic recombination checkpoint factors have been identified at this point. The fact that we isolated only a single new checkpoint factor after screening the deletion collection (which encompasses the entire non-essential yeast genome) is likely attributable to limits of this collection as well as the high stringency of our initial screen. First, a significant number of strains of the haploid deletion collection used in our screen are aneuploid or have accumulated other modifying mutations, which not only increases the noise

during screening (false positives), it can also mask true genetic interactors (false negatives) (Hughes et al., 2000; Pan et al., 2004). The accumulated abnormalities are a direct consequence of continued selective pressures that the cells were exposed to in the course of production and maintenance of this collection. Since the heterozygous diploid deletion collection is "covered" by a wild-type allele, it was not exposed to the same selective pressure, and was recently shown to yield screen data with a much reduced frequency of false positives and false negatives (Pan et al., 2004). Using the heterozygous deletion collection may therefore allow the isolation of additional checkpoint factors. An additional limit of the collection is that the collection only comprises deletions of non-essential genes, which presents a noticeable restriction, even when screening for factors involved in a non-essential process such as meiosis. For example, both *MEC1* and *GLC7* are essential under normal conditions (*mec1* mutants are viable in an *sml1* background) and could therefore only be isolated as conditional or meiosis-specific mutants.

The conclusion that our screen was very stringent is supported by the high rate of false-negatives. The requirement that bypass mutants had to form dityrosine-containing spores precluded the isolation of deletion mutants such as *rad17*, *ddc1*, *mec3*, or *rad24*, which, while they allow efficient bypass of the *dmc1* delay, do not restore dityrosine incorporation (Lydall et al., 1996; Pak and Segall, 2002). Notably, however, our inability to isolate this latter group of genes, gave a first indication that *FPR3* did not act in the same pathway as these factors.

Nonetheless, reducing the stringency, by repeating a *dmc1* suppressor screen using a reporter of G2/prophase exit, such as *pSPS4-lacZ* (Hepworth et al., 1998), rather than dityrosine incorporation, may allow isolation of a more comprehensive list of checkpoint factors.

The suppressor screen that led to the identification of *FPR3* can easily be modified to identify genetic interactions in a variety of meiotic processes (or other processes of interest in diploid cells), and may be a useful tool for isolating factors required for the meiotic response to microtubule perturbation (see below). Indeed, keeping in mind the above-mentioned limitations, the *Saccharomyces* deletion collection has several advantages. It provides good coverage without redundancy and therefore allows rapid screening of the entire non-essential yeast genome. It also circumvents the need for cloning mutations, because the deletions are arrayed in a defined order, which permits immediate identification of mutants of interest, and hence provides a formidable resource for genetic screens.

Defining a Checkpoint Role.

One general problem that has thus far hampered analysis of the checkpoints monitoring meiotic recombination, is that the majority of checkpoint factors are also directly involved in the control of meiotic DSB repair. First, several proposed checkpoint factors, in particular Rad17 and Rad24, as well as Pch2, appear to be directly required for meiotic recombination, because mutants lacking these factors exhibit a (transient) accumulation of DSB repair intermediates and a small but

detectable delay in meiotic G2/prophase (V. Borner, personal communication) (Hochwagen et al., 2005a; Shinohara et al., 2003). Second, the choice of repair template is often defective in checkpoint mutants. As a consequence increased levels of illegitimate repair from the sister chromatid or from ectopic positions are frequently observed (Grushcow et al., 1999; Niu et al., 2005; Schwacha and Kleckner, 1994; Schwacha and Kleckner, 1997; Thompson and Stahl, 1999; Wan et al., 2004; Xu et al., 1997). In particular, cells lacking the chromosomal proteins Hop1, Red1, and Mek1, and Dot1, can repair the majority of DSBs in a *DMC1*-independent manner (Niu et al., 2005; San-Segundo and Roeder, 2000; Wan et al., 2004; Xu et al., 1997), employing instead an alternative sister-directed repair pathway that requires the recombination factor Rad54 (Arbel et al., 1999; Niu et al., 2005; Xu et al., 1997). Thus, the question arises, whether mutations of *HOP1*, *MEK1*, *RED1* or *DOT1* bypass the delay of a *dmc1* mutant simply and solely because of increased sister repair. At least for *RED1* this appears unlikely, because *dmc1 red1* mutants do not delay meiotic cell cycle progression, even if *DMC1*-independent repair is eliminated by mutation of *RAD54* (D. Bishop, cited in(Xu et al., 1997)). Thus, at least Red1 also has a checkpoint function.

The separation of repair and checkpoint functions is, therefore, of general importance, when analyzing possible recombination checkpoint factors. In the past, several cytological assays, such as TUNEL staining or scoring the number Rad51 foci by immunofluorescence, have been employed to determine whether, as would be expected for proposed checkpoint mutants, recombination

intermediates persist into meiosis I (Bailis et al., 2000; Lydall et al., 1996). However, the cytological assays are often difficult to interpret, especially when the numbers of Rad51 or TUNEL foci are close to background levels, as would be the case, for example, in *dmc1 red1* mutants. To circumvent these problems, a genetic assay using strains without repair templates (haploid *MATa/α cdc6-mn* strains, lacking both homologous and sister chromosomes) has been established in this work that allows unambiguous detection of checkpoint roles (Hochwagen et al., 2005a). Any bypass of a cell cycle delay that is observed in the absence of repair templates should be the consequence of a checkpoint defect, because it cannot be due to the activation of illicit repair (except non-homologous end-joining, which can be controlled by deleting DNA ligase IV (Wilson et al., 1997)). This assay will help the evaluation of proposed checkpoint factors and, in combination with assays addressing a possible role in template choice, will be invaluable in classifying checkpoint factors and determining their role in the surveillance of meiotic recombination.

