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# Maintenance of genome integrity in gametes: Coping with accidental and programmed DNA double-strand breaks during meiosis

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# Maintenance of genome integrity in gametes: Coping with accidental and programmed DNA double-strand breaks during meiosis

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*ABSTRACT*

DNA double-strand breaks (DSBs) can arise at unpredictable locations after DNA damage or in a programmed manner during meiosis. DNA damage checkpoint response to accidental DSBs during mitosis requires the Rad53 effector kinase. On the other hand, the meiosis-specific Mek1 kinase, together with Red1 and Hop1, mediates the recombination checkpoint in response to programmed Spo11-dependent meiotic DSBs that are required for meiotic recombination to take place. In *Saccharomyces cerevisiae*, the Sae2 protein and the Mre11-Rad50-Xrs2 complex are necessary to remove the covalently attached Spo11 protein from the DNA ends of meiotic DSBs, which are then resected by so far unknown nucleases.

As several aspects of the control of the response to DSBs during meiosis are still obscure, I focused my research as Ph. D. student on two different aspects of this control: 1) the possible role of Rad53 and inter-relationships with Mek1 in the response to accidental and programmed DSBs during meiosis and 2) the mechanisms responsible for processing Spo11-induced meiotic DSBs.

1. We have provided evidence that exogenous DSBs lead to Rad53 phosphorylation during meiosis, whereas programmed meiotic DSBs do not. However, the latter can trigger phosphorylation of a protein fusion between Rad53 and the Mec1-interacting protein Ddc2, suggesting that the inability of Rad53 to transduce the meiosis-specific DSB signals might be due to its failure to access the meiotic recombination sites. Rad53 phosphorylation/activation is elicited when unrepaired meiosis-specific DSBs escape the recombination checkpoint. This activation requires homologous chromosome segregation and delays the second meiotic division. Altogether, these data indicate that Rad53 phosphorylation prevents sister chromatid segregation in the presence of unrepaired programmed meiotic DSBs, thus providing a salvage mechanism ensuring genetic integrity in the gametes even in the absence of the recombination checkpoint.

2. By using site directed mutagenesis and gene replacements, we have demonstrated that phosphorylation of Sae2 Ser-267 by cyclin-dependent kinase 1 (Cdk1) is required to initiate meiotic DSB resection by allowing Spo11 removal from DSB ends. This finding suggests that Cdk1 activity is required for the processing of Spo11-induced DSBs, thus providing a mechanism for coordinating DSB

resection with progression through meiotic prophase. Furthermore, we used different genetic and biochemical tools to demonstrate that the helicase Sgs1 and the nucleases Exo1 and Dna2 participate in lengthening the 5'-3' resection tracts during meiosis by controlling a step subsequent to Spo11 removal. Our findings suggest that, once Spo11 has catalyzed meiosis-specific DSB formation, it is removed from the DSB ends by endonucleolytic cleavage that necessitates CDK-mediated Sae2 phosphorylation and the nuclease activity of MRX. This cleavage is in turn required for resection of the break by either Exo1 or Dna2-Sgs1 activities, thus allowing completion of meiosis-specific DSB processing.

# INTRODUCTION

# MEIOSIS

Sexually reproducing organisms produce gametes that have half the normal cellular chromosome complement; in consequence, union of male and female gametes restores the normal cellular chromosome complement rather than doubling it. Meiosis is the process that accomplishes the requisite halving via a program in which a single round of DNA replication is followed by two successive rounds of chromosome segregation. A diploid meiotic cell thus yields four haploid meiotic products. In most animals, the meiotic products are transformed directly into gametes. In higher plants, they grow by mitotic divisions into gametophytes, which then yield pollen and ovules. In many fungi, such as the budding and fission yeasts, bryophytes, and pteridophytes, they constitute the haploid phase of the life cycle.

The term "meiosis" has very antique origins. In ancient Greece this word was used to indicate *reduction.* Today with such word we refer to a specific type of cellular division which generates haploid gametes from diploid cells.

Meiosis guarantees to every daughter cell a complete haploid genome although not identical to neither one of the maternal genomes due to the phenomenon known as "recombination".

Like mitosis, meiosis begins with one round of DNA replication, but while in mitosis only one round of chromosome segregation follows (fig.1a), in meiosis two rounds of chromosome segregation occur: MI (meiosis I) and MII (meiosis II), thus giving rise to cells containing only one copy of genetic material (fig. 1b) (Petroncski et al., 2003).

To both reduce chromosome number and ensure that gametes inherit a complete copy of the genome, maternal and paternal versions of each chromosome (homologous chromosomes) segregate in opposite directions at the first of the two meiotic divisions (Fig. 1b). Sister chromatids can then be segregated to the final haploid nuclei at the second meiotic division (Fig. 1b). For meiosis I to occur, homologs must pair and join prior to their segregation.

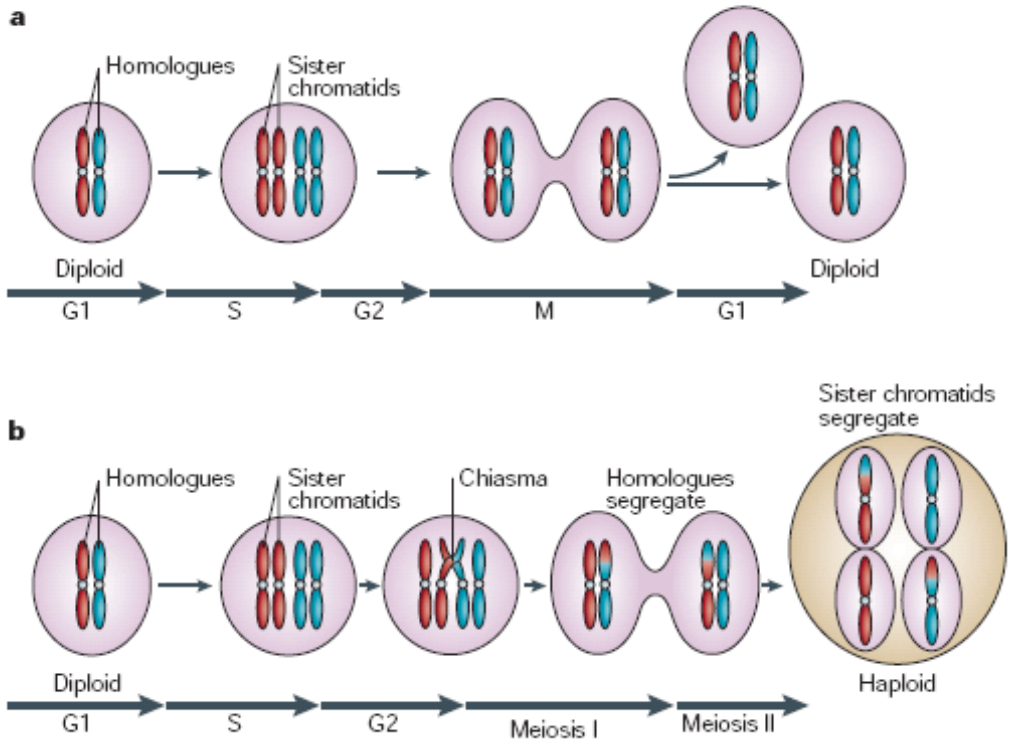



**Fig.1 Mitotic cell cycle and meiosis. a)** In mitosis, diploid cells replicate chromosomes during S phase and segregate sister chromatids at the opposite poles during M phase. This way diploid daughter cells are produced.
**b)** In meiosis, two chromosome-segregation phases, meiosis I and meiosis II, follow a single round of DNA replication. During meiosis I, paired homologous chromosomes (red and blue) are segregated to opposite poles. Then, sister chromatids segregate to opposite poles during meiosis II, which results in the formation of non-identical haploid gametes.

### *The phases and events of Meiosis*

Meiosis begins with cells in a premeiotic G1/G0 condition characterized by marked expansion of the nucleus (Rhoades MM., 1961). Premeiotic S-phase follows, with concomitant formation of intersister connections. This stage, like its mitotic counterpart, is characterized by diffuse chromatin within which strongly staining foci are often seen. The molecular events of S-phase in mitotic and meiotic cells are also closely analogous, with the important exception that meiotic S-phase always takes much longer (Holm PB., 1977). In some organisms, homologues are paired at premeiotic G1/G0, in which case pairing may be loosened or lost during meiotic S-phase and then restored (Weiner BM and Kleckner N., 1994).

- *Meiosis I*

Meiosis I is made up of four cytologically distinct phases that follow pre-meioic DNA replication, prophase I, metaphase I, anaphase I and telophase I (fig.2).

During **prophase I** chromosomes couple, synaps and recombine. This phase is made up by five stages: leptotene, zygotene, pachytene, diplotene and diakinesis. Just before leptotene, chromosomes of many organisms undergo a cycle of compaction (preleptotene).

The *leptotene* stage (Greek "leptos"= thin) is characterized by chromosomes that are discernibly individualized, appearing thin and thread-like (fig.3). Overall, the total array of chromosomes appears as a dense tangle of such threads. Next comes *zygotene* (Greek "zygos"=pair), at which stage chromosomes are shorter and fatter and homologs begin to come close together. Synapsis occurs, this is a state that refers to a tight continuous association along the chromosome length in which the four chromatids are aligned and held togheter by the synaptonemal complex, a proteinaceous structure formed between homologous chromosomes. Each synapsed couple of homologous chromosomes is made up by four cromatids creating a structure known as *tetrad* (fig.3).

By the next stage, *pachytene* (Greek "pakhus"= thick), synapsis is fully complete and synapsed homologous chromosomes are shorter,

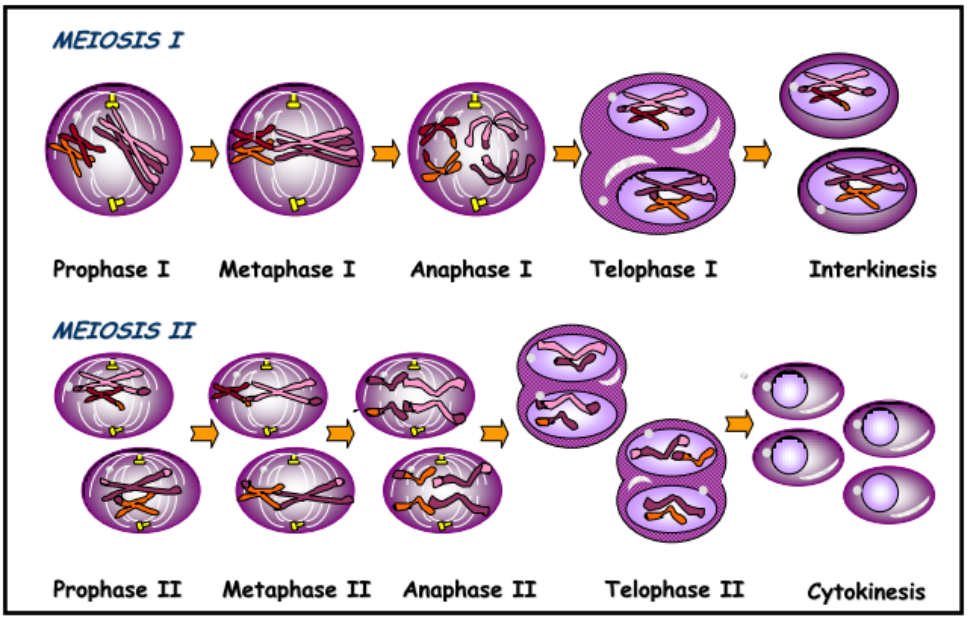



**Fig.2 Meiotic divisions.** In meiosis two nuclear division occur: meiosis I and meiosis II. For each division there are four phases, prophase, metaphase, anaphase and telophase. After meiosis I, two daughter cells containing a pair of sister chromatids for each chromosome are generated. In meiosis II, sister chromatids are separated giving rise to four daughter cells containing only one copy of each chromosome.

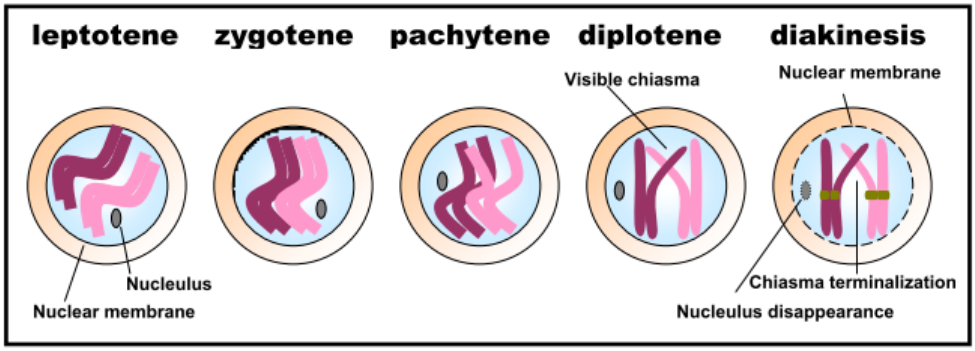



**Fig.3 Meiotic prophase I.** Prophase I is composed by five stages: leptotene, zygotene, pachytene, diplotene, diakinesis. At the end of prophase I the coupled homologs are ready to align on the metaphase plate in order to separate at opposite poles during anaphase.

thicker and tightly associated. At the end of this stage the synaptonemal complex is disassembled and chromosomes start to spread out (Fig.3). Pachytene is followed by *diplotene* (Greek "diploos"= double).

Homologs start to be widely separated (in repulsion) but remain held together at the chiasmata (Greek "chiasma"= beams arranged in a cross in the framework of a roof).

The final stage of prophase is *diakinesis* (Greek, "kinesis"= movement). During this stage, bivalents continue to shorten, and chiasmata move towards cromatids' ends (terminalization). The nucleulus disappears and the nuclear membrane is dissolved (fig.3).

**Metaphase I.** In this phase there is the total dissolution of the nuclear membrane, the initial disposition of tetrads on the cell's metaphase plate and the complete formation of the meiotic spindle (fig.2). In anaphase I homologue chromosomes have to be separated but sister chromatids need to be tightly united in order to be separated afterwards, in MII. For this reason, differently from mitosis, the kinetochores of sister chromatids are attached to microtubules with the same polarity. This phenomenon is known as kinetochore mono-orientation and is favoured by the "Monopolin complex". Such complex will dissociate from kinetochores in anaphase I. This way maternal centrosomes will separate from the paternal ones but sister chromatids will remain bound together thanks to their cohesion and chiasmata.

In **anaphase I** homologous chromosomes are separated and migrate to opposite poles where new nuclei will be formed (fig.2). At this point it is fundamental for the cell to separate homologs and not sister chromatids. Many proteins are involved in such control. In particular sister chromatids are kept together thanks to the cohesin protein complex, which is thought to form a ring structure around the DNA (Gruber et al., 2003). During meiosis several cohesin rings have to be assembled on the DNA both on the chromatid arms, to keep together homologue chromosomes, and on centromeric regions, to impede sister chromatid separation before Anaphase II. The fundamental element of the meiotic cohesin complex is the protein Rec8 (Klein et al., 1999; Watanabe et al., 2001).

In anaphase I, separase recognizes the phosphorylated Rec8 protein localized on the chromatid arms and degrades it (Clyne et al., 2003; Lee and Amon, 2003). Degradation of Rec8 causes loss of all the other proteins of the cohesin complex, thus allowing the separation of homologue chromosomes but not of sister chromatids, in which Rec8 is still present at the centromeres. Each chromosome in Anaphase I is made up of two sister chromatids, the so-called *diad.*

**Telophase I.** In this phase diads reach the opposite poles of the cell and new nuclear membranes start to form around each diploid nucleus (fig. 2).

Telophase is followed by **Citokinesis** in which two new nuclei are formed in two separate daughter cells. What follows is a brief interphase in which chromosomes are decondensed. Then, during the prophase of MII, they are immediately recondensed, without any additional DNA replication (fig.2).

- *Meiosis II*

The second meiotic division (MII) is quite similar to mitosis. In **prophase II** chromosomes are condensed and the nuclear membrane is dissolved, in **metaphase II** chromosomes are aligned on the metaphase plate with sister kinetochores attached to microtubule fibres originating from opposite poles. During **anaphase II** sister chromatids migrate to the opposite poles of the cell and in **telophase II** nuclear membranes are reconstructed (fig.2). The following **cytokinesis** gives rise to four haploid cells (fig.1b, fig.2).

## *Meiosis induction*

The decision to exit the proliferative state and begin meiosis marks the differentiation of progenitor cells into gametes. Like other developmental decisions, gamete production requires the execution of a unique transcriptional program. In meiosis the generation of haploid gametes requires both cell cycle regulators and meiosis-specific proteins. The decision to enter meiosis is achieved differently upon

organisms. In particular, in pluricellular organisms, stem cell differentation into germinal cells is thought to depend on external signals generated by nearby cells (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004).

In budding yeast, meiosis is induced in the presence of low nutrients and gives rise to spores. (Yamamoto, 1996; Honigberg e Purnapatre**,** 2003). In particular, the presence of low nutrients induces expression of two meiotic regulator genes *IME1* e *IME2* (inductors of meiosis). The *IME1* gene encodes for a transcriptional factor that enables the starting of a transcriptional program that regulates and induces numerous other regulators (Chu et al., 1998; Primig et al., 2000). In particular one of the targets of Ime1 is the *IME2* gene, that encodes for a meiosis-specific kinase homologous to CDK (cyclin-dependent kinase) that promotes the onset of pre-meiotic S phase through degradation of the inhibitor of the S phase cyclin-dependent kinase complexes (Dirick et al., 1998).

Ime2 has also the fundamental role of inhibitingubiquitin-dependent proteolysis, thus favouring the stabilization of B-type cyclins (Clb5 and 6). Stable B-type cyclins interact with the Cdc28 kinase, and are in turn necessary for the beginning of DNA replication and for chromosome segregation (Bolte et al., 2002). Hence, to start pre-meiotic DNA replication it is necessary to activate those S phase-CDK complexes composed by Clb5, Clb6 and Cdc28, which are also required during pre-mitotic S phase (Dirick et al., 1998; Stuart e Wittenberg, 1998; Benjamin et al., 2003).

Although pre-meiotic and pre-mitotic DNA replications appear quite similar, since they share not only the same regulators but also the same replicative mechanisms, they are indeed quite different (Newlon, 1988; Simchen, 1973). In fact, as previously mentioned, pre-meiotic S phase is much longer than pre-mitotic S phase and this is probably due to the presence of auxiliary factors needed to trigger the interactions between homolog pairs, which are necessary for a correct chromosome segregation at MI.

Not only the pre-meiotic S-phase but also meiotic progression is strictly regulated by CDK. As for vegetative cells, in which entry into anaphase depends on high levels of CDK activity and exit from anaphase depends on loss of this activity, meiotic cells require high

CDK activity for entering both anaphase I and anaphase II and loss of CDK activity for exiting such phases. In particular CDK activity is regulated by cyclins Clb1, Clb4 and Clb5 during MI and by Clb3 and Clb5 during MII (Carlile and Amon, 2008).

## MEIOTIC RECOMBINATION

Meiosis reduces cellular chromosome content from diploid to haploid. This involves two consecutive cell divisions, meiosis I and meiosis II, following a single round of DNA replication. The first meiotic division separates the maternal and paternal versions of each chromosome, known as homologous chromosomes, whereas sister chromatids segregate during meiosis II. For proper homologue segregation to occur, homologues must first pair with each other and then become physically joined, so that they orient together on the meiotic spindle. As previously mentioned, reciprocal recombination between homologous non-sister chromatids, combined with inter-sister cohesion, allows the establishment of physical connections (chiasmata) between homologous chromosomes that is essential for their correct alignment and segregation at meiosis I (Marston et al., 2004; Petronczki et al., 2003). Chromosomes that fail to crossover, frequently fail to disjoin properly, yielding aneuploid gametes.
In any case, for crossovers to be functional in promoting meiosis I segregation in yeast, they must occur in the context of the synaptonemal complex (SC). The SC is formed by condensation of the sister chromatids along protein cores called axial elements (AEs). Synapsis is complete when AEs of homologous chromosomes are connected by proteins in the central region to form a tripartite structure (Page and Hawley, 2004) .
The molecular events of meiotic recombination have been most thoroughly described in *S. cerevisiae*. DNA double-strand break (DSB) formation is the prerequisite for initiation of meiotic recombination and requires the products of meiotic-specific genes, including the evolutionary conserved topoisomerase-like enzyme Spo11. Also the MRX (Mre11-Rad50-Xrs2) complex is necessary for meiotic DSB formation independently of its catalytic nuclease activity

(Alani et al., 1990; Ivanov et al., 1992; Nairz and Klein, 1997; Usui et al., 1998). Chromatin immunoprecipitation studies have shown that Mre11 transiently binds during meiotic prophase to DSB specific regions, and this association does not require DSB formation (Borde et al., 2004). Indeed, Mre11 association to DSB sites requires Xrs2, but not Rad50, suggesting that an intact MRX complex is not required for meiotic DSB formation (Borde et al., 2004). However, *rad50Δ* cells are defective for this process (Borde et al., 2004). Notably, *rad50*-hook mutants are also deficient in DSB formation and this defect can be suppressed by artificially reestablishing the dimerization of Rad50-hook variants (Wiltzius et al., 2005). This suggests that the activity of MRX via the Rad50 coiled-coil domain might bridge sister chromatids in order to establish a proper architecture of the sisters to be cleaved.

### *Meiosis-specific double-strand break (DSB) formation*

Spo11, together with several further factors, breaks both strands of a DNA molecule, creating a DSB with covalent linkages between the newly created 5′ DNA ends and a Spo11 catalytic tyrosine residue (Keeney, 2001) (fig.4). Removal of covalently bound Spo11 occurs by endonucleolytic cleavage of a few bases away from the break site, which frees an oligonucleotide with attached Spo11 (Neale et al., 2005) (fig.4). The MRX complex, together with Sae2, is likely responsible for this activity. In fact, *rad50s* and *sae2Δ* mutants allow Spo11-mediated DSBs formation, but are totally defective in the endonucleolytic removal of Spo11 from the 5′ DNA ends (Keeney and Kleckner, 1995; McKee and Kleckenr, 1997; Alani et al., 1990; Prinz et al., 1997). Seven of the nine reported *rad50s* hypomorphic mutations cluster on the Rad50 crystal structure to a narrow surface patch that has been proposed to form a protein interaction site (Hopfner et al., 2000). This raises the possibility that the *rad50s* mutations affect the interaction between Sae2 and Rad50 (Rattray et al., 2001). Separation of function *mre11* mutations, allowing DSB formation but not Spo11 removal, have also been identified (Moreau et al., 1999; Nairz and Klein, 1997; Furuse et al., 1998; Tsubouchi and

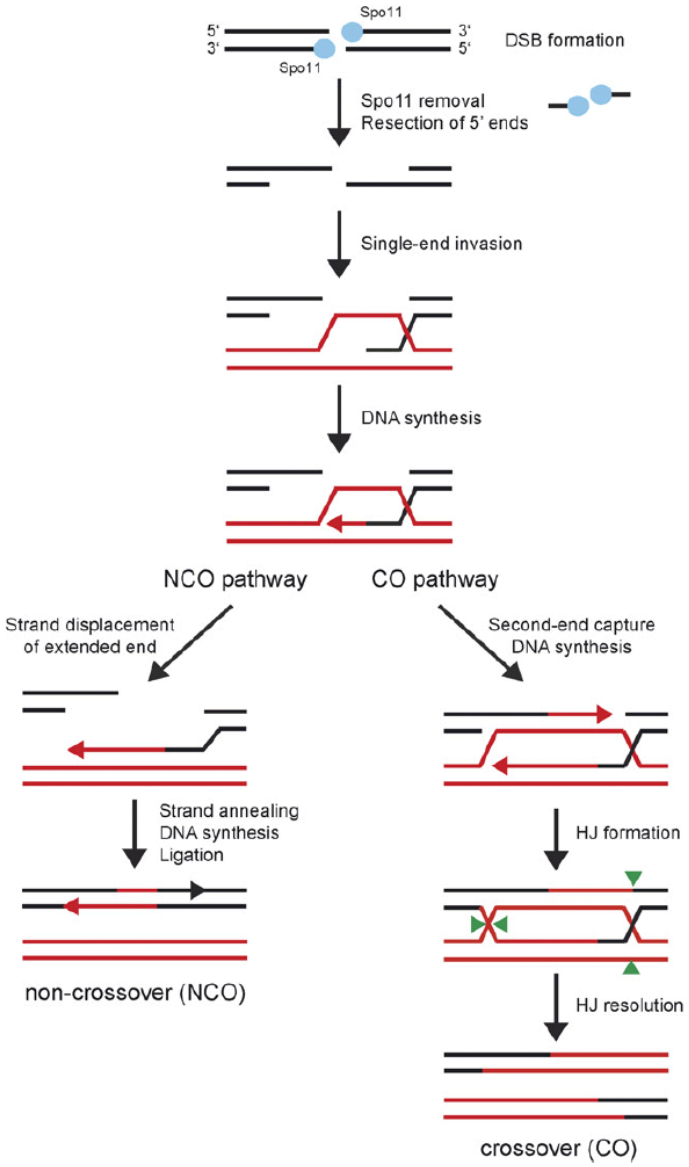



**Fig.4 The double-strand break repair model of meiotic recombination.** Homologs are indicated in black (paternal) and red (maternal). Spo11 generates a DSB in one of the parental chromatids. After Spo11 removal, DSB ends are resected to generate 3'- ended ssDNA tails and one 3'-ended ssDNA tail invades the duplex homologous DNA sequence (red lines). The intermediate generated in such a way is unstable and the decision to generate crossover (CO) or non-crossover (NCO) is made at this step of the process. In the NCO pathway, the transient strand invasion complex may be dissociated, allowing newly synthesized DNA to anneal to complementary ssDNA on the other side of the break. DNA synthesis and ligation yield a non-crossover product. In the CO pathway, the second 3' end invades or anneals with the displaced strand (second-end capture) and primes DNA synthesis. Ligation yields a double Holliday junction, whose resolution (green triangles) generates a mature product with exchanged flanking DNA.