Checkpoints Regulated by Fpr3 and PP1.

A number of checkpoints are active during meiotic recombination, and similar checkpoints also monitor the chromosomal integrity of mitotically dividing cells. Presumably, most, if not all, of these checkpoints can eventually be silenced even if damage or repair intermediates persist. In the case of the recombination checkpoint, and likely also the *rad50S* checkpoint, this adaptation is controlled by Fpr3 and PP1 (Bailis and Roeder, 2000; Hochwagen et al., 2005a). The

involvement of these factors in the silencing of other checkpoints remains to be analyzed. Interestingly, Fpr3 may not be involved in the regulation of the *zip1* checkpoint, although this checkpoint can be silenced by overexpression of PP1 (Bailis and Roeder, 2000; Hochwagen et al., 2005a). It is possible that the Fpr3 homolog Fpr4 is required for the regulation of PP1 in the context of the *zip1* checkpoint.

Both, Fpr3 and PP1 are not meiosis-specific proteins and could therefore also regulate checkpoints in mitosis. The observation that PP1 is required for the recovery from DNA damage checkpoint dependent cell cycle arrest in *Xenopus* egg extracts, supports this idea (Den Elzen and O'Connell, 2004). Furthermore, a deletion of *FPR3* partially rescues the sensitivity of *rad52-327* mutants to the topoisomerase I inhibitor camptothecin (J. Haber, personal communication). To investigate the role of Fpr3 in the control of the mitotic DNA damage checkpoint, we analyzed whether mutants lacking *FPR3* or both *FPR3* and *FPR4* are sensitive to DNA damaging agents such as the UV-mimetic 4-nitroquinoline-1-oxide (4-NQO) or the DSB inducing drug bleomycin. Exposure to 4-NQO did not reveal any obvious sensitivity of *fpr3* or *fpr3 fpr4* mutant cells compared to wild type, whereas repair-deficient *rad51* mutants were highly sensitive to this type of DNA damage (Figure 1). In contrast, *fpr3* mutants were more sensitive than wild type to high concentrations of bleomycin. Curiously, however, this sensitivity could be suppressed if both *FPR3* and *FPR4* were eliminated. In fact, *fpr3Δ fpr4Δ* double mutants exhibited resistance to bleomycin that exceeded even wild-type levels

(Figure 1). This may indicate competing roles of *FPR3* and *FPR4* in the response to chemically induced DSBs. Clearly, more experiments, including careful titrations and time-course experiments, will be necessary to determine the exact role of Fpr3 and Fpr4 in the mitotic response to DNA damage.

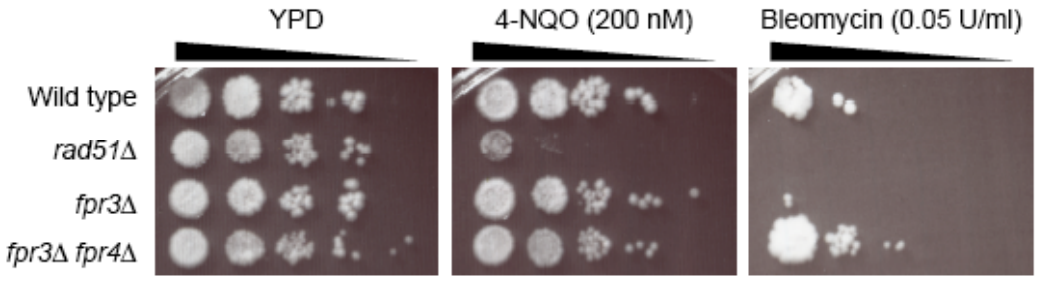

Figure 1: DNA damage sensitivity of *fpr3*Δ and *fpr3*Δ *fpr4*Δ mutants.
Wild type (A4841), *rad51*Δ (A7962), *fpr3*Δ (A6575), and *fpr3*Δ *fpr4*Δ (A7188) cultures were grown to mid-exponential phase (OD_{600} = 0.4) at 30°C. Cultures were diluted by factors of 10, and 3µl of the respective dilutions spotted in triplicate onto YPD, YPD + 200 mM 4-NQO, and YPD + 0.05U/ml bleomycin.

To address a potential role of Fpr3 in the adaptation of mitotic cells to DNA damage, more specialized assays will be necessary. Several such systems, which allow the controlled introduction of a DSB without the possibility of repair, have successfully been used for the characterization of other adaptation factors, such as the Polo kinase Cdc5, casein kinase II, Ku70/Ku80, as well as the phosphatases Ptc2 and Ptc3 (Lee et al., 1998; Lee et al., 2000; Leroy et al., 2003; Toczyski et al., 1997). All of these factors act as positive regulators of adaptation, in whose absence cells cannot adapt to a single DSB. However, the same assays, when

conducted as time course experiments, should also allow the analysis of potential negative regulators of adaptation, such as Fpr3, because accelerated adaptation should be readily observable.