Ogawa, 1998). These mutations, which alter residues in the N-terminal part of the protein containing the conserved phosphoesterase motifs, impair Mre11 nuclease activity (Moreau et al., 1999; Usui et al., 1998; Furuse et al., 1998). This suggests that Spo11 removal requires Mre11 nuclease activity that is known to be enhanced by Sae2. Moreover, it has been shown that also Sae2 exhibits an endonuclease activity (Lengsfeld et al., 2007), suggesting that this protein, possibly in cooperation with MRX, may allow Spo11 removal by mediating an endonucleolytic cleavage close to the DNA end.

Both MRX and Sae2 are involved in DSB processing also in mitotic cells. In particular, DSB resection is promoted by the activity of the cyclin-dependent protein kinase Cdk1 (Cdc28/Clb) during the S and $G_2$ cell cycle phases (Aylon et al., 2004; Ira et al., 2004) and this control relies on the phosphorylation of Sae2 Ser-267 by Cdk1 (Huertas et al., 2008), a mechanism that is conserved in the vertebrate homologue of Sae2, CtIP (Huertas and Jackson, 2009; Yun and Hiom, 2009). If the same regulation is also active for resection of programmed DSBs remains to be established. Moreover, *SAE2* or *MRE11* deletion impairs DSB resection in vegetative *S. cerevisiae* cells (Clerici et al., 2005; Ivanov et al., 1994; Clerici et al., 2006). Furthermore, Sae2 and MRX act in the same epistasis group to allow DSB resection in mitotic cells (Clerici et al., 2006), and are required to ensure efficient repair by single strand annealing (SSA) of both meiotic and mitotic DSBs (Clerici et al., 2005; Neale et al., 2002). Finally, they both participate in processing hairpin-containing DNA structures, and the Mre11 nuclease activity is essential for this process (Lobachev et al., 2002; Rattray et al., 2001). However, *SAE2* deletion only slows down resection at sites of clean DSBs in vegetative cells (Clerici et al., 2005; Ivanov et al., 1994), whereas it completely impairs resection of Spo11-induced DSBs (Nairz and Klein, 1997; Keeney and Kleckner, 1995; McKee and Kleckenr, 1997; Prinz et al., 1997). This is consistent with the hypothesis that Sae2 activity might be particularly important to initiate resection of DSB ends that are resistant to exonucleases because they bear protein–DNA crosslinks at their termini (such as Spo11-induced DSBs). It has been proposed recently that MRX and Sae2 catalyze a limited amount of DSB end resection in vegetative cells. The 3'-ended DNA ends are then rapidly

processed by either Exo1 or the RecQ helicase Sgs1, the latter acting in concert with the nuclease Dna2 (Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008). If the same resection pathways contribute to the formation of ssDNA in meiotic cells remains to be determined.

Interestingly, Sae2 undergoes checkpoint-mediated phosphorylation during meiosis, starting at the onset of premeiotic S phase, with a maximal peak at the time of meiotic DSB generation, and decreasing when DSBs are repaired by homologous recombination (Cartagena-Lirola et al., 2006). Moreover, failure to repair DSBs results in accumulation and persistence of phosphorylated Sae2 (Cartagena-Lirola et al., 2006). This phosphorylation depends on the DNA damage checkpoint kinases Mec1 and Tel1, the functional homologs of human ATR and ATM, respectively, whose simultaneous deletion also leads to the accumulation of unprocessed DSBs (Cartagena-Lirola et al., 2006). Mutation of the Sae2 S/T-Q motifs preferred for phosphorylation by Mec1/ATR and Tel1/ATM kinases abolishes Sae2 phosphorylation during meiosis and results in unprocessed DSB accumulation (Cartagena-Lirola et al., 2006). Although it is still unknown whether the unresected DNA ends in *mec1Δ tel1Δ* double mutants still have attached Spo11, this suggests that Tel1- and Mec1-mediated Sae2 phosphorylation is necessary for Sae2 nuclease activity and/or for the enhancement of the nuclease activity of Mre11, possibly by triggering a transient interaction between phosphorylated Sae2 and Rad50.

Putative orthologs of *S. cerevisiae* Sae2 have been identified in other organisms like *Schizosaccharomyces pombe* (Ctp1/Nip1), *Arabidopsis thaliana* (Com1/Sae2), *Caenorhabditis elegans* (Com1/Sae2) and *Homo sapiens* (CtIP). In humans, CtIP was originally identified as an interactor of the transcriptional repressor CtBP (Schaeper et al., 1998). It also interacts with the retinoblastoma protein RB (Fusco et al., 1998) and the tumour suppressor protein BRCA1 (Yu et al., 1998; Li et al., 1999). Like *S. cerevisiae* Sae2, both *S. pombe* Ctp1/Nip1 and human CtIP facilitate ssDNA formation at DSB ends in mitotic cells (Limbo et al., 2007; Sartori et al., 2007), suggesting that they might play a role similar to that of *S. cerevisiae* Sae2 also in meiosis. Consistent with this hypothesis, the lack of Ctp1/Nip1 affects *S.pombe*

spore viability (limbo et al., 2007) and causes accumulation of unrepaired meiotic DSBs (Akamatsu et al., 2008). Furthermore, *A. thaliana* and *C. elegans* Com1/Sae2 mutants are sterile, accumulate Spo11 during meiotic prophase and fail to form Rad51 foci despite the presence of unrepaired DSBs (Penkner et al., 2007; Uanschou et al., 2007).

### *Processing and repair of programmed meiosis-specific DSBs*

After Spo11 is removed from the 5' DNA ends by endonucleolytic cleavage, one or more nucleases resect the break to generate 3'-ended single-stranded DNA overhangs. The RecA like strand exchange proteins Rad51 and Dmc1 bind these tails to form presynaptic nucleoprotein filaments, which engage in the search for homologous template, with a strong preference towards the homologous chromosome rather than the sister chromatid (Bishop et al., 1992; Schwacha and Kleckner, 1997; Neale and Keeney, 2006). Meiotic DSBs ultimately yield two types of recombinants: crossovers (CO), with reciprocal exchange between homologue pairs, and non-crossovers (NCO), in which no reciprocal exchange occurs. These two types of recombinants can be generated by the cleavage of the dHJ (Fig. 4) in either the same or opposite direction.
However, some genetic data suggest that meiotic crossovers and non-crossovers derive from independent mechanisms. In fact, a number of *S. cerevisiae* mutants show reduced dHJ resolution and meiotic crossover formation without being affected in the formation of non-crossovers (Bishop and Zickler, 2004). Moreover, reducing the number of meiotic DSBs, and thus total recombination events, does not cause a parallel reduction in the number of crossovers (Martini et al., 2006). Instead, there is a tendency for crossovers to be maintained at the expense of non-crossovers, suggesting the existence of a genetically controlled mechanism to ensure formation of obligate crossovers (Martini et al., 2006). In fact, crossover formation is tightly regulated: each chromosome pair usually forms at least one (the "obligate crossover") and multiple crossovers on the same chromosome pair tend to be widely and evenly spaced (termed

"interference") (Bishop and Zickler, 2004). Failure to form a crossover and errors in crossover location are responsible for many of the inborn aneuploidies observed in humans (Hassold et al., 2007).
The decision to generate crossovers or non-crossovers is thought to occur well before Holliday junction resolution, prior to or during the formation of the first stable strand exchange intermediates (Bishop and Zickler, 2004; Allers and Lichten, 2001; Borner et al., 2004).
A model resembling the SDSA (Synthesis-dependent strand-annealing) mitotic recombination mechanism has been proposed (Fig. 4), in which one of the resected double-strand ends can initially invade the homologous duplex to form a displacement loop. This generates an asymmetric single-end invasion (SEI) intermediate, in which the invading end can be extended by limited DNA synthesis (Hunter and Kleckner, 2004). If the extended end of this intermediate is ejected, annealing of partner ends could lead to repair of the DSB, thus yielding to non-crossover products (Fig. 4, left). If the extended ends are not ejected, DNA synthesis primed by the invading strand extends the SEI intermediate, enabling it to anneal to the single-stranded tail on the other side of the break (second-end capture) (Fig. 4, right). Ligation then yields a double Holliday junction, which is resolved exclusively as crossovers.

## *The synaptonemal complex*

The pre-requisite for MI to occur properly is that homologous chromosomes pair and join prior to their segregation. During pachytene, homologous chromosomes are fully paired by a conserved structure called Synaptonemal complex (SC), whose proper formation requires meiotic recombination between homologs in many organisms. In fact, mutants defective in DSB formation or unable to process recombination intermediates have drastically reduced and/or abnormal SC (Keeney, 2001). Additionally, generation of exogenous DSBs by DNA damaging agents in the absence of normal meiotic DSBs allows partial SC formation in some cases (Celerin et al., 2000; Tesse et al., 2003; Romanienko et al., 2000) . However, the above relationship is not universal, as *D. melanogaster* and *C. elegans* form

normal SC even when unable to initiate meiotic recombination (McKim et al., 1998; Henderson et al., 2004). SC formation initiates unfrequently and fully synapsed chromosomes are rare in the absence of recombination in budding yeast (Celerin et al., 2000; Tesse et al., 2003; Henderson et al., 2004), indicating that DSB formation is required for the correct assembly of SC in this organism.

The SC begins to form during leptotene, when sister chromatids become organized into a linear loop of chromatin arrays that define a geometric axis. As leptotene proceeds, a small fraction of these bridges matures into structures known as axial associations or axial elements (fig.5). Afterwards, during pachytene, these structures bind the central element (CE) of the SC which function is to assemble and nearer the two axial elements and, therefore, the respective chromosomes, completing synapsis.

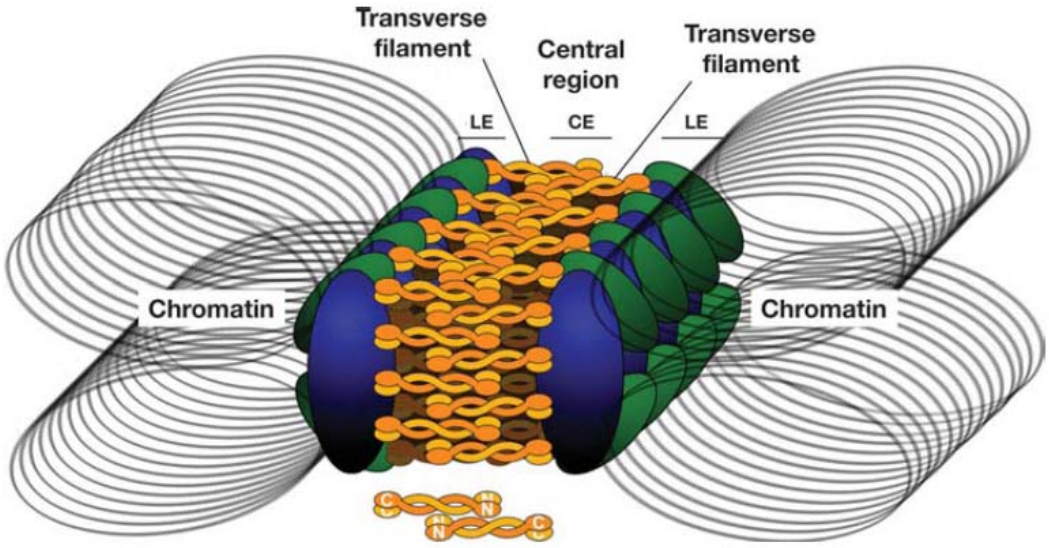



**Fig.5 Model of Synaptonemal Complex structure.** Shown is a cross section of a segment of the SC with lateral elements (LE), transverse filaments, central element (CE), and central region. The arrangement of transverse filament proteins, as determined experimentally for Zip1p is shown at bottom. Also shown is a hypothetical arrangement of cohesins/condensins (*blue ovals*) and other LE proteins (*green ovals*) along the LEs.

Once the SC is fully formed, the axial elements become lateral elements (LE) (fig.5) and the region between them is called the central region (CE) (fig.5). In this region dense structures called "nodules" are formed, which can be of two types: those present in zygotene (early nodules) and those present in pachytene (late nodules). The former are usually small and irregular in structure, while the latter are usually of bigger dimensions but of reduced number and could correspond to the total events of genetic cross-over events.
Many proteins of the SC have been characterized thanks to yeast mutants. In particular, synapsis between homologs is absent in *S. cerevisiae zip1* mutants, because of the lack of the major component of the SC, Zip1. (Sym et al., 1993). Moreover, the *S. cerevisiae* Red1 protein, which has been shown to associate with axial elements before synapsis formation and with lateral elements once the SC is formed (Smith e Roeder, 1997), is necessary for the formation of such elements and is localized on pachytene chromosomes (Smith e Roeder, 1997).
Many other proteins, among which the meiotic cohesin Rec8 (Klein et al., 1999) and the meiotic recombination checkpoint proteins Hop1 and Mek1 (Hollingsworth et al, 1989; Hollingsworth et al., 1997) contribute to the axial-structure formation. The absence of any of these proteins prevents proper assembly of the SC and therefore correct homolog pairing (Hollingsworth, 1989; Rockmill and Roeder, 1990; Loidl et al., 1994; Hollingsworth et al., 1997).

# MONITORING DNA DOUBLE-STRAND BREAKS (DSBs)

The genome of living organisms can suffer both spontaneous and induced DNA damage. Among the most deleterious DNA lesions are double-strand breaks (DSBs), whose failure to be repaired can lead to loss of genetic information and chromosome rearrangements. DSBs can arise accidentally during both mitosis and meiosis of eukaryotic cells, either by DNA replication problems or by exposure to environmental factors, such as ionising radiations or genotoxic drugs. Moreover, as previously described, they are introduced into the genome in a programmed manner to initiate meiotic recombination in germ cells. In both cases, DSBs can be repaired by homologous recombination (HR), which involves the interaction between DNA sequences with perfect, or near-perfect, homology. The primary function of HR in mitotic cells is to repair DSBs, whereas, during meiosis, HR is essential to establish a physical connection between homologous chromosomes, thus ensuring their correct disjunction at the first meiotic division. In addition, meiotic recombination promotes genetic diversity by creating new combinations of maternal and paternal alleles. DNA end processing and repair synthesis are required to ensure mitotic and meiotic DSB repair by HR, which also depends on the establishment of cohesin-dependent tethering between sister chromatids (Strom and Sjogren, 2007). Both accidental and programmed DSB repair are coupled to cell cycle progression by surveillance mechanisms, named DNA damage checkpoint and recombination checkpoint, which delay mitotic cell cycle progression and meiosis I, respectively, until DSB repair is achieved. These protective mechanisms are envisaged as signal-transduction cascades, where upstream sensors monitor and detect altered DNA molecules, while central transducers act in a protein kinase cascade to regulate a myriad of downstream effectors. In both DNA damage and recombination checkpoints, DSB detection is achieved by highly conserved protein kinases, including mammalian ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR), as well as their *Saccharomyces cerevisiae* orthologs Tel1 and Mec1 (Longhese et al., 2006; Shiloh, 2006). The checkpoint signal is then

propagated to the downstream targets in two different ways. While the DNA damage checkpoint requires the effector kinases Rad53 and Chk1, as well as their adaptor Rad9, the Mek1, Red1 and Hop1 meiotic proteins are specifically required to transduce the recombination checkpoint signal.

## THE DNA DAMAGE CHECKPOINT

In both yeast and humans, the DNA damage checkpoint serves at least two primary purposes: to arrest the cell cycle in response to DNA damage, thereby coordinating cell cycle progression with DNA repair capacity (Weinert et al., 1994), and to regulate transcription of DNA damage response genes, as well as activation and recruitment to damaged sites of various repair/recombination proteins (Longhese et al., 2006; Shiloh, 2006). This checkpoint can sense and signal the presence of accidental DNA lesions during both mitosis and meiosis. In fact, in *S. cerevisiae,* inactivation of the telomeric protein Cdc13, which causes the accumulation of large amounts of ssDNA at telomeres (Garvik et al., 1995), leads to checkpoint dependent cell cycle arrest in mitotic metaphase or in meiotic prophase (Garvik et al., 1995; Weber and Byers, 1992).

### *DNA damage checkpoint sensors*

In both yeast and human, DSB detection is achieved by protein kinases, including mammalian ATM and ATR, *S. cerevisiae* Tel1 and Mec1 and *S. pombe* Tel1 and Rad3 (Longhese et a., 2006; Shiloh et al., 2006). Both yeast Tel1 and human ATM appear to bind DNA through their interaction with the MRX and MRN complexes, respectively (Nakada et al., 2003; Falck et al., 2005). Rather than using MRX/MRN, Mec1, Rad3 and ATR function in a complex with Ddc2 (Paciotti et al., 2000; Rouse and Jackson, 2000; Wakayama et al., 2001), Rad26 (Edwards et al., 1999) and ATRIP (Cortez et al., 2001), respectively. These complexes seem to respond directly to DNA insults. In fact, Mec1 and Ddc2 are recruited to DNA damaged

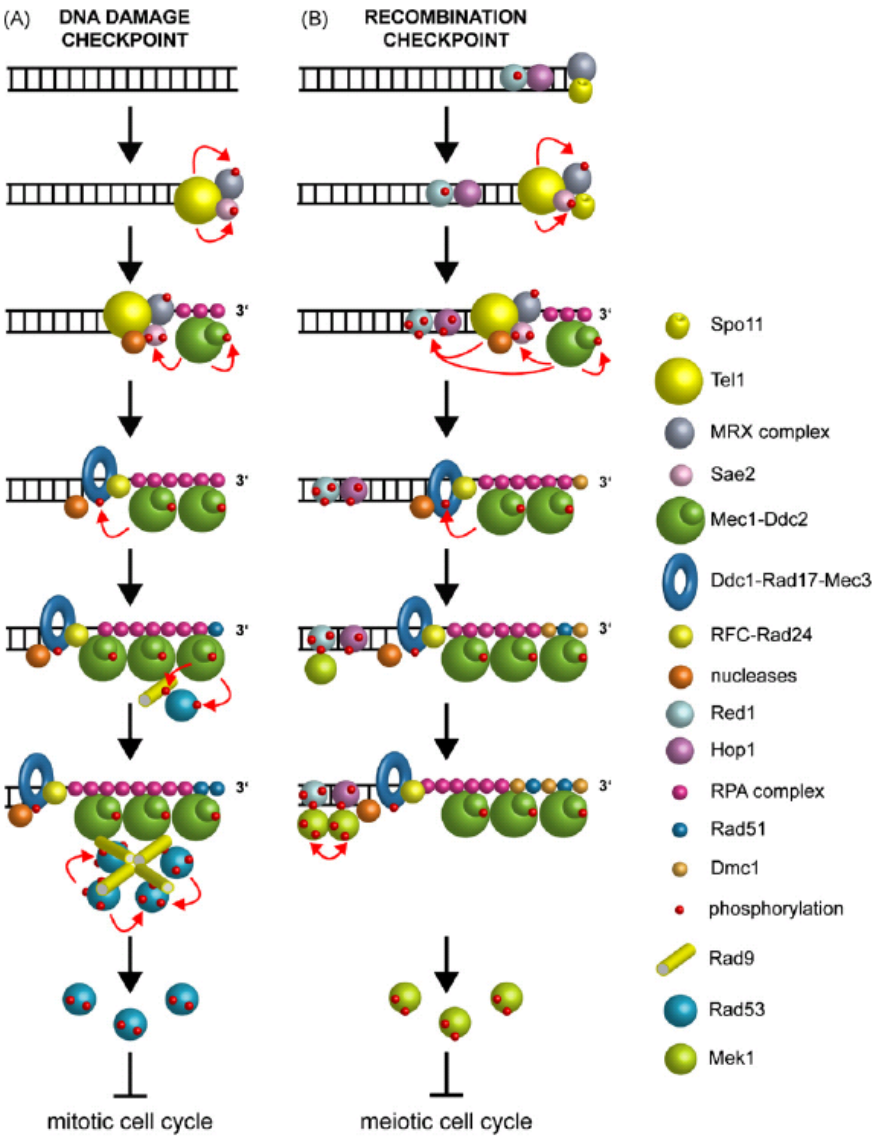
sites independently of other checkpoint proteins (Kondo et al., 2001; Melo et al., 2001), and *in vivo* Ddc2 and Rad26 phosphorylation does not require known checkpoint factors other than Mec1 and Rad3, respectively, suggesting a pivotal role for these kinases in sensing DNA alterations (Paciotti et al., 2000; Edwards et al., 1999). Two other important regulators of Mec1/ATR activity are a clamp and a clamp loader, which show homology to the replication clamp proliferating cell nuclear antigen (PCNA) and its loader replication factor C (RFC), respectively (Majka and Burgers, 2004). The *S. cerevisiae* checkpoint clamp is a heterotrimer consisting of the Ddc1, Rad17 and Mec3 subunits, whose human and *S. pombe* orthologues are called Rad9, Rad1 and Hus1 (Weinert et al., 1994; Longese et al., 1996; Longhese et al., 1997; Paciotti et al., 1998). The *S. cerevisiae* loader, Rad24-RFC, consists of the four small RFC subunits (Rfc2–5) that interact with Rad24, the orthologue of *S. pombe* and human Rad17. While the Mec1/Ddc2 and the ATR/ATRIP complexes can be recruited to the sites of DNA damage independently of the checkpoint clamp in both yeast and human cells (Kondo et al., 2001; Melo et al., 2001; Zou et al., 2002), the presence of the clamp stimulates the kinase activity of Mec1 (Majka et al., 2006). Thus, through a mechanism resembling the loading of PCNA by RFC during DNA replication, the Rad24-RFC complex recruits the checkpoint clamp Ddc1-Rad17-Mec3 complex onto DNA structure. When loaded onto ssDNA, this clamp enhances Mec1 ability to transmit and amplify the DNA damage signals, thus providing a mechanism to up regulate Mec1 kinase activity.