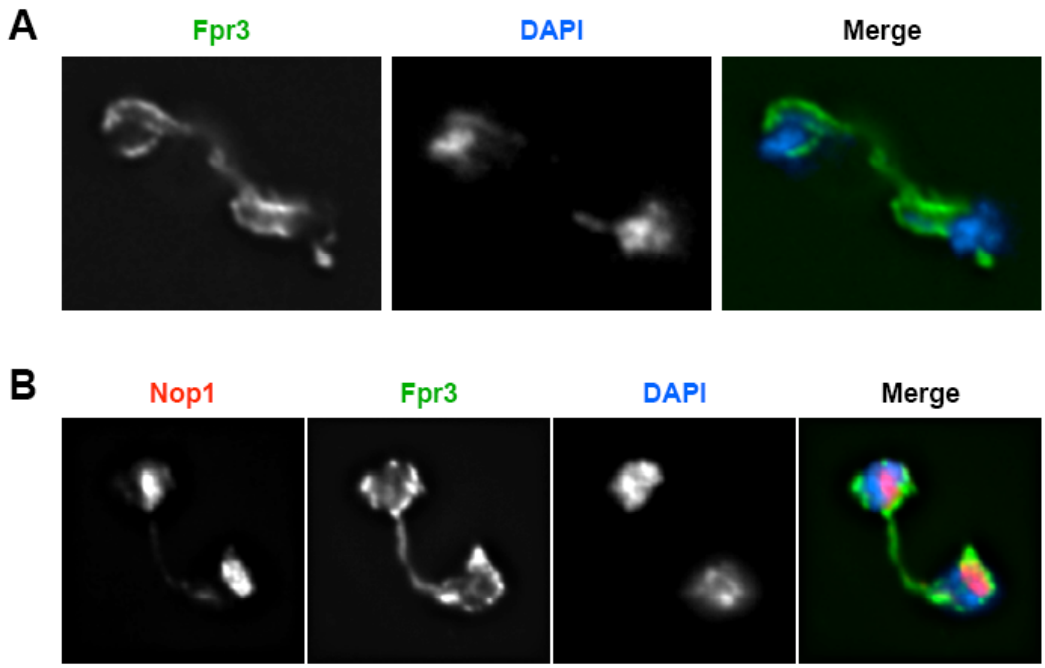

Figure 2: Localization of Fpr3 in telophase.
Deconvolved images (12 iterations). (A) Wild-type haploid cells (A10416) were arrested in G1 using alpha-factor pheromone and released. After 145 min, when most cells were in anaphase or telophase, cells were spread and probed by immunofluorescence. (B) Cycling wild-type diploid cells (A9671) were grown to mid-exponential phase, spread, and probed by immunofluorescence. In the Merge, Fpr3 is in green, DNA is in blue, and Nop1 is in red.

A final point of interest concerning a role of Fpr3 and PP1 in response to mitotic DNA damage concerns the nucleolar localization of these two proteins. Upon

entry into meiosis, both proteins leave the nucleolus and spread throughout the nucleus (Hochwagen et al., 2005a). It is not known whether this change in localization is necessary for the checkpoint functions of Fpr3 and PP1. In mitotic cells, Fpr3 remains sequestered in the nucleolus, adjacent to the Nop1-positive part of the nucleolus, throughout the cell cycle, including anaphase and telophase (Figure 2 A, B). However, it will be interesting to investigate possible changes in localization in response to DNA damage. Such DNA damage dependent relocalization has been observed for other checkpoint-associated factors, such as Sir3, which redistributes from telomeres to sites of DNA damage (Mills et al., 1999). Mitotic cells, which possess a much larger nucleolar compartment than meiotic cells, may provide a more amenable experimental system to investigate the role of nucleolar sequestration of Fpr3 and PP1.

FKBPs and Proline Isomerization.

A question raised again by this work is whether the in vitro proline isomerase activity of FK506 binding proteins is relevant in vivo. In the context of the recombination checkpoint, loss of the in vitro PPIase activity of Fpr3 did not correlate with the functionality of these mutants in vivo (Hochwagen et al., 2005a). A similar lack of correlation has also been observed for the PPIase activities of mutant human FKBP12 proteins and their activity in binding the ryanodyne receptor (Timerman et al., 1995). Strikingly, in the only crystal structure solved thus far of an FKBP bound to a substrate, the PPIase domain of FKBP12 binds to a Pro-Leu-Leu peptide of the type I TGF receptor (T R-I)

without actually interacting with the proline residue. Rather, in the crystal structure the two neighboring leucines occupy the hydrophobic pocket of the PPIase domain (Huse et al., 1999). This finding raises the possibility that proline isomerization is not the major activity of FKBPs in vivo. The FKBP12- T□R-I crystal structure indicates that the PPIase domain can function as a protein interaction domain. A binding role for PPIase domains could also explain the existence of proteins with several tandem PPIase domains (Breiman and Camus, 2002; Davies and Sanchez, 2005; He et al., 2004). Such a domain arrangement is common for adaptor proteins, which often harbor multiple protein interaction domains of the same family (e.g. tandem TPR domains or BRCT domains (D'Andrea and Regan, 2003; Manke et al., 2003)). Isotypic domains with catalytic function are rarely found in the same protein. As the PPIase domain is a largely hydrophobic pocket, it presumably associates preferentially with hydrophobic residues. An intriguing possibility is that PPIase domains bind methylated lysines or arginines. In molecular modeling simulations methylated lysine associates energetically very favorably with the hydrophobic pocket of the PPIase domain (P. Kolb, personal communication). In that case methylation and demethylation would provide a way to regulate binding of FKBPs. Analysis of the binding specificities of the PPIase domains therefore remains an intriguing avenue for future research.

Environmental Effects on Meiotic Progression.

Low temperatures trigger an arrest in meiotic G2/prophase (Hochwagen et al., 2005b). Even in the fast-sporulating SK1 strain background, the early stages of meiosis leading up to the first meiotic division consume at least 4 to 5 hours. Within such a time frame the ambient temperature can change drastically. Thus, the arrest response of meiotic cells low temperatures is likely a physiologically important safeguard of genomic integrity against unfavorable environmental change. The cell cycle arrest and concomitant downregulation of the meiotic gene expression program observed at low temperatures (10°C) are recapitulated when cells are exposed to the microtubule poison benomyl at room temperature (25°C) and partially reversed when a benomyl-resistant tubulin variant is used (Hochwagen et al., 2005b), suggesting that microtubule stability is the crucial parameter in this response. Microtubules are required for meiotic chromosome segregation. Our observation that the meiotic alignment of homologous chromosomes is impaired when microtubules are depolymerized, indicates that intact microtubules are also important prior to meiosis I, which may explain the existence of a meiotic mechanism monitoring microtubule destabilization. At this point, however, we cannot exclude that the failure to align chromosomes is a secondary consequence of the transcriptional response triggered by microtubule depolymerization. It therefore remains a possibility that microtubules do not serve an active role prior to meiotic chromosome segregation. During meiotic recombination, the establishment of stable strand invasion intermediates is strongly influenced by the ambient temperature (Borner et al., 2004). Low

temperatures (10°C) may negatively affect this temperature-sensitive step. If this were the case, microtubule destabilization may also serve as a temperature read-out to arrest cells in meiotic G2/prophase if conditions are not conducive for successful meiotic recombination. Analysis of the recombination proficiency of cold-resistant tubulin mutants should distinguish between these possibilities.

The molecular determinants that trigger the arrest of cells in meiotic G2/prophase in response to microtubule perturbation remain elusive. Activation of the mitotic DNA damage checkpoint induces the expression of a select set of damage response genes. Activation of the mitotic spindle assembly checkpoint, on the other hand, does not trigger major transcriptional changes (S. Biggins, personal communication). The effect on the meiotic gene expression program that we observed upon microtubule destabilization of meiotic cell, suggested that mechanisms other than the known meiotic checkpoints might be responsible. Indeed, we found that the arrest in G2/prophase was independent of the recombination checkpoint and did not require the spindle assembly checkpoint component Mad2 (Hochwagen et al., 2005b). However, in light of recent findings it would be interesting to revisit the question of an involvement of a subset of spindle assembly checkpoint components. In particular the spindle checkpoint factor Mad3, has recently been shown to act independently of Mad2 in meiosis. Furthermore, unlike Mad2, Mad3 serves to delay cells in meiotic G2/prophase (Cheslock et al., 2005). Thus, Mad3 may be an interesting candidate for a checkpoint factor involved in the meiotic response to microtubule perturbation. A

screen of the deletion collection asking for mutants that would not arrest in meiotic G2/prophase at low temperatures (perhaps using a *pSPS4-lacZ* reporter assay) should identify other factors required for this response.

Conservation and significance.

Several of the above processes may also be active in vertebrates. An early meiotic response to microtubule depolymerization has also been observed in mouse spermatogenesis, where colchicine treatment causes an arrest meiotic G2/prophase. Whether a transcriptional response comparable to the response observed in benomyl treated budding yeast cells also exists in mice remains to be analyzed. This meiotic arrest response to microtubule depolymerization may contribute to the efficacy of vinblastine (another miroctubule poison) in the treatment of testicular germ line cancers (Jordan and Wilson, 2004). The origin of germ line cancers is still debated (Browne et al., 2005). However, the observation of meiotic markers, such as the SC component HSYP1, in a subset of testicular germ line tumors suggests that at least some germ line cancers may be derived from meiotic cells (Oosterhuis and Looijenga, 2005), and may thus retain meiotic checkpoint activity.

The findings that PP1 and Fpr3 modulate the meiotic recombination checkpoint in budding yeast may also be relevant for vertebrate meiosis. In mice and *Xenopus*, PP1 has been shown to dephosphorylate several substrates during exit from meiotic G2/prophase, including the CDK regulator Cdc25 and the poly-

adenylation factor CPEB (Perdiguero and Nebreda, 2004; Tay et al., 2003). Moreover, the PP1c isoform, has been shown by targeted disruption to be required for mouse spermatogenesis (Varmuza et al., 1999). In budding yeast, Fpr3 serves to prevent premature silencing of the meiotic recombination checkpoint by PP1 (Hochwagen et al., 2005a). Could FKBPs also act as meiotic regulators higher eukaryotes? FKBPs are a highly conserved protein family and although no obvious homolog of Fpr3 exists in mice or humans, there are two FKBPs that may be Fpr3 paralogs. FKB3/FKBP25 is a nuclear FKBP that shares some of the domain structure of Fpr3 and like Fpr3 preferentially binds rapamycin over FK506 (Galat, 2004; Galat et al., 1992; Jin et al., 1992). The role of FKB3 in meiosis has not been explored. In contrast, the more distantly related FKBP6, at least in mice and rats, has a role in meiosis. Based on the sequence conservation of its PPIase domain, it is very likely that FKBP6 also binds rapamycin. Whether FKBP6 functions as a checkpoint factor, has not been addressed because FKBP6 is also required for meiotic progression (Crackower et al., 2003). Intriguingly, however, both *Fkbp6*$^{-/-}$ and *PP1c*$^{-/-}$ mice exhibit male-specific sterility (in both cases female homozygous mutants are fertile) (Crackower et al., 2003; Varmuza et al., 1999), which may point to a functional interaction between these two proteins in mouse spermatogenesis.