### *Detection of accidental DSBs and DNA damage checkpoint initiation*

Although Tel1/ATM and Mec1/ATR share some of their downstream effectors, the DNA damage signals that evoke these two kinases are distinct. While human ATM plays a primary role in the response to DSBs, Mec1/ATR controls the response to a much broader spectrum of DNA damage. Based on the finding that Tel1/ATM association to

the break site is transient and that Tel1 ability to sense and transduce the DSB signal is disrupted when DSB ends are subjected to 5'–3' exonucleolytic degradation (Mantiero et al., 2007), it has been proposed that Tel1/ATM and MRX/MRN are recruited to blunt or minimally processed DSB ends to initiate DSB signalling (Fig. 6A).

**Fig.6 DNA damage and recombination checkpoint pathways in budding yeast.**
**(a) Sensing and signalling accidental DSBs by the DNA damage checkpoint.** When a DSB occurs, the MRX complex and other factors are the first proteins that localize to the unprocessed break. Recognition of DSBs by MRX allows checkpoint activation by recruiting Tel1. Tel1 in turn hosphorylates Sae2, which is recruited to DSB ends independently of MRX. MRX, Sae2 and Tel1 contribute to resection of DSB ends by exonucleases to generate 3-ended ssDNA tails coated by RPA, which allow the loading of Mec1-Ddc2 and subsequent Mec1-dependent checkpoint activation. Mec1 activation is also supported by independent loading of the Ddc1-Rad17-Mec3 complex by Rad24-RFC. Once recruited to the DSB ends, Mec1 phosphorylates Rad9, which promotes the recruitment of inactive Rad53 in an FHA-dependent manner, thus allowing its activatory phosphorylation by Mec1. Phosho-Rad9 facilitates Rad53 in trans autophosphorylation by increasing the local concentration of Rad53 molecules. Active Rad53 kinase molecules are then released from the complex and can phosphorylate downstream targets to arrest mitotic cell cycle progression.
**(b) Sensing and signalling programmed meiotic DSBs by the recombination checkpoint**. Hop1-phospho-Red1 complexes are assembled onto DNA prior to DSB formation. Spo11, MRX and other proteins catalyze the formation of programmed meiotic DSBs. MRX allows checkpoint activation by recruiting Tel1, which in turn phosphorylates Sae2. Introduction of a DSB results in both Hop1 phosphorylation and additional Red1 phosphorylation. When Spo11 is removed from the DSB end, DSB resection by exonucleases generates 3-ended ssDNA tails coated by RPA, Rad51 and/or Dmc1, which allow the loading of Mec1 and subsequent Mec1-dependent checkpoint activation. Phospho-Red1 allows recruitment of Mek1 via the FHA domain. Phosphorylated Hop1 promotes dimerization of Mek1, which undergoes in trans autophosphorylation. Active Mek1 kinases are then released from the complex and can phosphorylate downstream targets to arrest meiotic cell cycle progression.


(A) DNA DAMAGE CHECKPOINT
(B) RECOMBINATION CHECKPOINT
3'
mitotic cell cycle
meiotic cell cycle
Spo11
Tel1
MRX complex
Sae2
Mec1-Ddc2
Ddc1-Rad17-Mec3
RFC-Rad24
nucleases
Red1
Hop1
RPA complex
Rad51
Dmc1
phosphorylation
Rad9
Rad53
Mek1

On the contrary, the versatility of ATR/Mec1 in the DNA damage response suggests that this pathway is likely able to sense a common signal generated by different types of DNA damage. A simple structure commonly generated at the sites of DNA repair and stressed replication forks is ssDNA-coated by RPA. *S. cerevisiae* Mec1-Ddc2 and human ATR-ATRIP complexes recognize RPA-coated ssDNA regions that can arise after DSB processing (Fig. 6A). In fact, ATR does not localize at damaged sites in the absence of RPA, which also stimulates *in vitro* ATRIP binding to ssDNA in human cells (Zou and Elledge; 2003). Similarly, Ddc2 and Mec1 are recruited to DSBs in an RPA-dependent manner (Zou and Elledge; 2003). Several lines of evidence indicate that the signalling event for DSB-induced checkpoint activation is the recruitment of the MRX/MRN complex to the break site (Fig. 6A). First, MRX/MRN binds directly to DNA and its initial recruitment at DSBs is transient and it occurs independently of any other DNA damage response protein examined so far (Lisby et al., 2004; Lukas et al., 2003; Moreno-Herrero et al., 2005). Moreover, it is required for Tel1/ATM association to DSB lesions through the interaction between the C-terminal motif of Xrs2/Nbs1 and Tel1/ATM (Nakada et al., 2007; Falck et al., 2005; You et al., 2005). Finally, both introduction of the hypermorphic *rad50s* allele and deletion of the *SAE2* gene prolong MRX occupancy at DSBs (Clerici et al., 2006; Lisby et al., 2004), and constitutively upregulate Tel1/ATM signalling (Clerici et al., 2006; Usui et al., 2001; Morales et al., 2005). The observation that also elimination of Mre11 nuclease activity results in this same phenotype (Clerici et al., 2006; Lisby et al., 2004) suggests that Sae2 limits MRX-mediated checkpoint activation by stimulating the nuclease activity of MRX and its subsequent dissociation from the DNA. Both the DNA repair and checkpoint functions of Sae2 appear to be regulated by Mec1- and Tel1-dependent phosphorylation after accidental DNA damage (Baroni et al., 2004). In fact, elimination of the predicted Mec1- and Tel1-dependent Sae2 phosphorylation sites leads to increased persistence of Mre11 at DSBs and upregulation of Tel1-dependent signalling, as it does deletion of *SAE2* (Clerici et al., 2006). Thus, Mec1 and Tel1, once activated, might limit MRX-dependent checkpoint signalling by phosphorylating Sae2. In humans, the MRN/ATM complex, once recruited to DSBs, not only initiates

the checkpoint response, but also promotes DSB resection and formation of ssDNA, the critical intermediate structure for both HR repair and ATR-dependent signalling (Falck et al., 2005; Adams et al., 2006; Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006). The checkpoint functions of the budding yeast ATM orthologue, Tel1, appear more furtive than those of ATM. In fact, Tel1 deficient cells do not show obvious hypersensitivity to DNA damaging agents and are not defective in checkpoint activation in response to a single HO-induced DSB, which depends primarily on Mec1 (Clerici et al., 2006; Mantiero et al., 2007; Ira et al., 2004). This suggests that DSB-induced checkpoint activation is seemingly different in human and *S. cerevisiae* cells. However, recent evidences indicate that Tel1 can activate the checkpoint response to DSBs independently of Mec1, although its signalling activity becomes apparent only in the presence of multiple DSBs (Mantiero et al., 2007). Moreover, it contributes to generate 3'-ended ssDNA leading to Mec1-dependent checkpoint activation (Mantiero et al., 2007). Thus, although Tel1 contribution to the checkpoint can be masked by the prevailing activity of Mec1, the mechanism governing ATM and ATR-dependent checkpoint activation in humans seems to operate also in *S. cerevisiae*. Generation of RPA-coated ssDNA overhangs at the DSB termini and ATR/Mec1-dependent checkpoint activation also requires the activity of cyclin-dependent kinases (CDK) (Jazayeri et al., 2006; Ira et al., 2004), whose sequential action determines cell cycle progression. In particular, inhibition of Cdk1 activity in G2-arrested cells prevents DSB processing and HO-induced DSB repair by HR (Ira et al., 2004; Aylon et al., 2004). This led to a model, where Cdk1 activation in S and G2 phases allows DSB resection, thus committing the break to HR. However, although low Cdk1 activity does not impair MRX and, presumably, Tel1 recruitment to DSB ends (Ira et al., 2004), DSBs in G1 are not capable to activate a Tel1-dependent checkpoint. This suggests that Clb/Cdk1 activity is necessary also to promote downstream events in the checkpoint cascades. Consistent with this hypothesis, active Clb/Cdk1 complexes are required to activate the Tel1-dependent checkpoint after replication of UV-damaged DNA (Clerici et al., 2004). Since the checkpoint adaptor Rad9 it is known to undergo a Cdk1-dependent

phosphorylation (Ubersax et al., 2003), it is possible that Cdk1 activity may influence checkpoint signal transduction by regulating Rad9 phosphorylation.

### *Activation of the DNA damage checkpoint*

Once DNA perturbations are sensed, the checkpoint signals are propagated through evolutionarily conserved protein kinases, which are called Rad53 and Chk1 in *S. cerevisiae*, and Chk2 and Chk1 in humans, respectively. While Rad53 is required for proper response to DNA damage in all the cell cycle phases, budding yeast Chk1 contributes only to the activation of the G2/M checkpoint (Sanchez et al., 1999). The activation of both Rad53 and Chk1 is not governed by their simple interaction with Mec1 or Tel1, but requires a stepwise process. In particular, it has been proposed that the Rad9 protein acts first as an adaptor to mediate the interaction between Mec1 and Rad53 (Sweeney et al., 2005), and then as a scaffold to allow Rad53 autophosphorylation and activation (Gilbert et al., 2001) (Fig. 6A). The current model proposes that Mec1 phosphorylates Rad9 after detection of DNA lesions and phosphorylated Rad9 recruits Rad53 to DNA lesions in a manner that depends on the Rad53 Fork Head Associated (FHA) domains (Sun et al., 1998; Durocher et al., 1999). Mec1 then phosphorylates on multiple sites Rad53 molecules that are bound to Rad9, thus allowing Rad53 to become active. Moreover, Mec1-dependent phospho-Rad9 completes the Rad53 activation process by facilitating in trans Rad53 autophosphorylation, perhaps by increasing the local Rad53 concentration on the Rad9 surface (Fig. 6A).
Active Rad53 kinase molecules are then released from the complex and can phosphorylate downstream targets to arrest mitotic cell-cycle progression.

## MEIOTIC RECOMBINATION CHECKPOINT

Chromosome segregation prior to the completion of meiotic DSB repair and recombination can result in loss or missegregation of entire chromosome arms and in the formation of aneuploid gametes, conditions frequently associated to birth defects. Not surprisingly, a pathway called recombination or pachytene checkpoint, ensures accurate segregation of homologous chromosomes at the first meiotic division (fig.6B). Similar to the DNA damage checkpoint pathway, which delays progression through mitosis in response to accidental DNA damage, the recombination checkpoint, once meiotic DSBs are formed, delays entry into anaphase I until completion of meiotic DSB repair, thus ensuring generation of viable gametes with balanced genetic information. The recombination checkpoint, which has been studied in more detail in *S. cerevisiae*, can be observed in many organisms, including *C. elegans*, *D. Melanogaster* and mice (Hochwagen and Amon, 2006). For example, mice lacking the meiotic HR proteins Dmc1, Hop2 or Msh5 experience a block in gametogenesis followed by apoptosis of germ cells (de Rooij and de Boer, 2003). Interestingly, inactivation of ATM in Dmc1−/− mouse spermatocytes does not result in the bypass of the meiotic cell cycle arrest, suggesting that ATM promotes meiotic DSB repair (Barchi et al., 2005; Di Giacomo et al., 2005). Moreover, cells of the female germline of *C. elegans* hermaphrodites with meiotic DSB repair defects are removed by apoptosis. This programmed cell death is induced in the pachytene stage of meiotic prophase, and requires the checkpoint proteins Rad-5, MRT-2 and HUS-1, the latter being the ortologues of the *S. cerevisiae* DNA damage checkpoint proteins Rad17 and Rad24, respectively (Gartner et al., 2000).

### *Detection of programmed meiotic DSBs and initiation of the recombination checkpoint*

Mechanistically, the recombination checkpoint is related to the DNA damage checkpoint. In fact, the protein kinases Mec1 and Tel1, as

well as their accessory complexes Rad24-RFC and Ddc1-Rad17-Mec3, known to be required for the DNA damage checkpoint, are necessary also for the recombination checkpoint (Lydall et al., 1996) (fig.6). Similar to the DNA damage checkpoint, DSB blunt ends or 3'-ended ssDNA arising from their processing activate the recombination checkpoint in a Tel1/ATM- and Mec1/ATR-dependent manner, respectively (Fig. 6B). Unlike in mitosis, where DSB resection cannot be completely abolished, unprocessed meiosis-specific DSBs are stably generated in *S. cerevisiae sae2Δ* or *rad50s* mutants. In fact, in the absence of Sae2 or in the presence of the Rad50s variants, Spo11 remains covalently attached to DSB ends that therefore
cannot be resected (Keeney and Kleckner, 1995; McKee and Kleckner, 1997; Alani et al., 1990; Prinz et al., 1997). These meiotic aberrant intermediates activate the meiotic recombination checkpoint, and this activation depends primarily on Tel1 (Usui et al., 2001). Thus, similar to the function of Tel1/ATM in sensing and signalling accidentally broken blunt ends (Mantiero et al., 2007), Tel1/ATM is capable of signalling unresected meiosis-specific DSBs (Fig. 6). The recombination checkpoint signal is then propagated primarily by the Mek1 kinase and its regulators Red1 and Hop1 (Xu et al., 1997).
Meiosis specific 3'-ended ssDNA are instead generated in *S. cerevisiae* cells lacking Dmc1, which is required for the initial strand-invasion step of meiotic recombination. These mutant cells are competent to remove Spo11 from the DSB ends, but they accumulate large amounts of hyper-resected DSBs with unusually long single-stranded tails, due to their failure to engage in inter-homologue repair (Bishop et al., 1992). Similarly to the DNA damage checkpoint, where generation of 3'-ended ssDNA results in Mec1/ATR recruitment and Mec1/ATR-dependent checkpoint activation (Zou and Elledge, 2003), activation of the recombination checkpoint in *dmc1Δ* mutants is dependent on Mec1 and on its activators Rad24, Ddc1 and Rad17 (Lydall et al., 1996) (Fig. 6). It remains to be determined whether ssDNA-coated by RPA is the signalling event for Mec1 activation also in meiosis. Because RPA can directly compete with Rad51 and Dmc1 for binding to ssDNA, Rad51 and/or Dmc1 nucleoprotein filaments may also constitute a signal for Mec1 (Fig. 6). Then, activation of the downstream targets requires Mek1, Red1 and Hop1, like in *sae2Δ* and

*rad50s* cells (Xu et al., 1997; Niu et al., 2005; Niu et al., 2007) (Fig. 6B). Apparently, Tel1 is not required to activate the recombination checkpoint in *dmc1Δ* cells. However, because Tel1 appears to detect unprocessed DSBs in both mitosis and meiosis (Mantiero et al., 2007; Usui et al., 2001) and the meiotic-specific DSB ends are rapidly resected, the time window for Tel1 to sense and signal meiotic DSBs in *dmc1Δ* cells is transient and can be masked by the prevailing activity of Mec1. Alternatively, Tel1 ability to sense and signal meiotic DSBs is minor compared to that of Mec1. Consistent with this hypothesis, despite the persistence of meiotic DSBs, *sae2Δ* and *rad50s* cells display a transient Tel1 dependent meiotic delay, whereas *dmc1Δ* cells exhibit a permanent Mec1-dependent meiotic block (Usui et al., 2001; Xu et al., 1997). Thus, whether DSBs are programmed or arise accidentally, the Tel1 and Mec1 kinases can detect them in a similar manner, leading to a model for both DNA damage and recombination checkpoint, in which Tel1/ATM can sense and signal unprocessed DSBs. Once DSB resection occurs, generation of 3'-ended ssDNA leads to Mec1/ATR recruitment and subsequent Mec1/ATR-dependent checkpoint activation (Fig. 6).

## *The meiotic recombination checkpoint signal transduction cascade*

The recombination checkpoint signals are transduced to the downstream effectors by the meiosis-specific Mek1, Red1 and Hop1 proteins (Xu et al., 1997) (fig.6B), which are structural components of the meiotic chromosome axes and physically interact with each other (Bailis and Roeder, 1998; de los Santos and Hollingsworth, 1999). Mek1 is a protein kinase, whose activity is necessary to maintain the checkpoint-dependent cell cycle arrest of meiotic recombination mutants (Bailis and Roeder, 1998; de los Santos and Hollingsworth, 1999; Wan et al., 2004). Its activation in response to DSBs shares similarities with activation of the checkpoint kinase Rad53 after accidental DNA damage, suggesting that Mek1 may be the meiotic paralogue of Rad53. Indeed, both Rad53 and Mek1 possess a

phosphospecific FHA domain, which is required for their activation as kinases and mediates their interactions with Rad9 and Red1, respectively (Sun et al., 1998; Durcher et al., 1999;Wan et al., 2004). Meiotic DSB formation leads to Hop1 phosphorylation (Niu et al., 2005), and phosphorylated Hop1, once bound to Mek1, promotes Mek1 dimerization, which in turn enables Mek1 kinase in trans autophosphorylation of T327 and T331 residues (Niu et al., 2005; Niu et al., 2007; Wan et al., 2004). Hop1-Mek1 interaction and Mek1 activation requires the FHA domain of Red1 (Bailis and Roeder, 1998; Wan et al., 2004), suggesting that the Mek1 FHA-phospho-Red1 interaction positions Mek1 molecules for autophosphorylation in the dimerized state (Fig. 6B). Hop1 has been recently shown to contain a functional conserved motif that is a [S/T]Q cluster domain (SCD) comprising three adjacent sites (S298, S311, T318) that are targeted by the Mec1/Tel1 kinases (Carballo et al., 2008). Elimination of Mec1/Tel1 mediated phosphorylation within the Hop1 SCD in $hop1^{SCD}$ mutants prevents chromosomal localization and phosphorylation of Mek1, consistent with Hop1 acting as an adaptor of Mek1 in the Mec1/Tel1 signalling pathway (fig.6B) (Carballo et al., 2008). Still to be resolved in this scenario is the function of Red1, which interacts with Hop1 (de los Santos et al., 1999; Woltering et al., 2000). Although Hop1 orthologs have been identified in other organisms ranging from fission yeast to humans (Lorenz et al., 2004; Chen et al., 2005), the [S/T]Q sites important in budding yeast meiosis are only conserved among the fungal and plant orthologs (Carballo et al., 2008). Similarly, Mek1 is found only among fungal species, suggesting that the mechanism underlying transduction of the meiotic recombination checkpoint signal may vary among different organisms.

### *Meiotic recombination checkpoint targets*

The Ndt80 (Hepworth et al., 1995 and 1998; Tung et al., 2000), Swe1 (Leu et al., 1999) and Sum1 (Lindgren et al., 2000) proteins have been shown to be targets of the meiotic recombination checkpoint. Ndt80 is a global transcriptional activator of the middle-sporulation-specific genes including *CLB1*, and controls entry into the meiotic divisions by

regulating the activity of Cdc28 (Xu et al., 1995) and of the polo-like kinase Cdc5 (Sourirajan et al., 2008). The absence of Ndt80 causes cells to arrest at the pachytene stage of prophase I. In particular it has been proposed that Cdc5 is the only member of the Ndt80-dependent transcriptome to be required for progression through meiosis (Sourirajan et al., 2008). In fact, *CDC5* expression in *ndt80Δ* cells efficiently promotes the exit from pachytene (Sourirajan et al., 2008). Moreover, Ndt80 activity seems to be dependent on phosphorylation. In fact, Ndt80 is phosphorylated during a normal meiosis, but it is less abundant and hypophosphorylated in cells arrested in pachytene, where Ndt80-dependent genes are not expressed (Tung et al., 2000; Pak and Segall, 2002; Benjamin et al., 2003).
The activity of Ndt80 is inhibited by Sum1, a DNA-binding repressor of Ndt80, which competes with Ndt80 for binding the promoters of middle-sporulation-specific genes (Xie et al., 1999). The amount of Sum1 protein decreases transiently during a normal meiosis midway through sporulation (Lindgren et al., 2000), while it is stabilized in *dmc1Δ* cells, but not in *dmc1Δ rad17Δ* cells, which bypass the pachytene arrest signal (Lindgren et al., 2000).
Swe1 is the third known target of the meiotic recombination checkpoint and encodes for a kinase that inhibits Cdc28 activity through its phosphorylation on the tyrosine 19 residue (Booher et al., 1993). Unlike the case of *S. pombe*, this inhibitory phosphorylation of Cdc28 is not required in *S. cerevisiae* for the mitotic cell cycle arrest that is mediated by the DNA damage checkpoint (Amon et al., 1992; Sorger et al., 1992). Moreover, also Swe1 is regulated by phosphorylation, as it has been found to be hyperphosphorylated in pachytene arrested cells (Leu et al., 1999).

### *Meiotic checkpoint proteins acting in the barrier to sister chromatid repair*

Several lines of evidence indicate that the DNA damage checkpoint components shared by the recombination checkpoint perform important functions in the normal recombination machinery, rather than just monitoring meiotic recombination defects. For instance, loss

of Mec1 functions leads to a number of meiotic defects, including reduced recombination frequency, reduced spore viability, loss of inter-homolog bias and of crossover control, and aberrant chromosome synapsis (Kato et al., 1994; Carballo et al., 2007). Moreover, ATM deficient mice cells show high frequency of spontaneous chromosomal aberrations, intrachromosomal recombination and error-prone recombination during meiosis (Xu et al., 1996; Pandita et al., 2004), while ataxia telangiectasia (A-T) patients display gonadal atrophy and spermatogenetic failure, a phenotype that is mirrored by ATM-deficient mice (Pandita et al., 2004; Richardson et al., 2004). Finally, mutations in the *Drosophila melanogaster* mei-41 gene, encoding the ATM ortholog, reduce meiotic recombination frequency (Carpenter et al., 1979).

The Mec1, Rad17, Rad24 and Mec3 checkpoint proteins are also involved in promoting meiotic recombination between non-sister homologous chromatids rather than between sister chromatids. In fact, mutations in *MEC1, RAD17, RAD24* and *MEC3* reduce inter-homologue recombination frequency, while they increase the rate of ectopic recombination events and of illegitimate repair using sister chromatids (Grushcow et al., 1999; Thompson et al., 2003; Shinohara et al., 2003).

Interhomologue bias during meiotic recombination also requires the meiosis-specific checkpoint proteins Mek1, Hop1 and Red1 (Schwacha et al., 1997; Xu et al., 1997; Niu et al., 1995; Thompson et al., 1999). In fact, conditional inhibition of Mek1 kinase activity impairs inter-homologue recombination and allows DSB repair using sister chromatids in *dmc1Δ* cells. Moreover, increased activity of the strand exchange protein Rad51 can suppress the inter-homologue recombination defects of *dmc1Δ* cells (Xu et al., 1997; Bishop et al., 1999; Tsubouchi et al., 2006) in a Mek1-dependent manner (Niu et al., 2005). It has also been recently demonstrated that Mek1-dependent phosphorylation of Rad54 decreases its interaction with Rad51, thus reducing the total number of Rad51/Rad54 complexes which are necessary for sister chromatid recombination (Niu et al., 2009).

These data indicate that Mek1 activation is required to ensure recombination between homologue non-sister chromatids by suppressing intersister chromatid recombination (Niu et al., 2005; Niu

et al., 2009). In this context, the fact that Mec1, Rad17, Rad24, Mec3, Red1 and Hop1 act upstream of Mek1 in the recombination checkpoint suggests that they could help to prevent sister chromatid recombination by activating Mek1.

## AIMS OF THIS STUDY

Both accidental and programmed DSB repair are coupled to cell cycle progression by surveillance mechanisms, named DNA damage checkpoint and recombination checkpoint, which delay mitotic cell cycle progression and meiosis I, respectively, until DSB repair is achieved. In both DNA damage and recombination checkpoints, DSB detection is achieved by highly conserved protein kinases, including mammalian ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR), as well as their *S. cerevisiae* orthologs Tel1 and Mec1. The checkpoint signal is then propagated to the downstream targets in two different ways. While the DNA damage checkpoint requires the effector kinase Rad53 and its adaptor Rad9, the recombination checkpoint signal is propagated through the meiosis specific proteins Mek1, Red1 and Hop1, which are central components of meiotic chromosomes and are involved in several aspects of meiotic recombination. Mek1 activation in response to meiotic DSBs shares similarities with activation of the checkpoint kinase Rad53 after accidental DNA damage, suggesting that Mek1 may be the meiotic Rad53 paralogue.