Rapamycin is a widely used immunosuppressive and anti-proliferative drug (Easton and Houghton, 2004). The doses used in treatments are generally several orders of magnitude lower than the ones used in this study (factor ~250x;

concentration used here: 10 M = 3.7 g/ml; clinical dose against graft rejection: 15 ng/ml 24-h whole blood through level). However, we observed noticeable checkpoint inhibition already at a rapamycin concentration of 0.25 M (6x higher than clinical levels). Moreover, graft patients may receive a lifelong treatment with this drug. If a checkpoint role for FKBPs exists in humans, long-term effects on fertility or an increased frequency of sperm with chromosomal abnormalities cannot be excluded (a possible adverse effect of rapamycin would only affect males, because oogenesis has already progressed past meiotic recombination at the time of birth). Thus, studies investigating sperm quality of graft recipients who receive rapamycin treatment and a search for alternative immunosuppressive regimens may be warranted.

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Appendix A

Control of Meiotic Double Strand Break Formation by Cyclin Dependent Kinases.

Introduction

The fragmentation of the genome in the course of meiotic recombination is an essential prerequisite for faithful meiotic chromosome segregation. It also poses a substantial risk for the integrity of the genome. As a consequence, the introduction of DNA double strand breaks (DSBs) is a strictly controlled process. In all organisms that undergo meiotic recombination, DSBs are introduced by the conserved topoisomerase-like factor Spo11 (Keeney, 2001). DSBs are formed by a transesterase reaction resulting in Spo11 covalently bound to the ends of the break (Keeney et al., 1997). The DSB-forming activity of Spo11 depends on a large number of accessory factors, including Ski8/Rec103, the Rec102/Rec104 complex, Mer2/Rec107, Rec114, Mei4, and the MRX complex (Mre11, Rad50, and Xrs2) (Keeney, 2001). In the absence of any of these proteins, DSBs do not form. However, the molecular functions of these additional factors remain elusive. All ten proteins form an interaction network by two-hybrid analysis (Arora et al., 2004). Rec102/Rec104 and Ski8 interact directly with Spo11 (Arora et al., 2004; Kee et al., 2004; Uetz et al., 2000), and Rec102/Rec104 and Rec114 are required for the association of Spo11 with meiotic chromosomes (Prieler et al., 2005). Conversely, Spo11 is necessary for the chromosome association of Ski8, Rec102/Rec104 and Mre11 (Arora et al., 2004; Borde et al., 2004; Kee et al., 2004).

DSBs are formed predominantly at so-called hotspots of recombination. Targeting Spo11 to sites outside of hotspots by fusing a sequence-specific Gal4 DNA

binding domain to Spo11, results in DSB formation at the targeted sites. However, Spo11 activity still depends on all nine accessory factors indicating that these factors regulate Spo11 independent of DNA binding and DSB site selection (Pecina et al., 2002).

DSB formation initiates after premeiotic DNA replication. Recent experiments indicate coupling between DNA replication and DSB formation in both *S. cerevisiae* and *S. pombe*. Treating meiotic cells with the ribonucleotide reductase inhibitor hydroxyurea stalls replication forks and inhibits the formation of DSBs (Borde et al., 2000; Tonami et al., 2005). Moreover, if replication of the left arm of chromosome III is delayed, DSBs are also introduced late on that chromosome arm, although they form with wild-type timing on the normally replicating right arm (Borde et al., 2000). This suggests that the coupling of DSB formation to replication is a local phenomenon, likely involving the passage of the replication fork. The coupling only occurs once origins of replication have fired, because mutants, which lack a functional version of the helicase loading factor *CDC6* (*Cdc18* in *S. pombe*), fail to initiate premeiotic DNA replication, but are still able to form meiotic DSBs with wild-type timing (Hochwagen et al., 2005; Murakami and Nurse, 2001). Thus, the initiation of DSB formation does not require the presence replicated sister chromatids. This suggests that the coupling of DSB formation to premeiotic DNA replication is the consequence of a surveillance mechanism that prevents DSB on unreplicated chromosomes while premeiotic DNA replication is in progress.

Cyclin dependent kinases (CDKs) are composed of the catalytic kinase subunit Cdc28 paired with an activating cyclin subunit. Clb5 and Clb6 are the cyclins of S phase CDKs in *S. cerevisiae*. Meiotic cells lacking *CLB5* and *CLB6* or harboring an allele of *CDC28* that can be chemically inhibited (*cdc28-as1*), fail to initiate premeiotic DNA replication (Benjamin et al., 2003; Dirick et al., 1998; Smith et al., 2001; Stuart and Wittenberg, 1998). Unlike *cdc6* mutants, however, they also do not form meiotic DSBs (Dirick et al., 1998; Smith et al., 2001; Stuart and Wittenberg, 1998). S phase CDKs therefore appear to play a dual role as activators of premeiotic DNA replication and activators of DSB formation. Here we investigate possible targets of S phase CDKs, whose phosphorylation may be required for DSB formation.