Although Mek1 is required to activate the recombination checkpoint, the issue of whether Rad53 still plays a role in transducing the checkpoint signals in response to programmed meiotic DSBs was never determined. The work done during the first year of my PhD aimed to study the role of Rad53 in responding to DSBs during meiosis as well as its inter-relationships with Mek1. Although mechanistically the meiotic recombination checkpoint resembles the DNA damage checkpoint, we show that Rad53 is not phosphorylated and activated as soon as programmed meiosis-specific DSBs occur, suggesting that such DSBs are hidden from the canonical Rad53-

dependent DNA damage checkpoint machinery. Instead, checkpoint signal arising from meiotic programmed DSBs is specifically propagated through the meiosis specific proteins Mek1, Red1 and Hop1, which are central components of meiotic chromosomes indicating that the system monitoring programmed meiotic DSBs is an integral part of the chromosome structure formed during meiosis. However, Rad53 phosphorylation is triggered when unrepaired meiotic DSBs escape the recombination checkpoint-mediated prophase I arrest. This Rad53 phosphorylation and activation result in the slowing down of meiosis II, indicating the presence of an additional checkpoint that might provide a salvage mechanism preventing chromosome rearrangements and/or loss in the gametes even in the absence of the recombination checkpoint.

Programmed DSB formation requires meiosis-specific gene products, including the evolutionary conserved topoisomerase-like enzyme Spo11, as well as the three components of the MRX complex (Mre11-Rad50-Xrs2) (Longhese et al., 2009). In particular, a Spo11 dimer coordinately breaks both DNA strands, creating a DSB with covalent linkages between the 5′ DNA ends and the catalytic tyrosine residue of each Spo11 monomer (Keeney and Kleckner, 1995). Then, Spo11 must be removed by endonucleolytic cleavage to allow further DSB end processing by 5′-3′ resection that is required to initiate homologous recombination (Neale et al., 2005). This event is promoted by the Sae2 protein and the MRX complex, which are required to catalyze the endonucleolytic removal of Spo11-linked oligonucleotides (Keeney and Kleckner, 1995; Usui et al., 1998; Uanschou et al., 2007). Because DSBs are highly hazardous for genome stability, commitment to DSB resection and meiotic progression must be tightly regulated to ensure proper DSB repair. In vegetative *Saccharomyces cerevisiae* cells, DSB resection is promoted by the activity of the cyclin-dependent protein kinase Cdk1 (Cdc28/Clb) during the S and $G_2$ cell cycle phases (Aylon et al., 2004; Ira et al., 2004). This control relies on the phosphorylation of Sae2 Ser-267 by Cdk1 (Huertas et al., 2008) in a mechanism that is conserved in the vertebrate homologue of Sae2, CtIP (Huertas and Jackson, 2009; Yun and Hiom, 2009). Because Cdk1 activity is required to generate Spo11-induced DSBs (Henderson et al., 2006;

Wan et al., 2008), its involvement in allowing their processing was never assessed. Moreover, after Spo11 removal from the 5′ DSB ends, one or more so far unknown nucleases have to resect the break to generate 3′-ended single-stranded DNA (ssDNA) overhangs to initiate homologous recombination. Candidates for such activity are the nucleases Exo1 and Dna2 and the helicase Sgs1, which all contribute to resect DSB and chromosome ends in mitotic *S. cerevisiae* cells (Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008; Bonetti et al., 2009).

During the last two years of my PhD we investigated if even the processing of meiosis-specfic DSBs is regulated by CDK through phosphorylation of Sae2 and we characterized the nucleases involved in such processing . We show that phosphorylation of the Ser-267 residue of *S. cerevisiae* Sae2 by Cdk1 is required to initiate resection of meiotic DSBs. In fact, substitution of Sae2 Ser-267 with a non-phosphorylatable residue severely impairs both Spo11 removal and DNA-end processing, which instead take place efficiently when an aspartic residue mimicking constitutive phosphorylation replaces Sae2 Ser-267. Moreover, we demonstrate that further processing of Spo11-induced DSB ends depends on the nuclease Exo1 and on the helicase Sgs1 that act in two different pathways and that the nuclease Dna2 appears to contribute mainly to long range resection.

The data obtained during my three-year PhD and described in this experimental thesis provided relevant new insight into both the dynamics of DSB signaling and the regulation of programmed DBS processing during meiosis. In particular, the results achieved were published in the two articles that are presented in the “Results” section and discussed integrally in the “Discussion” section. Moreover, some of them were reported in the two following reviews of which I am co-author:

Longhese MP, Bonetti D, Guerini I, Manfrini N and Clerici M (2009). DNA double-strand breaks in meiosis: Checking their formation, processing and repair. *DNA Repair* 8 1127–113.

Longhese MP, Bonetti D, Manfrini N and Clerici M (2010). Mechanisms and regulation of DNA end resection. *The EMBO Journal* 29, 2864–2874.

# RESULTS

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# Role of the *Saccharomyces cerevisiae* Rad53 Checkpoint Kinase in Signalling Double-Strand Breaks during the Meiotic Cell Cycle

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Chromosomal breaks can occur at unpredictable locations in the genome of eukaryotic cells as a result of ionizing radiation, radiomimetic chemicals, or DNA replication across nicked DNA. Moreover, they are introduced in a programmed manner to initiate meiotic recombination during meiosis, the specialized differentiation process in which two rounds of chromosome segregation follow one round of DNA replication (reviewed in Longhese et al., 2008; Marston and Amon, 2004). In the first meiotic division (meiosis I) homologous chromosomes pair and separate, whereas sister chromatids segregate from each other in the second division (meiosis II). Both unprogrammed and programmed double-strand breaks (DSBs) can be repaired by homologous recombination. The primary function of homologous recombination in mitotic cells is to repair DSBs, whereas, during meiosis, it is essential to establish a physical connection between homologous chromosomes, thus ensuring their correct pairing and subsequent segregation at the first meiotic division. In any case, for crossovers to be functional in promoting meiosis I execution in *Saccharomyces cerevisiae*, they must occur in the context of a proteinaceous tripartite structure, the synaptonemal complex, which connects the axes of homologs along their entire lengths via a close-packed array of transverse filaments (reviewed in Page and Hawley, 2004).

Programmed meiotic DSB formation requires the product of the meiosis-specific gene *SPO11*, which, together with several other factors, breaks both strands of a DNA molecule, creating a DSB with covalent linkages between the newly created 5′ DNA ends and a Spo11 catalytic tyrosine residue (Keeney et al., 1997; Keeney and Kleckner, 1995). In *S. cerevisiae*, the highly conserved Sae2 protein and the MRX (Mre11-Rad50-Xrs2) complex catalyze the endonucleolytic cleavage of Spo11 from the 5′ DSB ends (Furuse et al., 1998; McKee and Kleckner, 1997; Moreau et al., 1999; Nairz and Klein, 1997; Neale et al., 2005; Prinz et al., 1997). After Spo11 removal, one or more nucleases resect the break to generate 3′-ended single-stranded DNA (ssDNA) overhangs. The RecA-like strand exchange proteins Rad51 and Dmc1 bind such tails to form presynaptic nucleoprotein filaments, which engage in the search for homologous templates (reviewed in Neale and Keeney, 2006).

Both accidental and programmed DSB repair are coupled to cell cycle progression by surveillance mechanisms, named DNA damage checkpoint and recombination checkpoint, which delay mitotic and meiotic cell cycle progression, respectively, until DSB repair is achieved (reviewed in Hochwagen and Amon, 2006; Longhese et al., 2008; Longhese et al., 2006). Mechanistically, the two checkpoints are related to each other. In fact, DSB detection is accomplished in both cases by highly conserved protein kinases, among which mammalian ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR), as well as their *S. cerevisiae* orthologues Tel1 and Mec1. During the mitotic cell cycle, Tel1/ATM appears to bind unprocessed DSBs via the MRX/MRN complex, and its signaling activity is disrupted when DSB termini are resected (Mantiero et al., 2007; Nakada et al., 2003). By contrast, Mec1/ATR is thought to recognize ssDNA regions that arise after DSB processing (Zou and Elledge, 2003).

During meiosis, unrepaired programmed DSBs are stably generated in *S. cerevisiae sae2Δ* or *rad50s* mutants, where Spo11 remains covalently attached to the DSB ends that therefore cannot be resected (McKee and Kleckner, 1997; Prinz et al., 1997). These meiotic aberrant intermediates activate a Tel1-dependent recombination checkpoint, which slows down meiosis I (Usui et al., 2001; Xu et al.,

1997). Unrepaired meiosis-specific DSBs with unusually long single-stranded tails are instead generated in *S. cerevisiae* cells lacking the strand exchange protein Dmc1 (Bishop et al., 1992). These cells are competent to remove Spo11 from the DSB ends but are defective in strand invasion. Similarly to the DNA damage checkpoint, where generation of 3′-ended ssDNA results in Mec1 recruitment and Mec1-dependent checkpoint activation (Zou and Elledge, 2003), activation of the recombination checkpoint in *dmc1Δ* mutants is dependent on Mec1 and its regulators Rad24 and Rad17 (Lydall et al., 1996). Despite the persistence of unrepaired meiotic DSBs, *sae2Δ* and *rad50s* cells display only a transient Tel1-dependent delay of meiosis I, whereas *dmc1Δ* cells exhibit a permanent Mec1-dependent meiosis I block (Usui et al., 2001; Xu et al., 1997), suggesting that Tel1 can sense and signal meiotic DSBs less efficiently than Mec1. Consistent with this hypothesis, Mec1 responds to a single DSB in mitosis, whereas Tel1 signaling activity becomes apparent only when multiple DSBs are generated in the absence of Mec1 (Mantiero et al., 2007). Interestingly, Sae2 undergoes Mec1- and Tel1-dependent phosphorylation during meiosis, with a peak at the time of DSB generation (Cartagena-Lirola et al., 2006). Mutations altering the Sae2 [S/T]Q motifs preferred for phosphorylation by ATM/ATR-like kinases lead to the accumulation of unprocessed DSBs, as does the simultaneous absence of Mec1 and Tel1 (Cartagena-Lirola et al., 2006), suggesting that the latter may allow DSB resection by phosphorylating Sae2.

Propagation of the checkpoint signals to the downstream targets occurs in two different ways, depending on whether the checkpoint response is elicited by accidental DSBs or by programmed meiotic DSBs. In fact, DNA damage checkpoint activation requires the effector kinase Rad53 and its adaptor Rad9 (Gilbert et al., 2001; Sweeney et al., 2005). Rad9 first promotes Mec1-Rad53 interaction and Mec1-mediated Rad53 phosphorylation/activation (Sweeney et al., 2005) and then acts as a scaffold to facilitate in *trans* Rad53 autophosphorylation (Gilbert et al., 2001). Despite their essential role in activating the DNA damage checkpoint in response to mitotic DSBs, Rad9 and Rad53 do not appear to be involved in controlling meiosis I progression in response to meiotic programmed DSBs

(Lydall et al., 1996). This control instead requires the meiosis-specific proteins Mek1, Red1, and Hop1. In particular, meiotic DSB formation leads to Mec1- and Tel1-dependent Hop1 phosphorylation, which is required for Mek1 activation (Carballo et al., 2008; Niu et al., 2007; Niu et al., 2005; Wan et al., 2004). However, inactivation of *HOP1*, *RED1*, or *MEK1* in *dmc1Δ* cells leads to efficient repair of the breaks via intersister recombination, indicating that meiotic progression in these cells is a consequence of inappropriate repair rather than an arrest relief (Niu et al., 2007; Niu et al., 2005; Xu et al., 1997).
In this study, we investigated the role of Rad53 in responding to DSBs during meiosis. We show that Rad53 is not phosphorylated and activated as soon as programmed meiosis-specific DSBs occur, suggesting that such DSBs are hidden from the canonical Rad53-dependent DNA damage checkpoint machinery. However, Rad53 phosphorylation is triggered when unrepaired meiotic DSBs escape the recombination checkpoint-mediated prophase I arrest. This Rad53 phosphorylation and activation result in the slowing down of meiosis II.

***Rad53 phosphorylation in response to accidental or programmed DSBs during meiosis.***

Mec1- and Tel1-dependent phosphorylation of Mek1 and Rad53 is required for their activation as protein kinases and can be detected as changes in their electrophoretic mobility (Carballo et al., 2008; Gilbert et al., 2001; Niu et al., 2007; Sanchez et al., 1999; Sun et al., 1998; Sweeney et al., 2005). It is known that Mek1 phosphorylation is induced by programmed DSBs that occur during meiosis (Bailis and Roeder, 1998; Carballo et al., 2008), whereas exogenous DNA lesions trigger Rad53 phosphorylation during mitosis (Sanchez et al., 1999). Thus, we asked whether treatment with DSB-inducing agents of cells undergoing meiosis in the absence of programmed DSBs, due to the lack of Spo11, could trigger Rad53 and Mek1 phosphorylation. Because meiotic programmed DSBs occur after completion of premeiotic DNA synthesis (time = 210 min), *spo11Δ* cells were treated for 30 min with the radiomimetic drug phleomycin 210 min after meiosis induction (Fig. 7A). As expected, due to their inability to generate meiotic DSBs, *spo11Δ* cells failed to phosphorylate Mek1

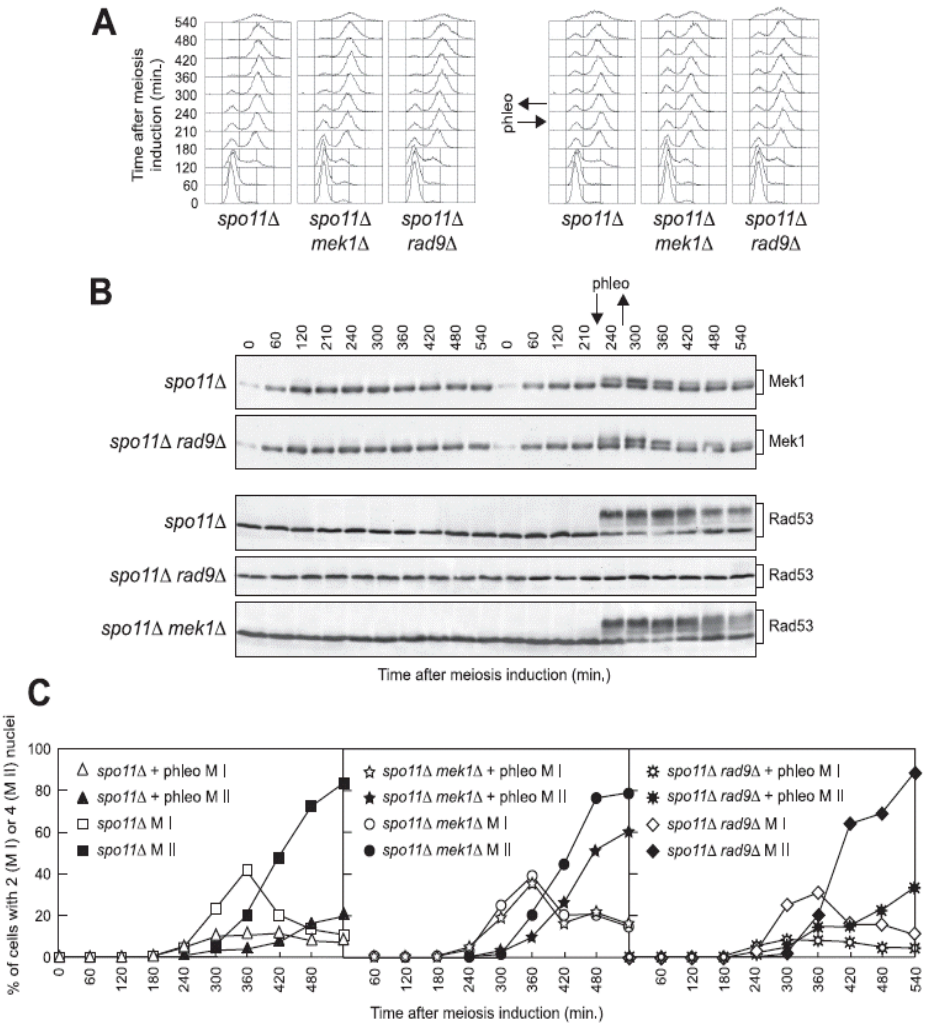


**FIG.7 Rad53 phosphorylation in response to chemically induced DSBs during meiosis I**. *spo11Δ* and *spo11Δ rad9Δ* diploid cells expressing Mek1-HA3 from the *MEK1* promoter, as well as *spo11Δ mek1Δ* diploid cells, were grown to stationary phase in YPA medium and then resuspended in SPM medium at time zero. At 210 min after transfer to SPM, half of each cell culture was incubated for 30 min in the presence of 5 µg/ml of phleomycin. Cell samples were collected at the indicated time points after transfer to SPM to analyze DNA content by fluorescence-activated cell sorting analysis (A); the phosphorylation pattern of Mek1 (B, top) and Rad53 (B, bottom) by Western blot analysis with anti-HA and anti Rad53 antibodies, respectively; and the percentages of binucleate (completed meiosis I [M I]) and tetranucleate (completed meiosis II [M II]) cells (C) by fluorescence microscope analysis of propidium iodide-stained cells. In all Western analysis, the same quantity of total protein extracts was loaded in each lane according to Coomassie blue staining.

after meiosis induction in the absence of phleomycin (Fig. 7B, top). In contrast, both Rad53 and Mek1 were phosphorylated after phleomycin addition in *spo11Δ* cells
(Fig. 7B), indicating that exogenous DNA damage during meiosis can trigger both Rad53 and Mek1 phosphorylation. Phleomycin-treated *spo11Δ* cells slowed down meiosis I compared to the untreated cells, and this delay was dependent on the recombination checkpoint (Fig. 7C). In fact, phleomycin-treated *spo11Δ mek1Δ* cells underwent meiosis I with kinetics similar to those of untreated *spo11Δ mek1Δ* cells and faster than those of phleomycin-treated *spo11Δ* cells (Fig. 7C). In contrast, when Rad53 phosphorylation was prevented by eliminating its regulator Rad9 (Fig. 7B, bottom), phleomycin-treated *spo11Δ rad9Δ* cells still slowed down meiosis I (Fig. 7C), indicating that Rad53 activation was not responsible for this delay. Thus, although Rad53 can be phosphorylated and activated in response to chemically induced DSBs, it does not induce arrest of meiosis I. Interestingly, although phleomycin-treated *spo11Δ mek1Δ* cells performed meiosis I with kinetics similar to those of the isogenic untreated cells, they still suffered a 60-min delay of meiosis II (Fig. 7C), suggesting that the phleomycin-induced meiosis II delay was not simply the consequence of the meiosis I delay. The finding that Rad53 can be phosphorylated in response to chemically induced DSBs during meiosis prompted us to ask whether it can be phosphorylated/activated in response to meiosis-specific DSBs. Because the inability to repair meiotic DSBs is known to activate the recombination checkpoint, we analyzed the pattern of Rad53 and Mek1 phosphorylation not only during an unperturbed meiosis but also when DSB repair was prevented by the lack of Dmc1 (Fig. 8A). We also monitored in the same samples the kinetics of DSB formation at the *THR4* hot spot (Fig. 8B) by Southern blot analysis of EcoRI-digested genomic DNA run on a native agarose gel. As shown in Fig.8, Mek1 was phosphorylated in both wild-type and *dmc1Δ* cells after transfer to sporulation medium, concomitantly with DSB formation (time = 210 min). Then, phosphorylated Mek1 decreased when DSBs were repaired in wild-type cells (time = 300 min), whereas it persisted until the end of the experiment in *dmc1Δ* cells (Fig. 8A, top), which remained arrested with undivided nuclei (data not shown) and

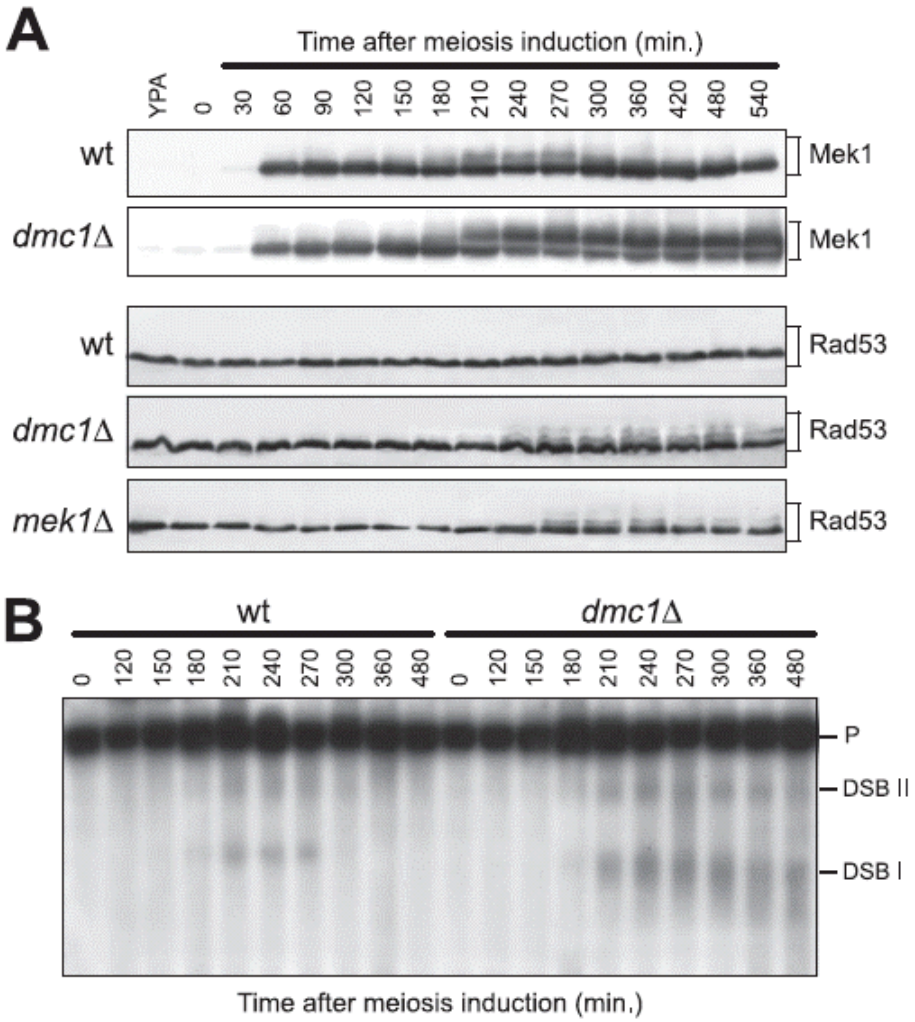


**FIG. 8 Rad53 phosphorylation in response to programmed DSBs during meiosis I**. Wild-type (wt) and *dmc1Δ* diploid cells expressing Mek1-HA3 from the *MEK1* promoter and *mek1Δ* diploid cells were grown to stationary phase in YPA medium and then resuspended in SPM at time zero. Cell samples were collected at the indicated time points after transfer to SPM to analyze phosphorylation of Mek1 (A, top) and Rad53 (A, bottom) as in Fig. 7B and meiotic DSB formation by Southern blot analysis (B). Southern blotting was performed on EcoRI-digested genomic DNA run on a native agarose gel, and the filter was hybridized with a probe complementary to the 5′ noncoding region of the *THR4* gene. This probe reveals an intact parental EcoRI fragment (P) of 7.9 kb and two bands of 5.7 and 7.1 kb corresponding to the two prominent meiotic DSB sites (DSB I and DSB II).

accumulated unrepaired hyperresected DSBs (Fig. 8B). In contrast, Rad53 was not phosphorylated in wild-type cells and only slightly in *dmc1Δ* diploid cells after transfer to SPM medium (Fig. 8A, bottom). The inability to phosphorylate Rad53 was not due to competition with Mek1 for Mec1- and Tel1-mediated phosphorylation. In fact, Rad53 was only slightly phosphorylated even in meiotic *mek1Δ* cells (Fig. 8A, bottom). Thus, meiosis-specific programmed DSBs fail to trigger Rad53 phosphorylation both in the presence and in the absence of Mek1.