Preliminary Results and Discussion

Both, meiotic depletion mutants of *CDC6* (*cdc6-mn*) and *clb5Δ clb6Δ* mutants fail to initiate premeiotic DNA replication. However, while *cdc6-mn* mutants still initiate DSB formation with wild-type timing, *clb5Δ clb6Δ* mutants do not form any DSBs (Hochwagen et al., 2005; Smith et al., 2001). The most likely explanation for this observation is that S phase CDKs have a replication independent role in DSB formation. Yet, it is also possible that *CDC6* is part of the mechanism that blocks DSB formation, when meiotic origins of replication are not fired due to the absence of S phase CDK activity. To distinguish between these possibilities we constructed mutants that lacked *CLB5* and *CLB6* and also

harbored a *cdc6-mn* mutation. If *CDC6* were part of a mechanism that prevents DSB formation in the absence of S phase CDK activity, then depletion of *CDC6* should restore DSB formation to *clb5Δ clb6Δ* mutants. We analyzed meiotic DSB formation by Southern blot at the well-characterized *HIS4LEU2* hotspot (Storlazzi et al., 1995). As observed previously, *cdc6-mn* mutants initiate meiotic DSBs with wild-type kinetics and, after a delay, repair most breaks by crossover recombination (Figure 1A, C, D). In contrast, *clb5Δ clb6Δ* mutants failed to form detectable amounts DSBs, and consequently no recombination products could be observed (Hochwagen et al., 2005; Smith et al., 2001). The *cdc6-mn clb5Δ clb6Δ* triple mutant behaved like a *clb5Δ clb6Δ* mutant (Figure 1A, C, D). However, as DSBs are turned over quickly when the meiotic repair machinery is active, we wished to confirm the absence of DSBs in the triple mutant in a *rad50S* background. A *rad50S* mutation causes unprocessed meiotic DSBs to accumulate (Alani et al., 1990), which permits a more precise measurement of meiotic DSB levels. Again, we observed that *cdc6-mn* mutants initiated DSB formation with wild-type kinetics, but cumulative DSB levels reached a plateau at about 70% of wild-type levels (Figure 1E, F). The reasons for the slightly lower DSB levels of *cdc6-mn* mutants are at present unclear. No DSBs could be detected in *clb5Δ clb6Δ rad50S* and *cdc6-mn clb5Δ clb6Δ rad50S* mutants (Figure 1E, F), confirming the observations made above in the *RAD50* (wild-type) background. Therefore, *cdc6-mn* is not epistatic to *clb5Δ clb6Δ*, indicating that *CDC6* does not prevent DSB formation in the absence of CDK activity. This suggests that Clb5-

CDK and Clb6-CDK activity is directly required for the initiation of meiotic DSB formation.

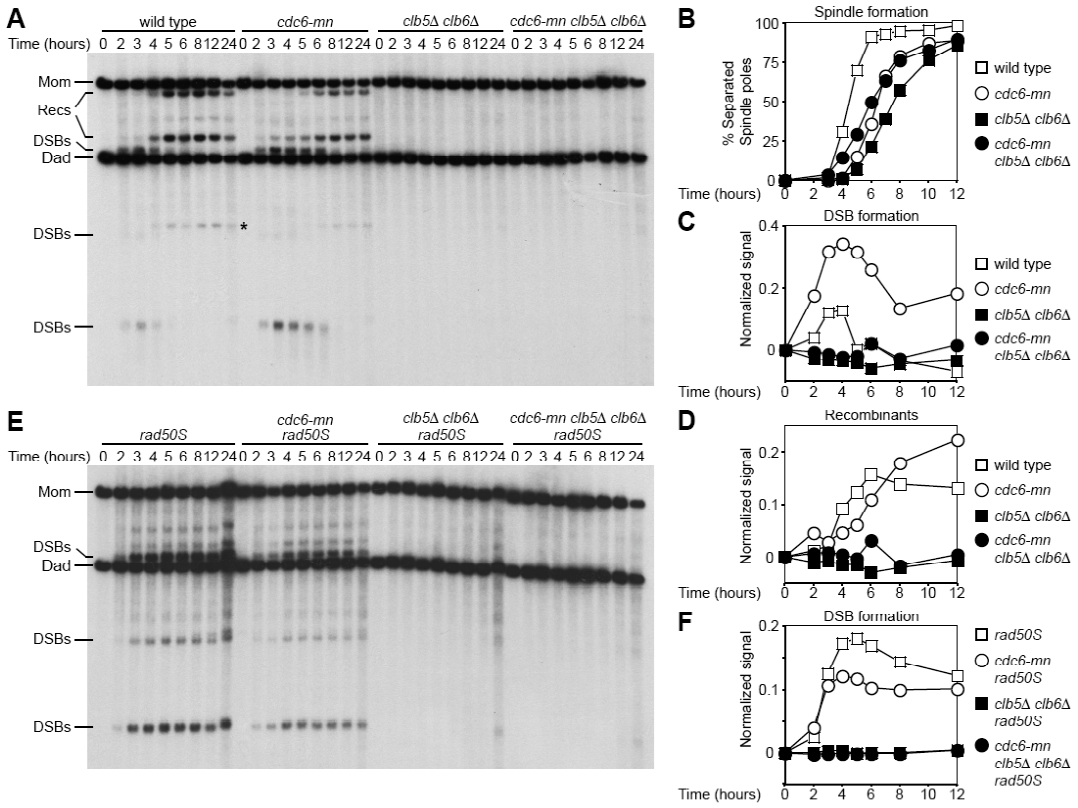

Figure 1: DSB formation of *cdc6-mn* and *clb5Δ clb6Δ* mutants.