***Targeting Rad53 to Mec1 results in Rad53 phosphorylation in response to meiotic DSB formation in both wild-type and dmc1Δ cells.***

Because Mec1 and Tel1 are required to phosphorylate and activate Mek1 in response to meiotic programmed DSBs, whereas chemically induced DSBs trigger Rad53 phosphorylation by the same kinases, the choice of using Mek1 instead of Rad53 in the recombination checkpoint cannot likely be exerted at the levels of Mec1- and/or Tel1-mediated DSB recognition. One possibility is that the DSB signals can be easily transduced to Mek1, Red1, and Hop1, because they are structural components of the meiosis-specific chromosome structure, whereas Rad53 activation might be prevented by its inability to detect Mec1/Tel1 signaling at meiotic DSBs. If this were the case, artificial targeting of Rad53 to Mec1 by fusing it with the Mec1 regulatory subunit Ddc2 (Paciotti et al., 2000) should result in Rad53 phosphorylation/activation in response to meiotic DSB formation. In order to analyze this possibility, wild-type and *dmc1Δ* cells were transformed with a plasmid carrying a *DDC2-RAD53*-FLAG in-frame fusion, whose expression was driven by the *RAD53* upstream regulatory sequences (Lee et al., 2004). The encoded Ddc2-Rad53 fusion protein was shown to be activated in a Mec1- and Tel1-dependent manner upon DNA damage and to circumvent the Ddc2, Rad24, Rad17, Mec3, and Rad9 requirement for Rad53 activation (Lee et al., 2004). It is known that Rad53 activation requires Mec1- and Tel1-dependent phosphorylation, which allows Rad53 molecules to undergo in *trans* autophosphorylation, thus completing the Rad53 activation process (Gilbert et al., 2001; Pellicioli et al., 1997; Sweeney

et al., 2005). We therefore monitored the activation of the Ddc2-Rad53 chimera by evaluating its electrophoretic mobility by Western blot analysis, as well as its autophosphorylation activity by an ISA (Pellicioli et al., 1999). As a control for Rad53 activation, we analyzed also isogenic cells expressing a Ddc2-Rad53kd fusion, where Ddc2 was fused to the kinase-defective Rad53K227A D339A variant (Lee et al., 2004). As shown in Fig. 9A, wild-type and *dmc1Δ* cells expressing either the Ddc2-Rad53 or the Ddc2-Rad53kd chimeras initiated and completed premeiotic S phase with similar kinetics. Strikingly, slowly migrating forms of the Ddc2-Rad53 fusion, presumably due to phosphorylation events, appeared in both wild-type and *dmc1Δ* cells (Fig. 9B concomitantly with meiotic DSB formation (time = 210 min; Fig. 9C). Then, they decreased in wild-type cells when DSBs were repaired (time = 360 min), whereas they were detectable as the predominant forms until the end of the experiment in *dmc1Δ* cells (Fig. 9B), where DSBs could not be repaired (data not shown). Consistent with the finding that Rad53 phosphorylation was not induced by meiotic DSB formation (Fig. 8A), the endogenous Rad53 protein did not show significant changes in electrophoretic mobility in either wild-type or *dmc1Δ* cells (Fig. 9B). When the same extracts were tested by ISA, a phosphorylated form corresponding to the Ddc2-Rad53 fusion protein was detected in both wild-type and *dmc1Δ* cells (time = 210 to 240 min) (Fig. 9D), and the corresponding phosphorylation reaction was dependent on Rad53 kinase activity. In fact, no phosphorylated forms were detectable by ISA in either wild-type or *dmc1Δ* cells expressing the Ddc2-Rad53kd fusion protein (Fig. 9D). Moreover, the simple proximity of Rad53 to Ddc2 in the Ddc2-Rad53 fusion was not sufficient to activate Rad53 in the absence of meiotic DSBs. In fact, similarly to the Ddc2-Rad53 mobility shift, Rad53 autophosphorylation activity became detectable in wild-type cells at the time of DSB formation (time = 210 to 240 min) and it decreased when DSBs were repaired (time = 360 min) (Fig. 9C and D), while it persisted until the end of the experiment in *dmc1Δ* cells that failed to repair meiotic DSBs (Fig. 9D) and arrested with undivided nuclei due to the presence of Mek1 (data not shown). Thus, targeting Rad53 to Mec1 through its fusion with Ddc2 triggers Rad53 activation in response to meiotic programmed DSBs. Consistent with

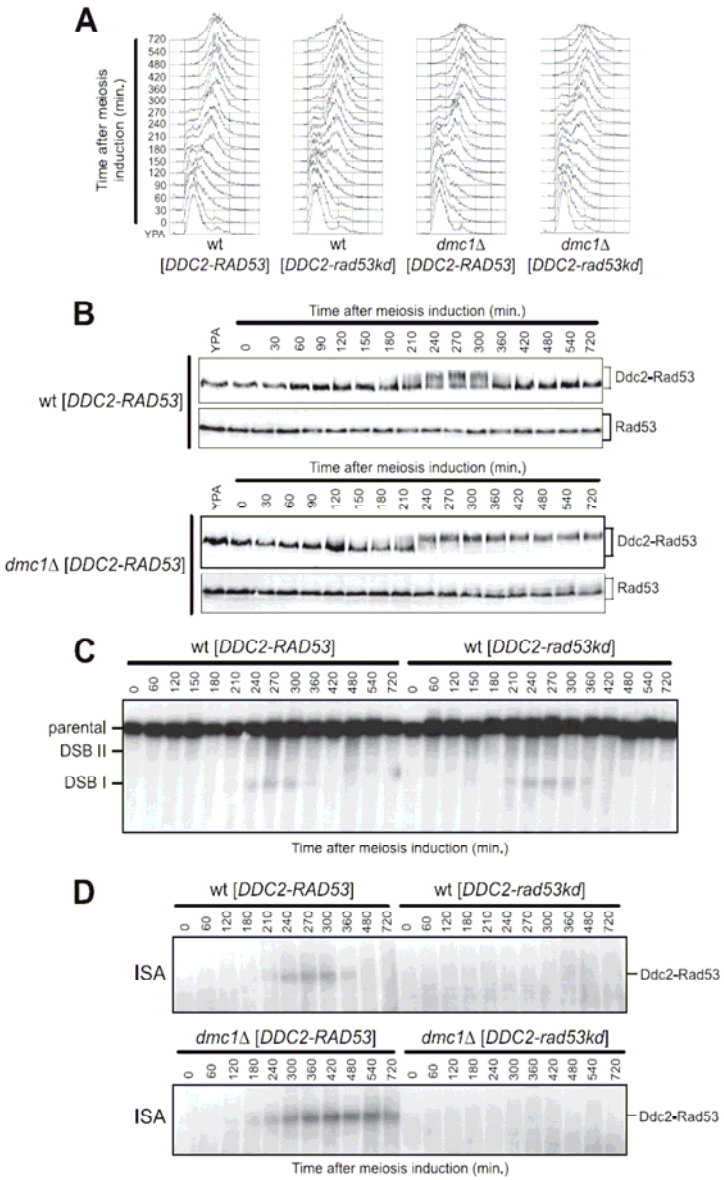


**FIG. 9 Targeting Rad53 to Mec1 results in Rad53 activation in response to m DSB formation**. Wild-type (wt) and *dmc1Δ* diploid cells, carrying the pRS316 *DDC2-RAD53-3FLAG* plasmid (*DDC2-RAD53*) or the pRS316 *DDC2-rad53K227A D339A-3FLAG* plasmid (*DDC2-rad53kd*), were grown to stationary phase in YPA and then resuspended in SPM at time zero. Cell samples were collected at the indicated time points after transfer to SPM to analyze DNA content by fluorescence-activated cell sorting analysis (A) and DSB formation (C) by Southern blot analysis on EcoRI-digested genomic DNA as described for Fig. 8B. Total protein extracts were prepared from the indicated strains and subjected to Western blot analysis with anti-FLAG and anti-Rad53 antibodies (B) and to ISA (D).

our previous observation that Rad53 activation by exogenous DSBs did not result in meiosis I delay, wild-type cells expressing the Ddc2-Rad53 or the Ddc2-Rad53kd fusion protein progressed through meiosis with similar kinetics (data not shown).

***Execution of meiosis I with unrepaired meiosis-specific DSBs triggers Rad53 phosphorylation.***

If the chromosome structure specifically formed during meiosis I inhibits Rad53 access to the meiotic DSB signals, unrepaired meiotic DSBs might be capable of inducing Rad53 phosphorylation once homologous chromosomes have separated from each other and cells enter meiosis II. Because the lack of Sae2 allows meiotic cells to perform meiosis I in the presence of unprocessed DSBs (Fig. 10C and D) (Usui et al., 2001; X et al., 1997), we monitored Rad53 phosphorylation in *sae2Δ* cells after meiosis induction (Fig. 10A and B). Rad53 phosphorylation was detectable in *sae2Δ* cells about 300 min after meiosis induction, and it was DSB dependent, because it was prevented in *spo11Δ sae2Δ* cells (Fig. 10B, top). Rad53 phosphorylation in *sae2Δ* cells occurred concomitantly with homologous chromosome segregation (time = 300 min) (Fig. 10B, top, and C), well after DSB formation (Fig. 10D), whereas Mek1 phosphorylation became detectable in the same *sae2Δ* cells at the time of meiotic DSB formation (time = 210 min) (Fig. 10B, bottom, and D). This suggests that unrepaired meiotic DSBs become capable of activating Rad53 after homologous chromosome segregation, whose inhibition might therefore prevent Rad53 phosphorylation in meiotic *sae2Δ* cells. To address this point, we monitored Rad53 phosphorylation in *sae2Δ* cells lacking the meiosis-specific transcription factor Ndt80, which is required to activate transcription of middle meiosis genes (Chu and Herskowitz, 1998) and whose lack causes meiotic cells to arrest at the pachytene stage of meiosis I (Xu et al., 1995). We found that Rad53 was not phosphorylated in *sae2Δ ndt80Δ* cells (Fig. 10B, top), which, as expected, failed to divide nuclei (Fig. 10C). The inability of *sae2Δ ndt80Δ* cells to phosphorylate Rad53 was not due to the failure to generate meiotic DSBs, because both these cells and *sae2Δ* cells phosphorylated Mek1 and accumulated unrepaired meiotic DSBs with similar kinetics (Fig.

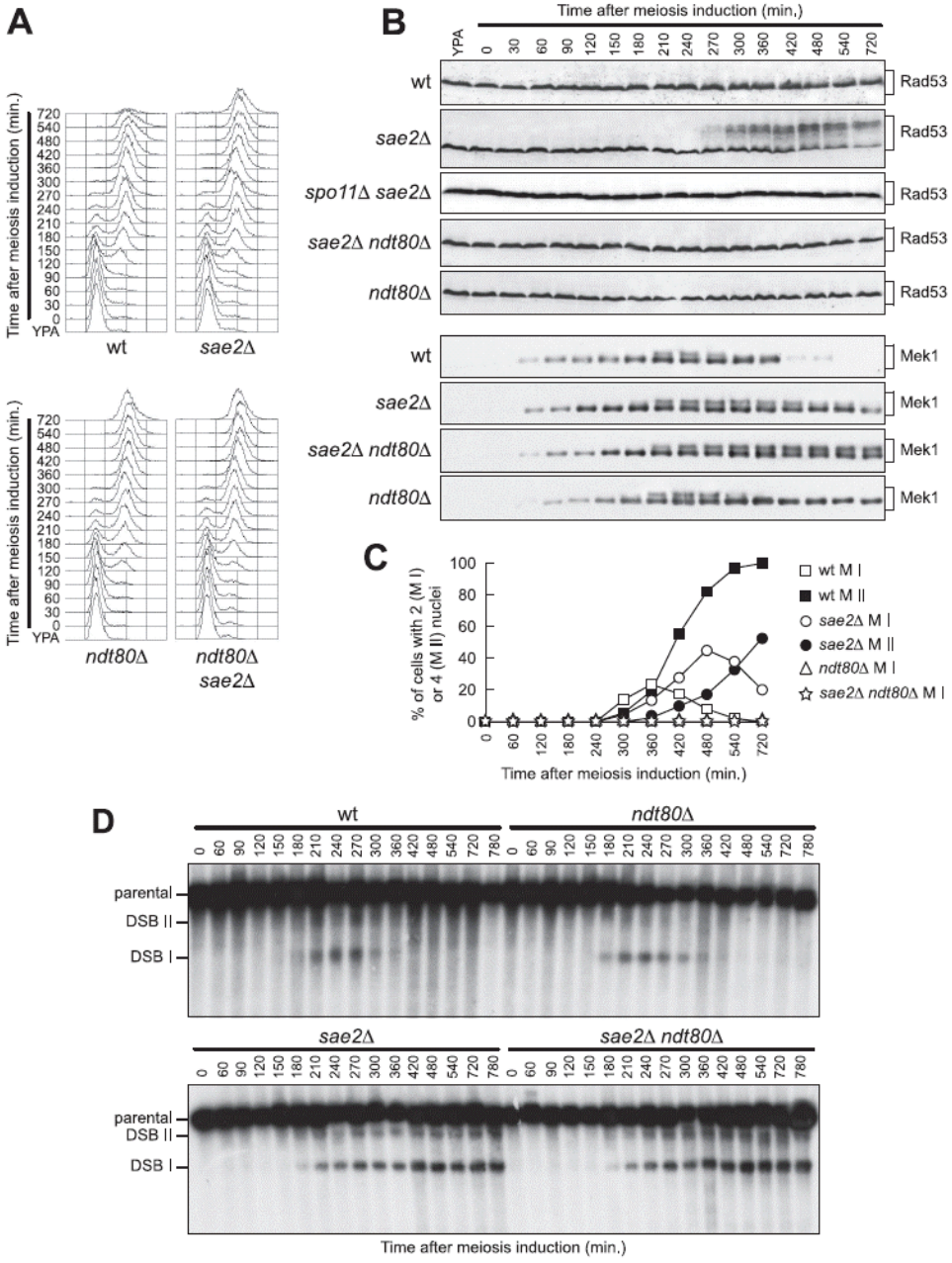


**FIG. 10 Rad53 phosphorylation in *sae2Δ* meiotic cells requires homologous chromosome segregation**. Wild-type (wt), *sae2Δ*, *sae2Δ spo11Δ*, *ndt80Δ*, and *ndt80Δ sae2Δ* diploid cells, all expressing Mek1-HA3 from the *MEK1* promoter, were grown to stationary phase in YPA and then resuspended in SPM at time zero. Cell samples were collected at the indicated time points after transfer to SPM to analyze DNA content (A); phosphorylation of Rad53 (B, top) and Mek1 (B, bottom); the percentages of binucleate (M I) and tetranucleate cells (M II) (C), as described for Fig. 7C; and DSB formation (D) as described for Fig. 8B.

10B, bottom, and D). Thus, unrepaired meiotic DSBs seem to induce Rad53 phosphorylation only after homologous chromosome segregation.

In order to further investigate this possibility, we asked whether Rad53 underwent phosphorylation in *dmc1Δ* cells that were allowed to segregate their homologous chromosomes due to the lack of Mek1. Because *MEK1* deletion allows repair of meiotic DSBs in *dmc1Δ* cells by using sister chromatids (Niu et al., 2005), we also deleted the *RAD54* gene that is required for this repair process in *dmc1Δ mek1Δ* cells (Niu et al., 2005). As shown in Fig. 11, Rad53 phosphorylation became detectable in *rad54Δ dmc1Δ mek1Δ* cells at the time of homologous chromosome segregation (time = 300 min). In contrast, it was under the detection level in *dmc1Δ* cells (Fig. 11A), which failed to complete meiosis I (Fig.11B), and in *dmc1Δ mek1Δ* cells (Fig. 11A), where meiotic DSBs were repaired (Niu et al., 2005; data not shown). Therefore, unrepaired meiotic DSBs induce Rad53 phosphorylation after execution of meiosis I.

***The meiosis II delay in sae2Δ cells depends on Rad53 activation.***

Both *sae2Δ* cells and *dmc1Δ mek1Δ rad54Δ* cells exhibit a delay in segregating sister chromatids during meiosis II (Fig. 10C and 11B). Since meiosis II is functionally equivalent to mitosis, we asked whether Rad53 activation was responsible for the *sae2Δ*-induced meiosis II delay. We were unable to synchronize *rad53Δ sml1Δ* meiotic cell cultures, because the lack of Rad53 impairs cell viability and mitotic cell cycle progression even in the absence of Sml1. We therefore inactivated Rad53 by deleting the *RAD9* gene, whose product is required for Rad53 activation in response to DNA damage during the mitotic cell cycle (Gilbert et al., 2001; Sweeney et al., 2005), while it is not involved in the recombination checkpoint (Lydall et al., 1996). Rad9 is known to undergo phosphorylation by cyclin-dependent kinases in the mitotic cycle (Ubersax et al., 2003) as well as Mec1- and Tel1-dependent hyperphosphorylation in response to DNA damage (Vialard et al., 1998). We found that Rad9 was hyperphosphorylated about 240 min after meiosis induction in *sae2Δ* cells (asterisk in Fig. 12A), similarly to Rad53 in the same cells (Fig. 12C). Furthermore, meiotic *sae2Δ rad9Δ* cells did not phosphorylate

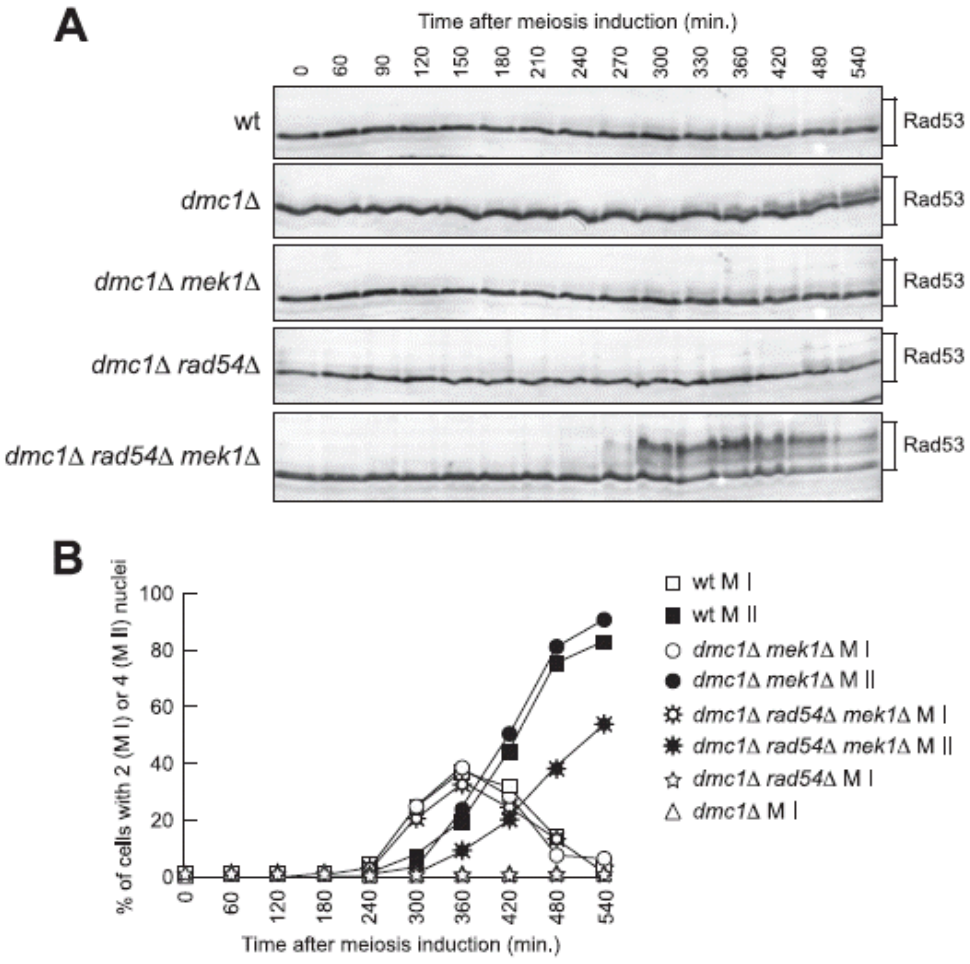


**FIG. 11 Rad53 phosphorylation after segregation of homologous chromosomes carrying unrepaired meiotic DSBs.** Wild-type (wt), *dmc1Δ*, *dmc1Δ mek1Δ*, *dmc1Δ rad54Δ*, and *dmc1Δ mek1Δ rad54Δ* diploid cells were grown to stationary phase in YPA and then resuspended in SPM at time zero. Cell samples were collected at the indicated time points after transfer to SPM to analyze Rad53 phosphorylation (A) and the percentages of binucleate (M I) and tetranucleate cells (M II) (B) as described for Fig. 7C.

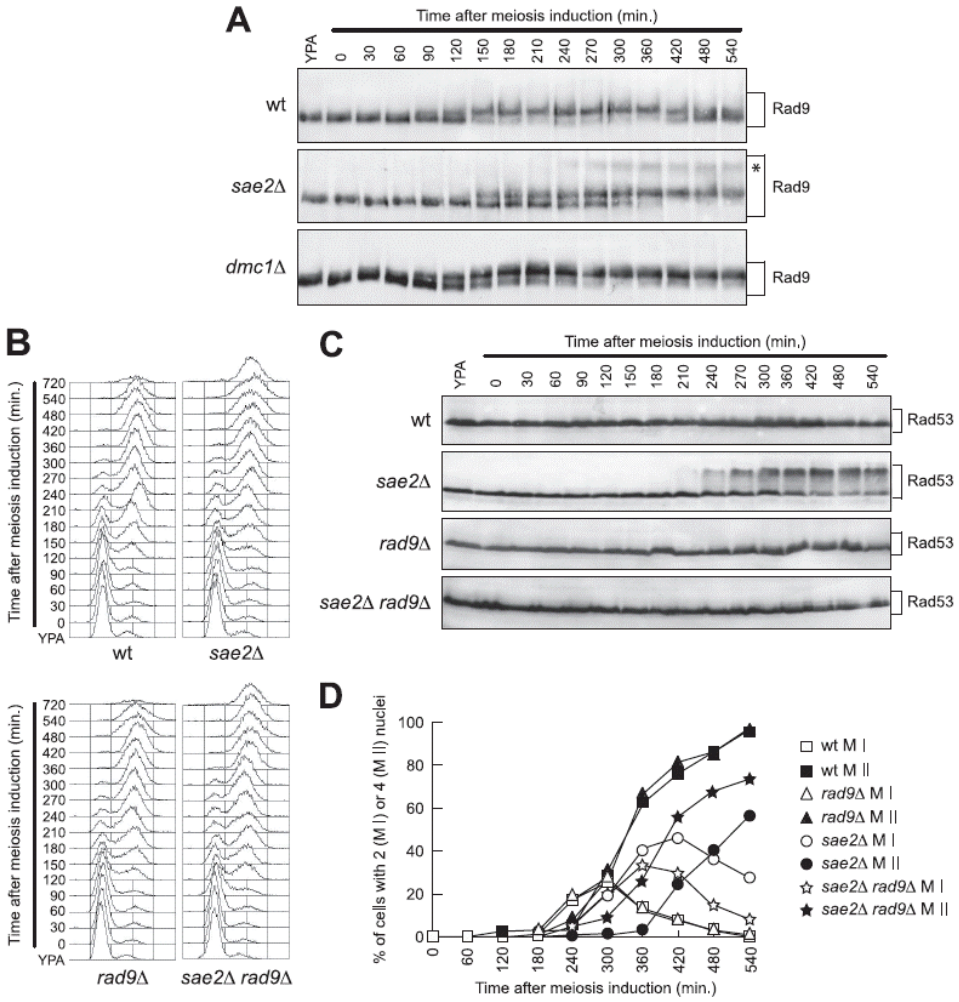


**FIG. 12 Progression through meiosis in *sae2Δ* cells lacking Rad9**. (A) Wild-type (wt), *sae2Δ*, and *dmc1Δ* diploid cells were grown to stationary phase in YPA and then resuspended in SPM at time zero. Total protein extracts were prepared from the indicated strains and subjected to Western blot analysis using anti-Rad9 antibodies. The asterisk points out hyperphosphorylated Rad9. (B to D) Wild-type, *sae2Δ*, *rad9Δ*, and *sae2Δ rad9Δ* diploid cells were grown to stationary phase in YPA and then resuspended in SPM at time zero. Samples were taken at the indicated time points for fluorescence-activated cell sorting analysis of DNA content (B), Western blot analysis of protein extracts with anti-Rad53 antibodies (C), and determination of the percentages of binucleate (M I) and tetranucleate (M II) cells (D).