(A-D) Wild-type (NKY1551), *cdc6-mn* (A10912), *clb5Δ clb6Δ* (A14033), and *cdc6-mn clb5Δ clb6Δ* (A13913) cells were induced to undergo meiosis and samples were taken at the indicated times to analyze recombination at the *HIS4LEU2* hotspot (Storlazzi et al., 1995) by Southern blot analysis (A) and the percentage of cells with separated SPBs (B). The Southern blot was probed with probe A. * indicates a *RAD52*-dependent DNA fragment that is likely the result of an intrachromosomal inversion event between the *HIS4LEU2* locus and the *leu2::hisG* locus. Quantification of the slower migrating recombinant band and of the fastest migrating DSB band is shown in (C) and (D), respectively. To obtain the normalized signal the measured signal was divided by the signal of the

parental band. The value of the 0 hour time point was then subtracted from all later time points to eliminate non-specific signal. (E, F) *rad50S* (A11675), *cdc6-mn* (A11265), *clb5Δ clb6Δ* (A14209), and *cdc6-mn clb5Δ clb6Δ* (A14212) cells were induced to undergo meiosis and samples were taken at the indicated times to analyze recombination. (F) Quantification of the fastest migrating DSB band.

Interestingly, despite the absence of sister chromatids, *cdc6-mn* mutants are able to engage in crossover recombination, which indicates that the failure in crossover formation of mutants lacking the cohesin subunits *REC8* or *SMC3* (Klein et al., 1999) is not solely due to the absence of links between sister chromatids. Indeed, crossover levels of *cdc6-mn* mutants eventually rise above wild-type levels, presumably because the homolog is the only available repair template (Figure 1C). However, not all DSBs are repaired (Figure 1D). It is possible that some DSBs cannot be repaired from the homolog. However, *cdc6-mn* mutants only delay in meiotic G2/prophase for a short time (Figure 1B), suggesting that the unrepaired DSBs are not efficiently recognized by any of the checkpoints monitoring meiotic recombination. Therefore, the failure to complete DSB repair in *cdc6-mn* mutants may be the consequence of precocious entry into meiosis I and the segregation of unrepaired chromosomes.

Search for CDK substrates required for DSB formation.

In the course of DNA replication, Clb5-CDK phosphorylates a number of targets that regulate origin firing and prevent re-replication before the cell has completed

mitosis (Diffley, 2004; Loog and Morgan, 2005; Masumoto et al., 2002; Wilmes et al., 2004). Similarly, S phase CDKs likely also phosphorylate multiple substrates involved in the formation of meiotic DSBs. A large-scale proteomic approach identified Spo11 as a possible CDK target, but with the exception of Rec114, did not analyze other factors required for meiotic DSB formation (Ubersax et al., 2003). To analyze possible CDK-dependent phosphorylation of factors involved in the initiation of DSB formation, we fused multiple copies of the hemagglutinin (HA) or myc epitopes to the carboxy terminus of Spo11, Rad50, Xrs2, Mei4, Rec102, Ski8/Rec103, Rec104, Rec114, and Mer1. Phosphorylation of a protein frequently changes its electrophoretic mobility in SDS polyacrylamide gels. We therefore compared the electrophoretic mobility of these epitope-tagged factors in wild-type cells and *clb5Δ clb6Δ* mutants that had been induced to undergo meiosis for 2 or 4 hours. Spo11, Rad50, Rec102, Ski8/Rec103, Rec114, and Mer1 did not exhibit obvious mobility differences in the two backgrounds (Figure 2A). On the other hand, slower-migrating bands of both Rec104 and Xrs2 were eliminated or strongly reduced in *clb5Δ clb6Δ* mutants suggesting that these two proteins undergo Clb5/Clb6-CDK dependent phosphorylation (Figure 2A). Wild-type cells also exhibited a slower migrating form of Mei4 that was absent in *clb5Δ clb6Δ* mutants. Because of its low abundance, this band has not been analyzed further at this point. Also, for technical reasons, mobility shifts of Mre11 and Mer2/Rec107 have not yet been analyzed.

The mobility shifts of Rec104 and Xrs2 were investigated further. Rec104 is a small protein of 182 amino acids that contains only a single consensus CDK target sequence at the very C-terminus of the protein: an SP site at positions 181 and 182. We therefore constructed epitope-tagged versions of Rec104 that either lacked these two amino acids or had Ser181 replaced by alanine and analyzed the meiotic electrophoretic mobility of these mutants. Both mutants exhibited wild-type mobility (Figure 2B), indicating that it is not the consensus CDK phosphorylation site of Rec104 that is phosphorylated in a CDK dependent manner.

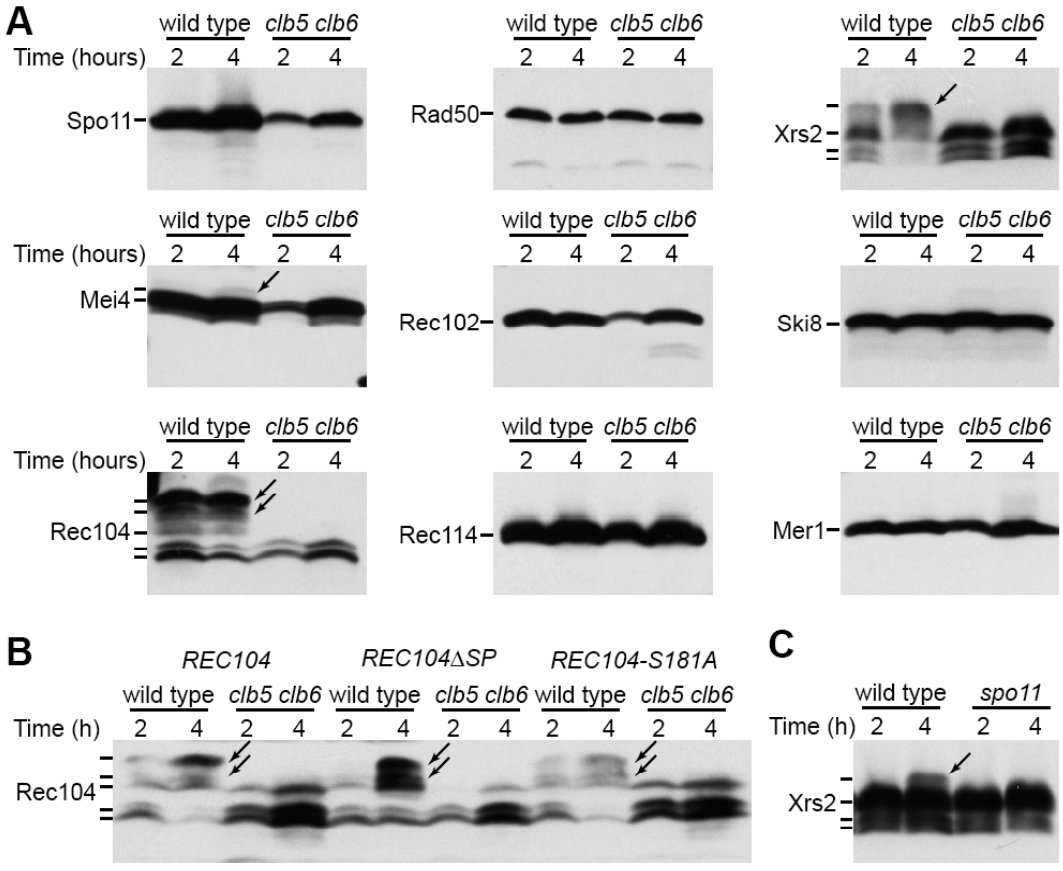

Figure 2: CDK dependent mobility shifts of DSB factors.

(A) Wild-type cells and *clb5Δ clb6Δ* mutants harboring *SPO11-18myc* (A12605 and A12601), *RAD50-6HA* (A13705 and A13711), *XRS2-13myc* (A13719 and A13697), *MEI4-6HA* (A13707 and A13694), *REC102-6HA* (A13712 and A13695), *SKI8-6HA* (A13713, and A13696), *REC104-6HA* (A13714 and A13708), *REC114-13myc* (A13717 and A13702), or *MER1-13myc* (A13718 and A13709) were induced to enter meiosis. Samples were taken at the indicated times and analyzed by electrophoresis in low-bisacrylamide gels (acrylamide : bisacrylamide = 150:1) and Western blotting. (B) Wild-type cells and *clb5Δ clb6Δ* mutants harboring *REC104-6HA* (A13714 and A13708), *REC104ΔSP-6HA* (A14285 and A14283), or *REC104-S181A-6HA* (A14286 and A14284) were induced to enter meiosis and processed as in (A). (C) Wild-type cells and *spo11Δ* mutants harboring *XRS2-13myc* (A13719 and A14432) were induced to enter meiosis and processed as in (A). Arrows indicate *CLB5 CLB6* dependent bands.

Xrs2 was previously described as a phospho-protein that undergoes DNA damage checkpoint dependent phosphorylation (D'Amours and Jackson, 2001). It is possible that a similar modification of Xrs2 occurs in response to meiotic DSB formation. This would mean that the change in mobility of Xrs2 in *clb5Δ clb6Δ* mutants was due to the inability of these mutants to form DSBs. To address this possibility, we analyzed the electrophoretic mobility of Xrs2 extracted from *spo11* mutant cells, which are unable to form DSBs despite active S phase CDKs. The slow-migrating form of Xrs2 was absent in *spo11* mutants (Figure 2C). This suggests that Xrs2 is not a direct S phase CDK target but rather becomes phosphorylated as a consequence of DSB formation.

In summary, our findings suggest that Clb5/Clb6-CDKs have a replication independent role in the initiation of meiotic DSBs. Two possible targets, whose CDK dependent phosphorylation may affect their role in DSB formation, are Rec104 and Mei4. However, which residues of these two proteins are phosphorylated and whether this phosphorylation is functionally important for the initiation of DSB formation remains to be determined. The CDK dependent electrophoretic mobility shift of Xrs2, on the other hand, appears to be a secondary consequence of meiotic DSB formation induced by Clb5/Clb6-CDKs. Xrs2 is selectively phosphorylated by the checkpoint kinase Tel1 in response to DNA damage both in vitro and in vivo (D'Amours and Jackson, 2001; Mallory et al., 2003). It is possible that Tel1 also phosphorylates Xrs2 in response to meiotic DSBs. Since both Tel1 and Xrs2 are specifically involved in the *rad50S* checkpoint (Usui et al., 2001), the electrophoretic mobility shift of Xrs2 may provide a useful marker to observe the activation of this checkpoint.

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