Rad53 (Fig. 12B and C), confirming that Rad53 activation after segregation of unrepaired homologous chromosomes depends on Rad9. Consistent with the knowledge that the Mek1-dependent checkpoint is responsible for the meiosis I delay of *sae2Δ* cells (Xu et al., 1997), both *sae2Δ* and *sae2Δ rad9Δ* cells showed similar delays in meiosis I execution compared to wild-type and *rad9Δ* cells (Fig. 12D). In contrast, meiosis II started earlier in *sae2Δ rad9Δ* cells than in *sae2Δ* cells (Fig. 12D), although it was still delayed compared to wild-type cells, due to the Mek1-dependent slowing down of meiosis I. Thus, Rad9-dependent activation of the Rad53 kinase is likely responsible for the meiosis II delay in *sae2Δ* cells.

***Both Mec1 and Tel1 promote Rad53 phosphorylation after execution of meiosis I.***

The *sae2Δ* mutation prevents Spo11 removal from meiotic DSB ends, leading to the accumulation of unresected DSBs, which in turn triggers a delay of both meiosis I and meiosis II (Alani et al., 1990; Cartagena-Lirola et al., 2006). This delay is dependent on both Mec1 and Tel1, with Tel1 playing the major role (Usui et al., 2001). In contrast, Tel1 is dispensable for the meiosis I arrest of *dmc1Δ* cells, which are known to accumulate unrepaired DSBs with unusually long single-stranded tails that are monitored by Mec1 (Bishop et al., 1992; Lydall et al., 1996).

To investigate whether phosphorylation of Mek1 and Rad53 in meiotic *sae2Δ* cells had the same genetic requirements, we monitored such phosphorylation events after meiosis induction in *sae2Δ tel1Δ* and *sae2Δ mec1Δ* cells, the latter being kept viable by *SML1* deletion (Zhao et al., 1998). The absence of Mec1 or Tel1 did not affect the kinetics of either premeiotic DNA replication (data not shown) or DSB accumulation (Fig.13B). Although inactivation of either Mec1 or Tel1 affected both Mek1 and Rad53 phosphorylation in *sae2Δ* cells, their effects were quantitatively different. In fact, Mek1 phosphorylation was dramatically reduced in *sae2Δ tel1Δ* cells compared to *sae2Δ* cells, whereas it was only slightly affected in *sae2Δ mec1Δ* cells under the same conditions (Fig. 13A, left). In contrast, Rad53 phosphorylation was undetectable in *sae2Δ mec1Δ* cells, whereas its amount was reduced in *sae2Δ tel1Δ* cells compared

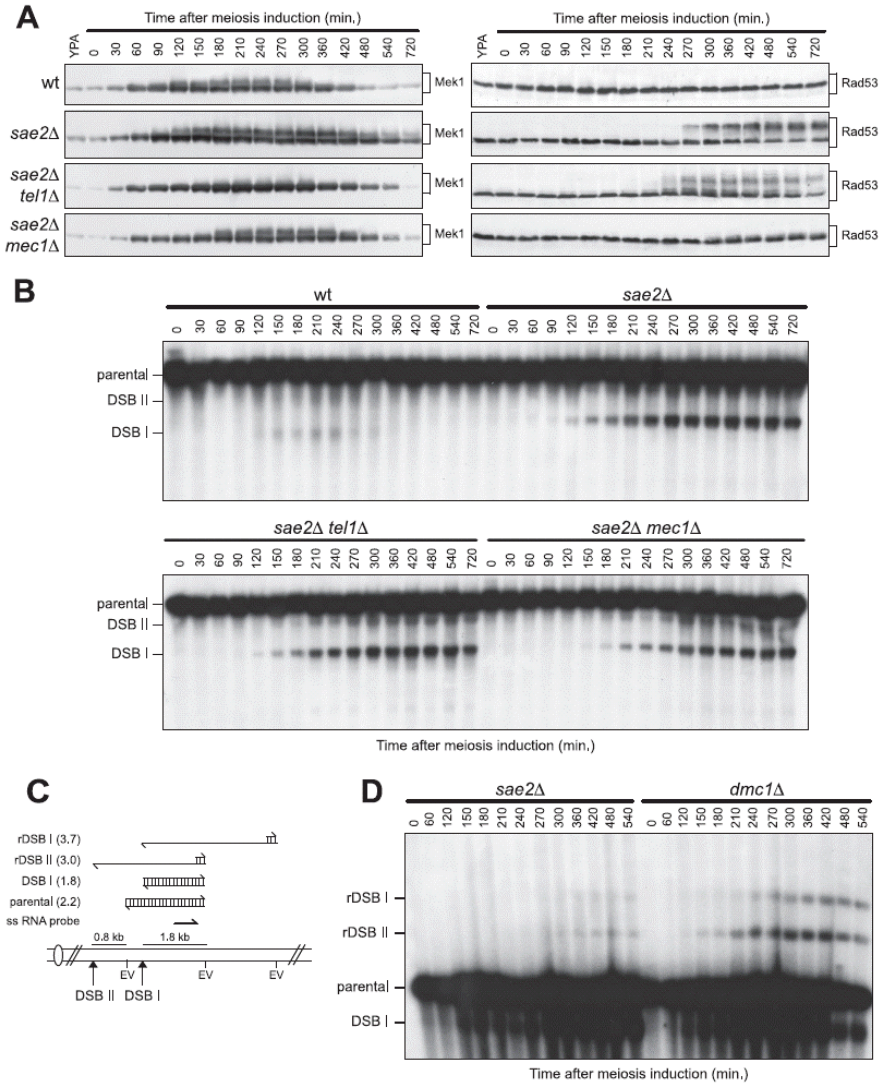


**FIG. 13 Rad53 phosphorylation in meiotic *sae2Δ* cells requires the checkpoint kinases Mec1 and Tel1.** (A and B) Wild-type (wt), *sae2Δ*, *sae2Δ tel1Δ*, and *sae2Δ mec1Δ* diploid cells, all expressing Mek1-HA3 from the *MEK1* promoter, were grown to stationary phase in YPA and then resuspended in SPM at time zero. Cell samples were collected at the indicated time points after transfer to SPM to analyze phosphorylation of Mek1 (A, left) and Rad53 (A, right) as described for Fig. 7B and DSB formation (B) as described for Fig. 8B. (C and D) Diploid cells carrying the *sae2Δ* or *dmc1Δ* allele were grown to stationary phase in YPA and then resuspended in SPM at time zero. (C) 5′-to-3′ resection eliminates EcoRV sites located 0.8 kb centromere-distal from DSB II and 1.8 kb centromere-distal from DSB I, producing larger EcoRV fragments (rDSB II and rDSB I) of 3 kb and 3.7 kb, respectively, detected by the probe. (D) Genomic DNA prepared from aliquots taken at the indicated times after transfer in SPM was digested with EcoRV and separated on an alkaline agarose gel. Gel blots were hybridized with a single-stranded RNA probe specific for the 5′ noncoding region of the *THR4* gene, which reveals Spo11-cut and uncut fragments of 1.8 kb and 2.2 kb, respectively.

to *sae2Δ* cells (Fig. 13A, right). Thus, Tel1 has a major role in triggering Mek1 phosphorylation in *sae2Δ* cells, while Rad53 phosphorylation in the same cells is primarily dependent on Mec1, suggesting that the signals eliciting Mek1 and Rad53 phosphorylation in *sae2Δ* cells undergoing meiosis I and meiosis II, respectively, are different. Because Mec1 is known to detect and signal ssDNA (Zou and Elledge, 2003), we wondered whether *sae2Δ* unresected DSB ends might undergo some processing after homologous chromosome segregation, thus allowing their detection by Mec1 and subsequent Rad53 phosphorylation. To test this hypothesis, we monitored ssDNA formation at two meiosis-specific DSBs within the *THR4* locus (Fig. 13C). As a control for DSB end resection, we also analyzed *dmc1Δ* cells, which are known to accumulate hyperresected DSBs. As shown in Fig. 13D, 3′-ended ssDNA resection products could be detected in meiotic *sae2Δ* cells at both DSBs (rDSB I and rDSB II), although they appeared later and in lower amounts than in *dmc1Δ* cells under the same conditions.

The ssDNA regions appeared in *sae2Δ* cells about 270 to 300 min after meiosis induction, at the time of Rad53 phosphorylation, when most cells had completed meiosis I (data not shown). Therefore, segregation of the homologous chromosomes containing unrepaired DSBs in *sae2Δ* cells may allow some DSB processing by unknown mechanisms, thus generating ssDNA regions that can be detected by Mec1 and induce Rad53 phosphorylation.

Altogether, the results obtained indicate that whereas accidental DSBs induce a Rad53-dependent DNA damage response during both mitosis and meiosis, meiotic DSB repair is monitored by a meiosis-specific checkpoint mechanism involving integral components of the chromosomal structures specifically formed during meiosis. On the other hand, when meiosis I takes place despite unrepaired meiotic DSBs, the latter can trigger a Rad53-dependent DNA damage checkpoint that slows down the second meiotic division.

Note: Materials and Methods related to the above experiments are described in the “Materials and Methods” section while references are listed in the “References” section.

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# Processing of Meiotic DNA Double Strand Breaks Requires Cyclin-dependent Kinase and Multiple Nucleases

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During the first meiotic division, homologous maternal and paternal chromosomes are segregated. In most organisms, homologs must be physically connected to ensure their proper segregation (Petronczki et al., 2003). By virtue of cohesion between sister chromatids, the exchange of chromosome arms through chiasmata formation provides the physical connections between homologous chromosomes. Chiasmata are generated by recombination events, which are initiated by the formation of self-inflicted DNA double strand breaks (DSBs) (Keeney and Kleckner, 1995). DSB formation requires meiosis-specific gene products, including the evolutionary conserved topoisomerase-like enzyme Spo11, as well as the three components of the MRX complex (Mre11-Rad50-Xrs2) (Longhese et al., 2009). In particular, a Spo11 dimer coordinately breaks both DNA strands, creating a DSB with covalent linkages between the 5′ DNA ends and the catalytic tyrosine residue of each Spo11 monomer (Keeney and Kleckner, 1995). Then, Spo11 must be removed by endonucleolytic cleavage to allow further DSB end processing by 5′-3′ resection that is required to initiate homologous recombination (Neale et al., 2005). This event is promoted by the Sae2 protein and the MRX complex, which are required to catalyze the endonucleolytic removal of Spo11-linked oligonucleotides (Keeney and Kleckner, 1995; Usui et al., 1998; Uanschou et al., 2007). In fact, budding yeast *sae2*Δ cells and *rad50s* separation-of-function mutants allow DSB formation but are totally defective in Spo11 removal from DSB ends (Keeney and Kleckner, 1995; Usui et al., 1998; Alani et al., 1990; McKee and Kleckner, 1997; Prinz et al., 1997). Similarly, *mre11* alleles impairing

Mre11 nuclease activity allow Spo11-induced DSB formation, but not Spo11 removal (Furuse et al., 1998; Tsubouchi and Ogawa, 1998; Moreau et al., 1999), suggesting that the latter may take place by Mre11-catalyzed endonucleolytic cleavage and that Sae2 participates in this process. As recently shown, also Sae2 exhibits an endonuclease activity (Lengsfeld et al., 2007), suggesting that this protein, possibly in cooperation with MRX, may allow Spo11 removal by mediating an endonucleolytic cleavage close to the DNA end. Because DSBs are highly hazardous for genome stability, commitment to DSB resection and meiotic progression must be tightly regulated to ensure proper DSB repair. In vegetative *Saccharomyces cerevisiae* cells, DSB resection is promoted by the activity of the cyclin-dependent protein kinase Cdk1 (Cdc28/Clb) during the S and $G_2$ cell cycle phases (Aylon et al., 2004; Ira et al., 2004). This control relies on the phosphorylation of Sae2 Ser-267 by Cdk1 (Huertas et al., 2008), a mechanism that is conserved in the vertebrate homologue of Sae2, CtIP (Huertas and Jackson, 2009; Yun and Hiom, 2009). Because Cdk1 activity is required to generate Spo11-induced DSBs (Henderson et al., 2006; Wan et al., 2008), its involvement in allowing their processing has not been assessed. After Spo11 removal from the 5′ DSB ends, one or more so far unknown nucleases have to resect the break to generate 3′-ended single-stranded DNA (ssDNA) overhangs to initiate homologous recombination. Candidates for such activity are the nucleases Exo1 and Dna2 and the helicase Sgs1, which all contribute to resect DSB and chromosome ends in mitotic *S. cerevisiae* cells (Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008; Bonetti et al., 2009). Consistent with this hypothesis, *EXO1* deletion has been shown to impair repair of meiotic DSBs and to reduce meiotic crossing over (Tsubouchi and Ogawa, 2000). Here we show that phosphorylation by Cdk1 of the Ser-267 residue of *S. cerevisiae* Sae2 is required to initiate resection of meiotic DSBs. In fact, substitution of Sae2 Ser-267 with a non-phosphorylatable residue severely impairs both Spo11 removal and DNA-end processing, which instead take place efficiently when an aspartic residue mimicking constitutive phosphorylation replaces Sae2 Ser-267. Moreover, we demonstrate that further processing of Spo11-induced DSB ends

depends on the nuclease Exo1 and the helicase Sgs1 that act in two different pathways.

***Sae2 Ser-267 is phosphorylated by Cdk1 in meiosis.***

Effective DSB resection in vegetative *S. cerevisiae* cells is promoted by Cdk1 activity during the S and G2 phases of the cell cycle (Aylon et al., 2004; Ira et al., 2004). Because Cdk1 activity is required to generate meiosis-specific DSBs (Henderson et al., 2006; Wan et al., 2008), we could not assess directly its involvement in Spo11-induced DSB resection. To overcome this problem, we exploited the fact that Cdk1-mediated control of DSB resection during mitosis relies on the phosphorylation of Sae2 Ser-267 by Cdk1 (Huertas et al., 2008). Thus, we asked whether Spo11-induced DSB resection requires Cdk1-mediated phosphorylation of Sae2 Ser-267. First, we examined if Sae2 is phosphorylated on Ser-267 during meiosis by using a phosphospecific antibody against this site (anti-γS267, kindly provided by S. Jackson, University of Cambridge, UK). Synchronous meiosis (Fig. 14A) was induced in diploid cells expressing either Sae2-HA or the Sae2-S267A-HA variant, where Ser-267 was substituted by a non phosphorylatable alanine residue. Western blot analysis of anti-HA immunoprecipitates revealed that the anti-γS267 antibody specifically detected wild-type Sae2-HA concomitantly with premeiotic S phase onset, but not Sae2-S267A-HA (Fig. 14B). By contrast, anti-HA antibodies detected both Sae2-HA and Sae2-S267A-HA (Fig. 14B). Thus, Sae2 Ser-267 is phosphorylated in a Cdk1-dependent manner after meiosis induction. Notably, both Sae2-HA and Sae2-S267A-HA underwent electrophoretic mobility shifts (Fig. 14B), known to be due to Mec1- and Tel1-dependent phosphorylation events that take place concomitantly with premeiotic DNA replication and increase with Spo11-induced DSB formation (Cartagena-Lirola et al., 2006). Thus, Ser-267 phosphorylation does not influence Sae2 mobility under this electrophoretic condition. This finding is consistent with previous data showing that DSB- and S phase-induced Sae2 electrophoretic mobility shifts during both meiosis (Cartagena-Lirola et al, 2006) and mitosis (Baroni et al., 2004) are undetectable in

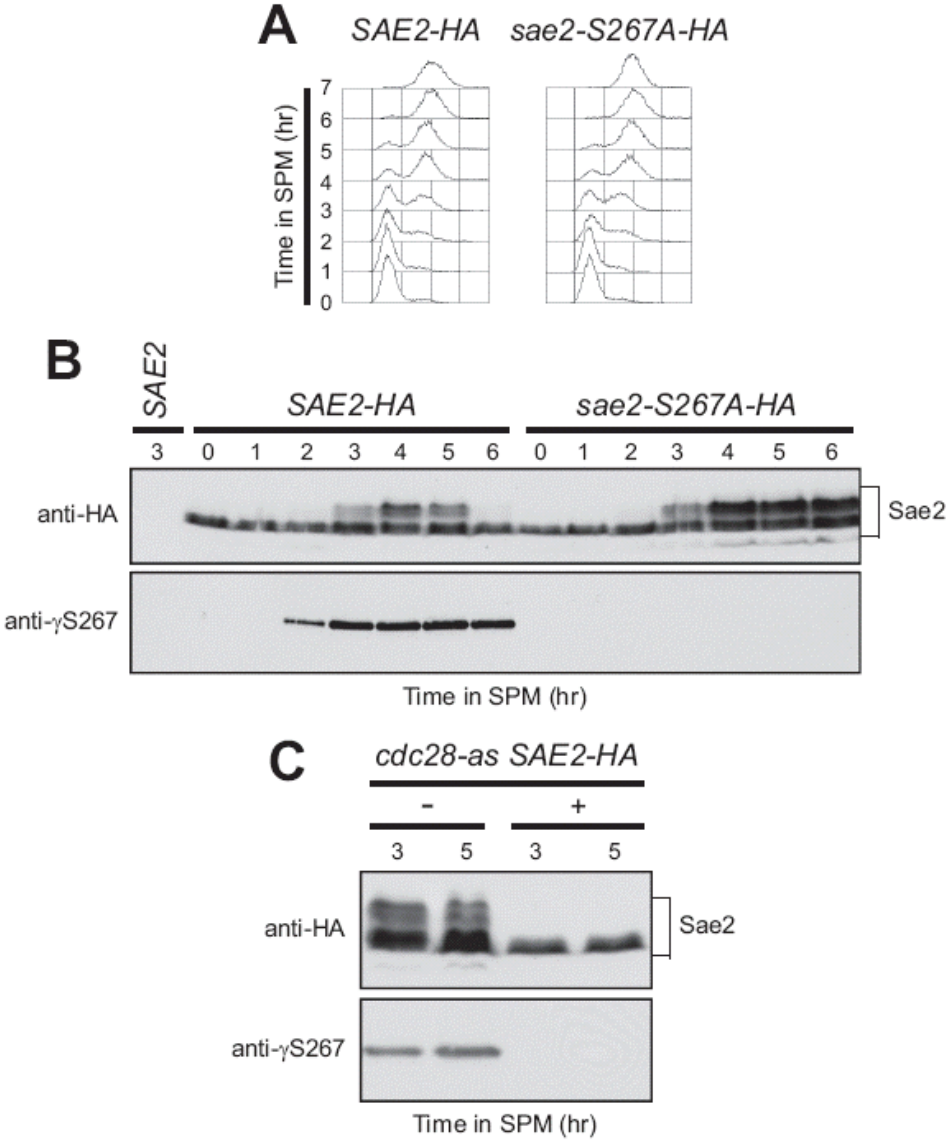


**FIG. 14 Cdk1-dependent phosphorylation of Sae2 Ser-267 during meiosis.** *A* and *B*, *SAE2-HA* and *sae2-S267A-HA* diploid cells were transferred to sporulation medium (*SPM*). *A*, at the indicated times after meiosis induction, DNA content was analyzed by FACS. *B*, protein extracts were immunoprecipitated with anti-HA antibody and subjected to Western blot analysis with anti-HA and anti-γS267 antibodies. Immunoprecipitation was also performed on untagged diploid cells (*SAE2*) 3 h after transfer to SPM. *C*, *cdc28-as SAE2-HA* diploid cells were transferred to SPM in the absence (−) or presence (+) of 5 μm 1-NM-PP1. At the indicated times after meiosis induction, protein extracts were immunoprecipitated with anti-HA antibody and subjected to Western blot analysis as in *B*.

both *mec1*Δ *tel1*Δ double mutants, and in cells carrying multiple changes to alanine of the five serine or threonine residues (Ser-73, Thr-90, Ser-249, Thr-279, and Ser-289) located in the (S/T)Q Sae2 motifs, which are favored for phosphorylation by Mec1/Tel1. We then evaluated Ser-267 phosphorylation dependence on Cdk1 by using the analogue-sensitive Cdk1 version Cdc28-as, which can be inactivated *in vivo* by the adenine analogue 1-NM-PP1 (Bishop et al., 2000). As shown in fig. 14C, *cdc28-as* meiotic cells allowed Sae2-HA Ser-267 phosphorylation in the absence of 1-NM-PP1, but not when the latter was added to the sporulation medium. In fact, anti-γS267 failed to detect wild-type Sae2-HA in immunoprecipitates from 5 μm 1-NM-PP1-treated *cdc28-as* cells (Fig. 14C). According to the knowledge that 5 μm 1-NM-PP1 prevents both DNA replication and DSB formation (Henderson et al., 2006), the anti-HA antibody failed to detect Mec1- and Tel1-dependent Sae2-HA mobility shifts in 1-NM-PP1-treated *cdc28-as* immunoprecipitates (Fig. 14C). Altogether, these data indicate that Sae2 Ser-267 phosphorylation during meiosis is Cdk1-dependent, suggesting that Cdk1 might regulate processing/repair of Spo11-induced DSBs through Sae2 phosphorylation.

***Meiotic DSB repair requires Sae2 Ser-267 phosphorylation.***

Because Sae2 is required to repair Spo11-induced DSBs by allowing Spo11 removal and generation of 3′-ended ssDNA (Keeney and Kleckner, 2005), we asked whether Cdk1-dependent Sae2 Ser-267 phosphorylation is required for this meiotic function. Thus, we analyzed the kinetics of DSB repair at the natural *YCR048W* meiotic recombination hotspot in cells expressing Sae2 variants where Ser-267 was substituted by either a non-phosphorylatable alanine residue (Sae2-S267A) or an aspartic residue mimicking constitutive phosphorylation (Sae2-S267D). The DSB appeared in all cell cultures undergoing synchronous meiosis as soon as cells completed premeiotic DNA replication (Fig. 15, A and B). However, the DSB signal disappeared at 360 min after transfer to sporulation medium in wild-type and *sae2-S267D* cells, whereas it persisted until the end of

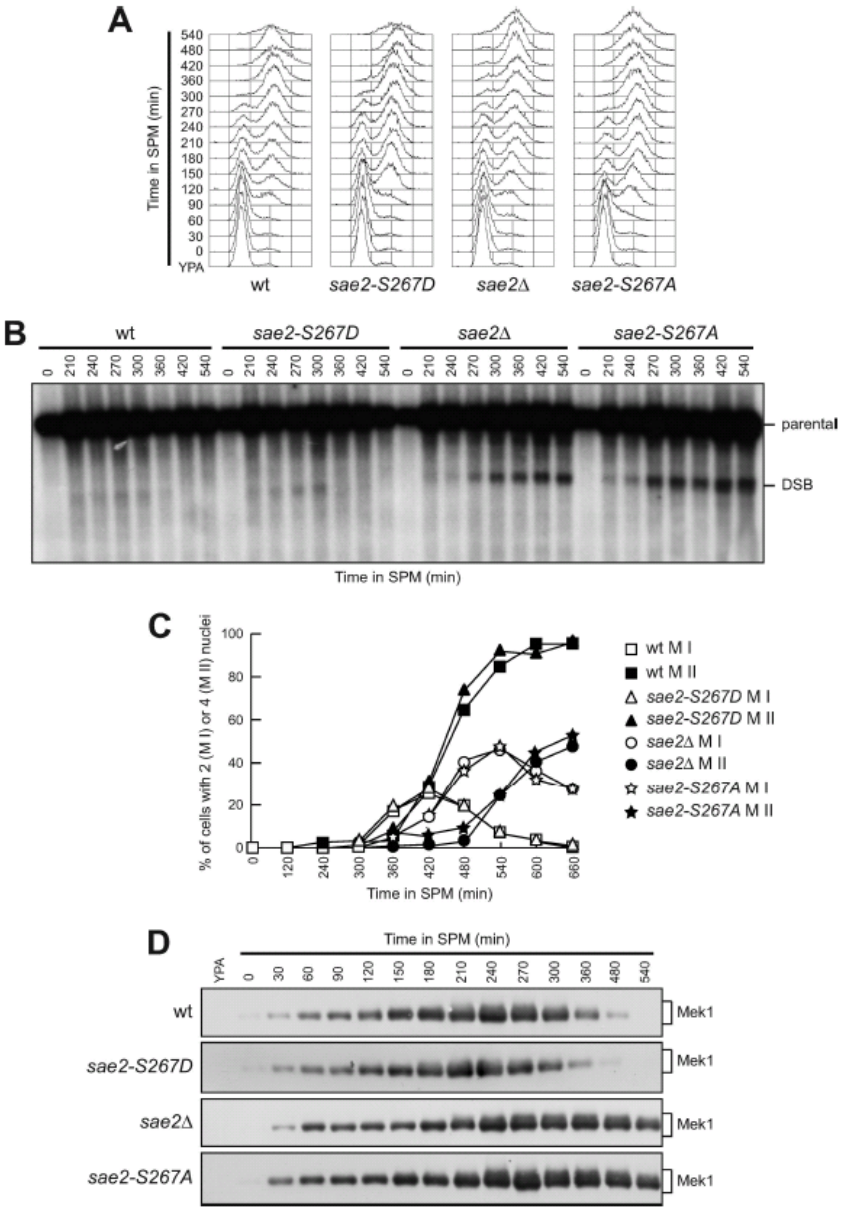


**FIG. 15 Sae2 Ser-267 phosphorylation is required for DSB repair.** Synchronous meiotic cultures of cells with the indicated genotypes and expressing Mek1-HA from the *MEK1* promoter were analyzed at the indicated times for DNA content by FACS (*A*), for DSB formation/repair at the *YCR048W* hotspot by Southern blot (*B*), for the percentages of binucleate (completed meiosis I, *MI*) and tetranucleate (completed meiosis II, *MII*) cells (*C*), and for Mek1 amount/phosphorylation by Western blot analysis with anti-HA antibody (*D*). The Southern blot in *B* was probed with a DNA fragment complementary to the 5′ non-coding region of the *YCR048W* gene, which reveals an intact parental EcoRI fragment (parental) of 7.9-kb and a band of 5.7-kb corresponding to the prominent meiotic DSB site (*DSB*).

the experiment in both *sae2*Δ and *sae2-S267A* cells (Fig. 15B). The inability of *sae2*Δ cells to repair meiotic DSBs is known to cause the hyperactivation of the Mek1-dependent recombination checkpoint that delays progression through meiosis (Cartagena-Lirola et al., 2006). Strikingly, similarly to *sae2*Δ cells, *sae2-S267A* cells started to undergo meiosis I and II 60 and 120 min later, respectively, than wild-type and *sae2-S267D* cells (Fig. 15C). Moreover, this delay correlated with Mek1 phosphorylation, whose amount remained constant until the end of the experiment in both *sae2Δ* and *sae2-S267A* cells, while it decreased in both wild-type and *sae2-S267D* cells within 360 min after transfer to sporulation medium (Fig. 15D). Consistent with hyperactivation of the recombination checkpoint, Mec1- and Tel1-dependent phosphorylation of Sae2-S267A persisted longer after meiosis induction than that of wild-type Sae2 (Fig. 14B). Thus, phosphorylation of Sae2 Ser-267 by Cdk1 is required for repair of Spo11-induced DSBs, which in turn allows deactivation of the meiotic recombination checkpoint.

***Meiotic DSB resection requires Sae2 Ser-267 phosphorylation.***

Because Sae2 is known to be required for Spo11 removal, we asked whether the Sae2-S267A variant might prevent Spo11 dissociation from the meiotic recombination *YCR048W* hotspot. To verify this hypothesis, we performed chromatin immunoprecipitation (ChIP) with anti-Myc antibody in strains expressing a fully functional Myc-tagged Spo11 variant, followed by quantitative real-time PCR to monitor coimmunoprecipitation of DNA fragments located either 162 bp (DSB) or 2319 bp (CON) distal to the natural *YCR048W* recombination hotspot. In all cell cultures, Spo11 associated with the DNA fragment closest to the *YCR048W* hotspot during the course of meiosis, as measured by an increase of the DSB/CON ratio (Fig. 16A). This Spo11-hotspot association decreased 6 h after meiosis induction in both wild-type and *sae2-S267D*, whereas it persisted in both *sae2*Δ and *sae2-S267A* cells (Fig. 16A). Thus, Spo11 removal requires phosphorylation of Sae2 Ser-267. Because Spo11-DNA

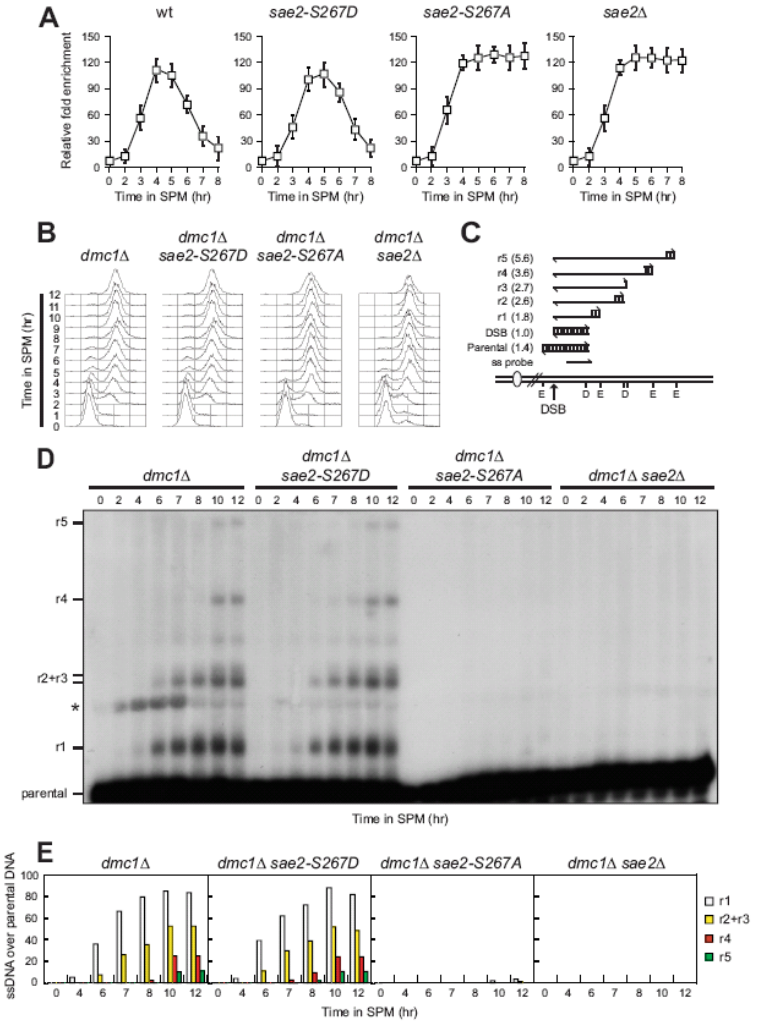


**FIG. 16 Phosphorylation of Sae2 Ser-267 is essential for both Spo11 removal and DSB resection.** *A*, Spo11-DNA association. Chromatin samples taken at different time points after meiosis induction were immunoprecipitated with anti-Myc antibody. Coimmunoprecipitated DNA was analyzed by quantitative real-time PCR using primer pairs located 162 bp (*DSB*) and 2319 bp (*CON*) distal to the DSB site of the *YCR048W* hotspot. Data were expressed as the -fold enrichment of DSB over CON signal after normalization to input signals for each primer set. The data presented are the mean of those obtained in three independent experiments. *Error bars* indicate ±S.D. *B*, synchronous meiotic cultures of cells with the indicated genotypes were analyzed at the indicated times for DNA content by FACS. *C*, scheme of the system used to detect DSB resection at the *YCR048W* hotspot. Genomic DNA was digested with both DraIII (*D*) and EcoRV (*E*), and DNA fragments were separated on alkaline agarose gel. Gel blots were hybridized with a single-stranded RNA probe, which reveals an uncut fragment of 1.4-kb (parental). DSB formation and subsequent 5′-to-3′ resection eliminate DraIII and EcoRV sites, thus producing larger DNA fragments (*r1*, *r2*, *r3*, *r4*, and *r5*) detected by the probe. *D*, genomic DNA prepared from samples taken at the indicated times during the experiment in *B* was analyzed for ssDNA formation as described in *C*. The *asterisk* points out an unspecific signal. *E*, densitometric analysis of the representative experiment shown in *D*. Values are expressed as arbitrary units. Three independent experiments were performed with very similar results.

association occurs independently of DSB formation (Prieler et al., 2005), we followed more directly the kinetics of 3′-ended ssDNA formation by Southern blot analysis of genomic DNA that was run on an alkaline agarose gel, followed by hybridization with a single-stranded RNA probe specific for the *YCR048W* gene (Fig. 16C). To ensure the visualization of all the resection products, all the strains carried the deletion of the *DMC1* gene, thus preventing the disappearance of the 3′-ended ssDNA regions due to homologous recombination between the homologous non-sister chromatids. Strikingly, after transfer to sporulation medium (Fig. 16B), 3′-ended ssDNA resection products were below the detection level in both *sae2-S267A dmc1*Δ and *sae2*Δ *dmc1*Δ cells, whereas they accumulated in both *dmc1*Δ and *sae2-S267D dmc1*Δ cells (Fig. 16 D and E). Altogether, these data indicate that Cdk1-dependent phosphorylation of Sae2 Ser-267 is required to resect Spo11-induced DSB ends. It has been shown that the *sae2-S267A* allele causes a strong reduction in spore viability (Uanschou et al., 2007; Huertas et al., 2008) (Table 1).

| Allele | Spore viability | No. viable spores/total |
|---|---|---|
| | % | |
| *SAE2* | 96 | 146/152 |
| *sae2*Δ | 1 | 2/156 |
| *sae2-S267A* | 15 | 30/200 |
| *sae2-S267D* | 95 | 122/128 |
| *sae2-S134A* | 75 | 81/108 |
| *sae2-S134D* | 95 | 76/80 |
| *sae2-S267A-S179A* | 16 | 19/120 |
| *sae2-S267A-S134A* | 3 | 8/264 |
| *sae2-S267A-S134D* | 16 | 22/136 |

**TABLE 1 Spore viability in *sae2* mutants.** Diploid strains homozygous for the indicated *SAE2* alleles were allowed to sporulate, and tetrads were dissected on YEPD plates. Spore viability was determined by scoring colony-forming spores after incubation at 28 °C for 3 days.

However, although both 3′-ended ssDNA and Spo11 removal were under the detection level in both *sae2*Δ and *sae2-S267A* cells, spore viability was reduced to a lesser extent in *sae2-S267A* cells compared with *sae2*Δ cells (Table 1). These findings suggest that full Sae2 activity might require Cdk1-dependent phosphorylation of additional residues. Besides Ser-267, Sae2 contains two other potential Cdk1 target sites, Ser-134 and Ser-179, with Ser-134 receiving a higher score for predicted phosphorylation site (Huertas et al., 2008). When Ser-179 was substituted with a non-phosphorylatable alanine residue, *sae2-S267A-S179A* and *sae2-S267A* mutant cells showed similar spore viability (Table 1), suggesting that Ser-179 does not contribute to support Sae2 activity. By contrast, a slight reduction in spore viability was caused by the *sae2-S134A* mutation (Table 1). Furthermore, *sae2-S267A-S134A* spore viability was reduced compared with *sae2-S267A*, and it was similar to that caused by *SAE2* deletion (Table 1). This loss of viability was likely due to the lack of Ser-134 phosphorylation and not to protein folding alterations, as *sae2-S134D* cells showed wild-type spore viability and *sae2-S267A-S134D* spore viability was similar to that of *sae2-S267A* cells (Table 1). Altogether, these data suggest that, in addition to Ser-267, Ser-134 phosphorylation might contribute to support Sae2 function in promoting meiotic DSB resection.

***Exo1 and Sgs1 are involved in meiotic DSB resection.***

After Spo11 removal by Sae2, the 3′-ended DNA strands are rapidly processed through a still unknown mechanism. Possible candidates for such activity are Exo1, Dna2, and/or the helicase Sgs1, because they contribute to resect chromosome ends that are trimmed by Sae2 and MRX in vegetative cells (Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008; Bonetti et al., 2009). We then monitored the kinetics of 3′-ended ssDNA generation at the natural *YCR048W* meiotic recombination hotspot in cells lacking Exo1, Sgs1, and/or Dna2. Because *sgs1Δ* single mutant cells displayed vegetative growth defects and the simultaneous deletion of *SGS1* and *EXO1* caused cell lethality in the SK1 background (data not shown), we

constructed a meiosis-specific *pCLB2-SGS1* conditional allele by replacing the native *SGS1* promoter with the *CLB2* promoter, which is strongly repressed during meiosis (Lee and Amon, 2003). Normal vegetative growth phenotypes and efficient premeiotic synchronization were observed in both *pCLB2-SGS1 dmc1*Δ and *pCLB2-SGS1 exo1*Δ *dmc1*Δ cells (Fig. 17A), where *DMC1* was deleted to ensure visualization of the resection products. Although DSB formation occurred with similar kinetics in all cell cultures after meiosis induction (data not shown), generation of 3′-ended ssDNA resection products was delayed in *exo1*Δ *dmc1*Δ compared with *dmc1*Δ cells, indicating that Exo1 contributes to meiotic DSB processing (Fig. 17, B and C). The residual resection of the Spo11-induced DSB in *exo1*Δ cells depends partially on Sgs1 activity. In fact, resection of this DSB was severely reduced in *pCLB2-SGS1 exo1*Δ *dmc1*Δ compared with *exo1*Δ *dmc1*Δ, although it was not defective in *pCLB2-SGS1 dmc1*Δ cells (Fig. 17, B and C). Thus, meiotic DSB end processing is controlled by at least two distinct mechanisms involving Sgs1 and Exo1, respectively, with Exo1 playing the major role. The inability to remove Spo11 from the DSB ends inhibits their processing, prompting us to ask whether the resection defects of cells crippled for both Sgs1 and Exo1 activities might be due to persistence of Spo11 binding to DSB ends. To test this hypothesis, we monitored Spo11 association to the meiotic *YCR048W* recombination hotspot by ChIP analysis with anti-Myc antibody from cells expressing Myc-tagged Spo11. A transient association of Spo11 to the DNA fragment located 162 bp distal to the natural *YCR048W* recombination hotspot was observed in both wild-type and *pCLB2-SGS1 exo1*Δ cells (Fig. 17D), indicating that Sgs1 and Exo1 are not involved in terminating Spo11-hotspot interaction. Thus, Sgs1 and Exo1 appear to participate in DSB processing by controlling a step subsequent to Spo11 removal. In addition to Exo1 and Sgs1, resection of chromosome ends in vegetative cells depends also of the nuclease/helicase Dna2 (Zhu et al., 2008; Bonetti et al., 2009). Thus, we analyzed whether Dna2 also promotes resection of meiotic DSBs by using a strain where its essential function in cell viability is bypassed by the *pif1-M2* mutation, which specifically impairs Pif1 nuclear function (Budd et al., 2006).

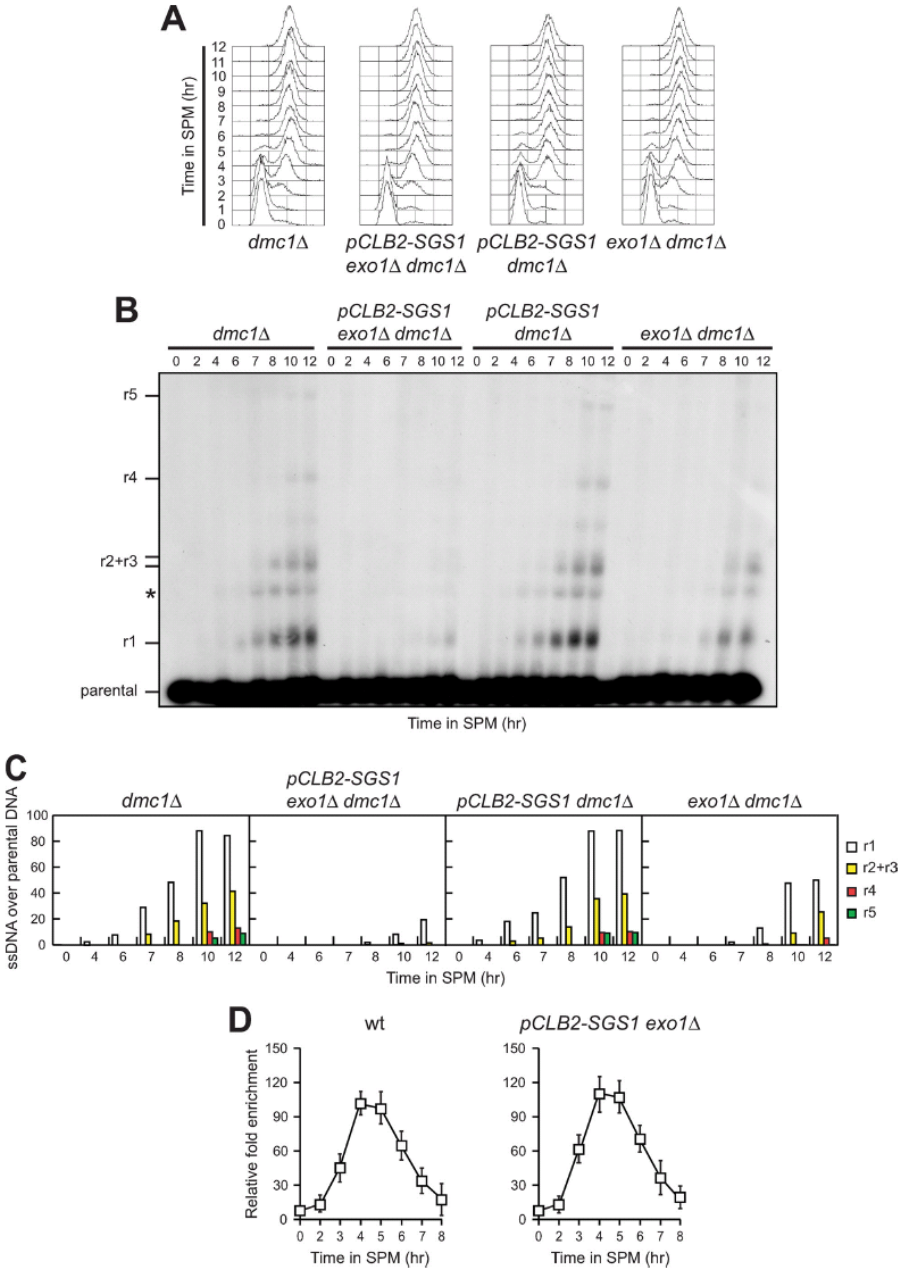


**FIG. 17 Meiotic DSB resection involves both Exo1 and Sgs1.** *A–C*, synchronous meiotic cultures of cells with the indicated genotypes were analyzed at the indicated times for DNA content by FACS (*A*) and for DSB resection by Southern blot (*B*) as described in Fig. 16C. *C*, densitometric analysis of the representative experiment shown in *B*. Values are expressed as arbitrary units. Three independent experiments were performed with very similar results. *D*, Spo11-DNA association. Synchronous meiotic cultures of cells with the indicated genotypes were analyzed at the indicated times by ChIP and quantitative real-time PCR as described in Fig. 16A.

We found that initiation of *YCR048W* DSB resection seems to occur with similar kinetics in *dmc1*Δ, *pif1-M2 dmc1*Δ, and *dna2*Δ *pif1-M2 dmc1*Δ cells (Fig. 18).

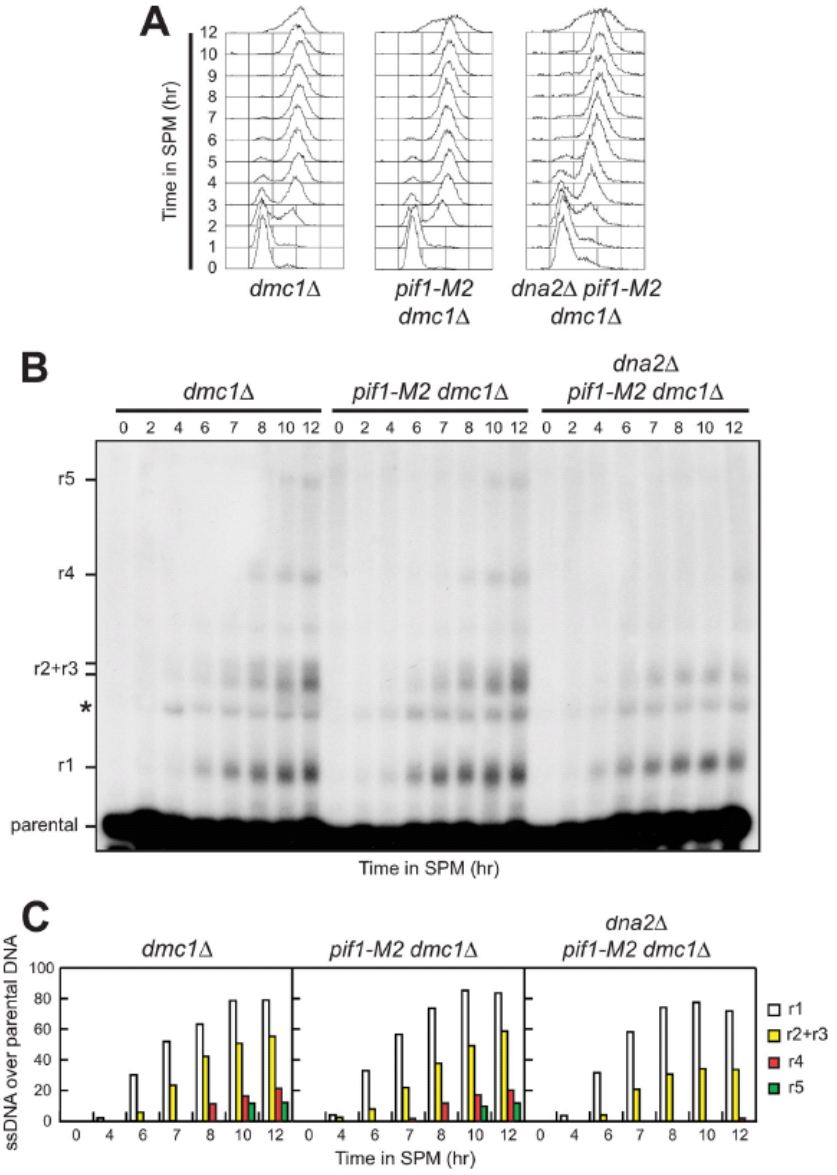


**FIG. 18 Dna2 participates in meiotic DSB resection.** Synchronous meiotic cultures of cells with the indicated genotypes were analyzed at the indicated times for DNA content by FACS (*A*) and for DSB resection by Southern blot (*B*) as described in Fig. 16C. *C*, densitometric analysis of the representative experiment shown in *B*. Values are expressed as arbitrary units. Three independent experiments were performed with very similar results.

However, generation of the longest resection products (*r4* and *r5*) was defective in *dna2*Δ *pif1-M2 dmc1*Δ cells compared with *dmc1*Δ and *pif1-M2 dmc1*Δ cells (Fig. 18, B and C). This finding suggests that Dna2 might contribute to the formation of long ssDNA tails, in agreement with the notion that Dna2 is involved in long range resection of DSB ends in vegetative *Saccharomyces cerevisiae* cells (Zhu et al., 2008). Unfortunately, we were unable to assess whether Dna2 could resect Spo11-induced DSBs in the absence of Exo1 or Sgs1, because both *dna2*Δ *pif1-M2 exo1*Δ and *dna2*Δ *pif1-M2 pCLB2-EXO1* cells were unviable, and *dna2*Δ *pif1-M2 pCLB2-SGS1* cells did not enter meiosis synchronously (data not shown). Taken together these data indicate that, just like the processing of accidental DSBs during mitosis, even the processing of Spo11-induced DSBs requires CDK-mediated Sae2 phosphorylation on Ser-267. In addition to Ser-267, Ser-134 phosphorylation might contribute to support Sae2 function in promoting meiosis-specific DSB resection. Moreover, the further processing of accidental and programmed DSBs have also similar requirements in terms of nucleases as both the processing events need the exonuclease Exo1, the nuclease Dna2 and the helicase Sgs1.

Note: Materials and Methods related to the above experiments are described in the “Materials and Methods” section while references are listed in the “References” section.

# DISCUSSION

## Role of the *Saccharomyces cerevisiae* Rad53 Checkpoint Kinase in Signaling Double-Strand Breaks during the Meiotic Cell Cycle.

Accumulation of unrepaired meiosis-specific DSBs is known to activate the recombination checkpoint that arrests the meiotic cell cycle prior to meiosis I (Fig. 19B). On the other hand, the DNA damage checkpoint senses and signals DSBs that arise at unpredictable locations as a consequence of DNA damage, thus delaying the mitotic $G_2$/M transition (reviewed in Longhese et al., 2008). Although these two checkpoint mechanisms share the sensor kinases Mec1/ATR and Tel1/ATM, the meiosis-specific Mek1 kinase is the effector of the recombination checkpoint, while the Rad53 kinase is known to be essential for transducing the DNA damage checkpoint signals during mitosis. How this specific use of Mek1 instead of Rad53 is achieved is currently unknown.

Here, we provide evidence that Rad53 can be phosphorylated during the meiotic cell cycle after generation of chemically induced DSBs, indicating that Rad53 can be activated in response to DNA damage also during meiosis. However, Rad53 activation does not result in meiosis I delay, suggesting that the regulators of meiosis I progression cannot be targeted by Rad53. Interestingly, neither Rad53 nor its activator Rad9 are phosphorylated and activated when programmed meiosis-specific DSBs arise during meiosis I, even when their repair is prevented by the lack of Dmc1. However, targeting Rad53 to Mec1 by a Ddc2-Rad53 chimera allows its phosphorylation and activation in response to meiotic programmed DSBs. Thus, the reason why Rad53 activation by meiotic programmed DSBs is prevented may be that Rad53 and/or Rad9 is not reachable by Mec1 signaling from the meiotic recombination sites.

On the other hand, a Rad53-dependent checkpoint response, which causes a delay of the second meiotic division, is elicited when unrepaired meiosis-specific DSBs escape the recombination checkpoint-mediated prophase I arrest (Fig. 19C and D). In fact, Rad53 is phosphorylated in *dmc1Δ mek1Δ rad54Δ* cells, which fail to repair meiotic DSBs due to the absence of Rad54, but are allowed to segregate homologous

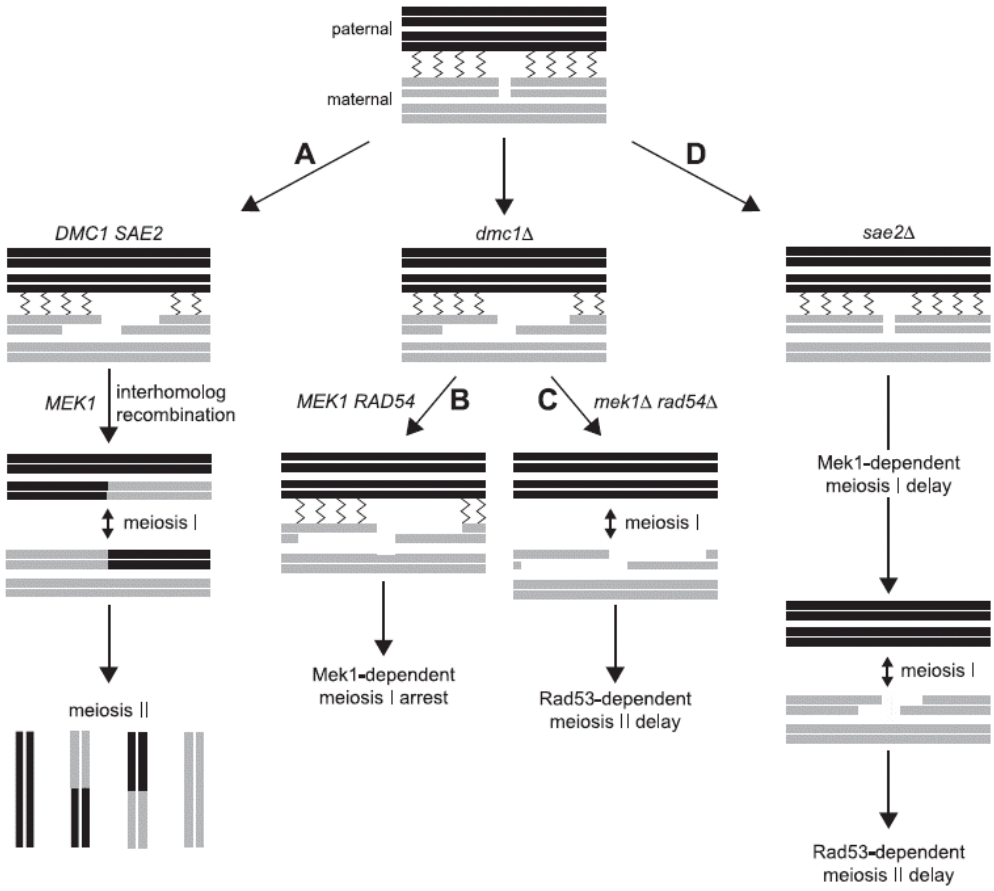


**FIG. 19 Detection of meiotic DSBs by the checkpoint machineries.** Homologs are indicated in black (paternal) and gray (maternal). Zigzag lines represent the meiosis-specific chromosome structure(s). In wild-type cells, DSB repair is accomplished via interhomolog recombination (A). In *dmc1Δ* cells, the inability to repair meiotic DSBs leads to Mek1 phosphorylation and a meiosis I arrest (B). Unprocessed meiotic DSBs in *sae2Δ* cells lead to a Mek1-dependent slowing down of meiosis I (D). When homologous chromosomes with unrepaired meiotic DSBs segregate from each other, these DSBs elicit a Rad53-dependent checkpoint that delays meiosis II (C and D).

chromosomes containing hyperresected DSBs due to the absence of Mek1. Moreover, Rad53 is phosphorylated in *sae2Δ* cells, which are known to perform meiosis I in the presence of unprocessed DSBs. This phosphorylation requires DSB formation and causes a delay of the second meiotic division. Unlike Mek1, whose phosphorylation is detectable concomitantly with meiotic DSB generation, Rad53 phosphorylation in both *sae2Δ* and *dmc1Δ mek1Δ rad54Δ* cells occurs at the time of meiosis I completion. Moreover, Rad53 phosphorylation is prevented in *sae2Δ* cells by eliminating Ndt80, which causes a meiosis I arrest at late prophase. This suggests that unrepaired meiosis-specific DSBs can elicit a Rad53-dependent checkpoint only when homologous chromosomes segregate from each other, and this checkpoint causes a delay in sister chromatid separation at meiosis II (Fig. 19C and D). The Rad53-dependent delay of meiosis II is transient even if meiotic DSBs are not repaired. Because meiotic cells progress through meiosis and form spores even if a DSB remains unrepaired (Malkova et al., 1996), one possibility is that the checkpoint mechanism is less responsive to DNA damage during meiosis than during mitosis.

Tel1 is thought to recognize unprocessed DSBs, whereas Mec1 senses and signals ssDNA regions that arise after DSB processing and are covered by the replication protein A complex (Mantiero et al., 2007 Zou and Elledge, 2003).

Consistent with previous data showing that the lack of Sae2 impairs DSB processing (Clerici et al., 2006), Tel1 has the major role in triggering Mek1 phosphorylation in meiotic *sae2Δ* cells. In contrast, Mec1 has a more critical role than Tel1 in triggering Rad53 phosphorylation in the same cells. These results suggest that a subset of meiotic DSBs are processed after homologous chromosome segregation in *sae2Δ* cells, thus generating ssDNA regions that are detected by Mec1.

How can Rad53 activation in response to programmed meiotic DSBs be prevented? Inhibition of Rad53 phosphorylation during meiosis I recalls the ability of meiotic cells to generate the so-called “barrier to sister chromatid repair” (Niu et al., 2007). In fact, one of the differences between mitosis and meiosis is that meiotic DSBs are repaired using an

intact homologous nonsister chromatid, whereas mitotic recombination occurs preferentially between sister chromatids (Kadyk and Hartwell, 1992; Schwacha and Kleckner, 1994). The Mek1, Hop1, and Red1 proteins, which are structural components of the meiosis-specific chromosome structures that favor the association between homologous chromosomes, are essential to establish the correct meiotic recombination partner choice (Carballo et al., 2008; Niu et al., 2007; Niu et al., 2005; Schwacha and Kleckner, 1997; Wan et al., 2004). Thus, one possibility is that this meiosis-specific structure (or some specific components) may not only suppress intersister DSB repair but also hide programmed meiosis-specific DSBs from being signaled as DNA damage to the Rad53 kinase, thus preventing activation of the Rad53-dependent DNA damage checkpoint during meiosis I. When homologous chromosome segregation takes place and interhomolog bias is abolished, meiotic DSBs that are not yet repaired could then be monitored as DNA damage by the canonical Rad53-dependent DNA damage checkpoint machinery (Fig. 19, C and D). Exogenous DSBs during meiosis I may in turn cause a local disruption of the meiosis-specific chromosome structure, thus allowing Rad53 to be phosphorylated and activated.

Given that DSB-induced Mek1 activation is required to ensure the formation of interhomolog crossovers (Niu et al., 2005), the meiosis-specific propagation of the checkpoint signals through Mek1, Red1, and Hop1 instead of Rad53 is likely critical for the formation of genetically balanced gametes. In fact, reduced Mek1 phosphorylation would allow meiosis to proceed without the correct repair partner choice and formation of chiasmata, which are critical for proper meiotic chromosome segregation.

The meiosis-specific large-scale structure does not prevent sensing and signaling of meiotic programmed DSBs by Mek1, Red1, Hop1, Mec1, and Tel1, possibly because they are part of the normal recombination machinery. In fact, Mek1, Red1, and Hop1 proteins are structural components of the meiotic chromosome axes (Bailis and Roeder, 1998; Woltering et al., 2000; Xu et al., 1997). Moreover, Mec1 loss of function leads to a number of meiotic defects, including aberrant chromosome

synapsis, reduced recombination frequency and spore viability, and loss of interhomolog bias and of crossover control (reviewed in Carballo and Cha, 2007). In higher eukaryotes, both ATR and ATM associate with different sites along meiotically pairing chromosomes (Keegan et al., 1996), and ATM-deficient mice show aberrant synapsis with unpaired axial cores and fragmented synaptonemal complexes (Barlow et al., 1998; Xu et al., 1996). Finally, mutations in the *RAD17*, *RAD24*, or *MEC3* gene, encoding regulators of Mec1 activity, reduce meiotic interhomolog recombination frequency, while increasing the frequency of ectopic recombination events and of illegitimate repair from the sister chromatids (Aylon and Kupiec, 2003; Grushcow et al., 1999; Shinohara et al., 2003; Thompson and Stahl, 1999).

In conclusion, whereas accidental DSBs induce a Rad53-dependent DNA damage response during both mitosis and meiosis, meiotic DSB repair is monitored by a meiosis-specific checkpoint mechanism involving integral components of the chromosomal structures specifically formed during meiosis. On the other hand, when meiosis I takes place despite unrepaired meiotic DSBs, the latter can trigger a Rad53-dependent DNA damage checkpoint that slows down the second meiotic division, which is functionally equivalent to mitosis. The possibility of activating this checkpoint might provide a salvage mechanism preventing chromosome rearrangements and/or loss in the gametes even in the absence of the recombination checkpoint, thereby further protecting the offspring from birth defects and cancer predisposition.

## Processing of Meiotic DNA Double Strand Breaks Requires Cyclin-dependent Kinase and Multiple Nucleases.

DNA DSBs are highly hazardous for genome integrity, but meiotic cells deliberately introduce them into their genome to initiate homologous recombination. To minimize the risk of deleterious effects, meiotic DSB formation, processing, and repair must be tightly regulated to occur only at the right time and place. We have investigated the mechanism by

which *S. cerevisiae* cells control Spo11 removal and resection of meiosis-specific DSBs. Overall, our data indicate that the requirements for resecting Spo11-induced DSBs, in terms of nucleases and Cdk1-dependent Sae2 phosphorylation, are similar to those of the processing events at an accidental DSB, indicating that cells have evolved the same mechanism to process both programmed and un-programmed DSBs.

***Regulation of Spo11 Removal from Meiotic DSBs.***

Cdk1 activity accumulates during premeiotic S phase, increases through prophase, and peaks at about meiosis I (Marston and Amon, 2004). We show that Sae2 Ser-267 is phosphorylated in a Cdk1-dependent manner during meiosis. Moreover, substitution of Ser-267 with a non-phosphorylatable residue causes phenotypes comparable to those of *sae2* null mutants, including severely impaired Spo11 removal and DNA-end processing. These defects are caused by the inability of Cdk1 to phosphorylate Sae2 Ser-267, because the same processes take place efficiently when an aspartic residue mimicking constitutive phosphorylation replaces Sae2 Ser-267. Thus, Cdk1-dependent phosphorylation of Ser-267 is required for Sae2 function in Spo11 removal from meiotic DSB ends and subsequent resection of the latter (fig.20). This finding implies that Cdk1 activity is required not only for generation of meiotic DSBs, but also for their resection, thus providing a mechanism for coordinating DSB resection with progression through meiotic prophase.

Although formation of single-stranded DNA at Spo11-induced DSBs is undetectable in *sae2-S267A* cells, spore viability of the latter is still 15% compared with 1% of *sae2*Δ cells. One possibility is that additional Cdk1-dependent phosphorylation events on Sae2 might be needed for optimal resection. In agreement with this hypothesis, we found that *sae2-S267A-S134A* cells displayed a strong reduction in spore viability compared with *sae2-S267A* cells. This loss of viability was likely due to the lack of Ser-134 phosphorylation, because *sae2-S267A-S134D* spore viability was similar to that of *sae2-S267A* cells. Although we were unable to demonstrate that Sae2 Ser-134 is phosphorylated *in vivo* by using phosphospecific antibodies, these observations suggest that, in addition to

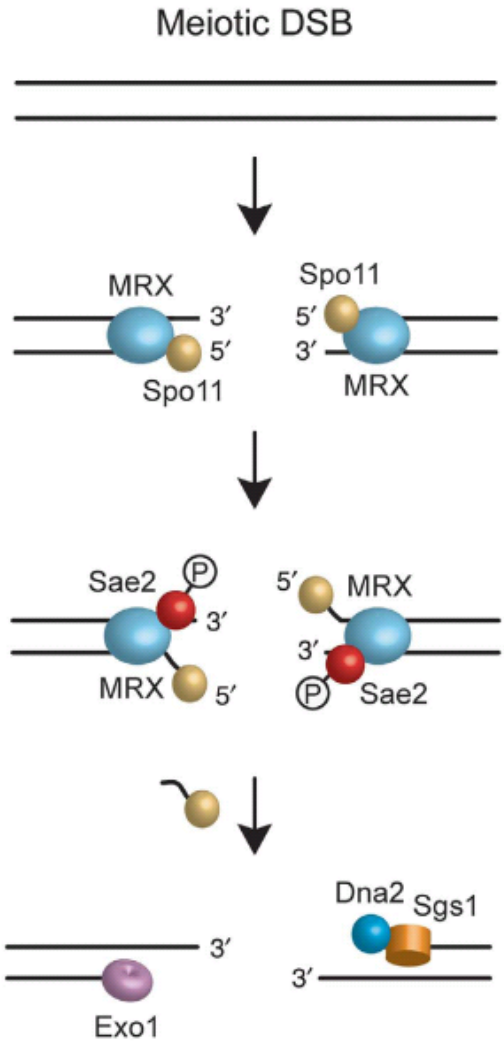


**FIG. 20 Resection of meiosis-specific DSBs.** Spo11, MRX and other proteins catalyse the formation of a meiosis-specific DSB. Upon phosphorylation of Sae2 by Cdk1, MRX and Sae2 catalyse the removal of Spo11 by endonucleolytic cleavage. Spo11 removal allows the processing of the 5'-strand by either Exo1 or Dna2–Sgs1.

Ser-267, phosphorylation of Ser-134 might be important for Sae2 meiotic functions.

How phosphorylation of Sae2 modulates Spo11 removal is still unknown. Sae2 has been shown to be an endonuclease that acts cooperatively with the MRX complex *in vitro* (Lengsfeld et al., 2007). These *in vitro* results were obtained in the absence of phosphorylation events, suggesting that phosphorylation of Sae2 is not absolutely required for its observed biochemical activity. Nevertheless, Sae2 function during meiosis and mitosis *in vivo* requires both Cdk1-dependent and checkpoint-dependent phosphorylation events (this study, Huertas et al., 2008; Cartagena-Lirola et al., 2006; Baroni et al., 2004). These apparent differences between the *in vivo* and *in vitro* data may suggest that unknown proteins inhibit Sae2 activity within the cell, such that its function is only exhibited upon phosphorylation events that relieve this inhibition. Alternatively, or in addition, Sae2 activity might be enhanced *in vivo* by positive regulators of DSB resection requiring Cdk1-dependent phosphorylations to exert their actions.

***DSB Processing after Spo11 Removal.***

It has been recently shown that Sae2, in conjunction with the MRX complex, functions in the initial trimming of accidental DSBs to generate short 3′ overhangs (Mimitou and Symington, 2008; Zhu et al., 2008). Then, a secondary processing, redundantly executed by either the Sgs1 helicase and the Dna2 nuclease or the 5′-3′ exonuclease Exo1, exposes extensive 3′ single-stranded tails (Mimitou and Symington, 2008; Zhu et al., 2008). The lengthening of single-stranded DNA tracts at Spo11-induced meiotic DSBs appears to have similar requirements in terms of nucleases as the processing events at accidental DSBs. In fact, both Sgs1 and Exo1 turned out to be involved in 3′-ended ssDNA generation after the initial endonucleolytic removal of Spo11, likely controlling two distinct but partially complementary pathways (fig.20). On the contrary, Exo1 and Sgs1 are not required for Spo11 removal, indicating that initiation and lengthening of meiotic DSB resection are controlled by different sets of nucleases.

Moreover, we demonstrate that generation of ssDNA at Spo11-induced DSBs depends also on the nuclease Dna2 (fig.20), which appears to contribute mainly to long range resection. Unfortunately, we could not establish the epistatic relationships between Dna2, Exo1, and Sgs1, because both *dna2*Δ *pif1-M2 exo1*Δ and *dna2*Δ *pif1-M2 pCLB2-EXO1* cells were unviable, and *dna2*Δ *pif1-M2 pCLB2-SGS1* cells did not enter meiosis synchronously. Nonetheless, the finding that resection of Spo11-induced DSBs is reduced to a lesser extent in *exo1*Δ than in *exo1*Δ *pCLB2-SGS1* cells indicates that Exo1 is not the only nuclease that can be targeted by Sgs1. Thus, we speculate that, although Exo1 can act independently of Sgs1, the helicase activity of the latter might unwind DSB ends to yield a fayed structure with both 5′ and 3′ single-stranded regions, thus facilitating nuclease access. Exo1 and/or other nuclease(s) such as Dna2 could then digest the 5′-terminal strand, resulting in a 3′ tail that can be engaged into homologous recombination. The combined use of enzymes with helicase and nuclease activities has been found also in bacteria, where the RecQ helicase and the RecJ 5′-3′ exonuclease function in DSB resection in the absence of the dominant RecBCD activity (Amundsen and Smith, 2003). There are compelling evidences that premeiotic DNA replication and DSB formation are coupled by a still unknown mechanism. In fact, delaying replication of the left arm of chromosome III locally delays DSB formation by the same margin, without affecting timing on the right arm (Borde et al., 2000; Murakami et al., 2003). One possibility is that replication fork passage (or associated processes) promotes installation of chromosome features that constrain subsequent DSB formation. Because Dna2 is involved in the removal of the Okazaki fragments during DNA replication (Bae et al., 2001), it is tempting to speculate that its function in the processing of Spo11-induced DSBs might be exerted only after the passage of the replication fork, thus linking premeiotic DNA replication not only with formation of DSBs, but also with their subsequent processing.

These findings led to the proposal of a model for the processing of meiosis-specific DSBs (Fig. 20) in which once Spo11 has catalysed DSB formation, it is removed from the DSB ends by endonucleolytic cleavage.

This cleavage requires CDK-mediated Sae2 phosphorylation and the nuclease activity of MRX and allows resection of the break which depends on either Exo1 or Dna2-Sgs1 activities.

# MATERIALS AND METHODS

# YEAST AND BACTERIAL STRAINS

## *Yeast strains*

Yeast strains used for this work are listed in Table 2.

**TABLE 2.** *S. cerevisiae* strains used in this study

| Strain | Relevant genotype |
|---|---|
| YLL1539/1C | *sae2Δ::KANMX4/sae2Δ::KANMX4 spo11Δ::hisG/spo11Δ::hisG* |
| YLL1573/2C | *MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL1600/1A | *dmc1Δ::KANMX4/dmc1Δ::KANMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL1602/2C | *sae2Δ:::NATMX4/ sae2Δ::NATMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL1672/2C | *mek1Δ::HPHMX4/mek1Δ::HPHMX4* |
| YLL1672/6A | *dmc1Δ::KANMX4/dmc1Δ::KANMX4 mek1Δ::HPHMX4/mek1Δ::HPHMX4* |
| YLL1788/75B | *spo11Δ::NATMX4/spo11Δ::NATMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL1970/2B | *tel1Δ::HPHMX4/tel1Δ::HPHMX4 sae2Δ::KANMX4/sae2Δ::KANMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL1973/21B | *spo11Δ::NATMX4/spo11Δ::NATMX4 mek1Δ::HPHMX4/mek1Δ::HPHMX4* |
| YLL1986/13C | *mec1Δ::NATMX4/mec1Δ::NATMX4 sml1Δ::HPHMX4/sml1Δ::HPHMX4 sae2Δ::KANMX4/sae2Δ::KANMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL2014/1B | *ndt80Δ::HPHMX4/ndt80Δ::HPHMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL2014/11A | *sae2Δ::NATMX4/sae2Δ::NATMX4 ndt80Δ::HPHMX4/ndt80Δ::HPHMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL2025 | [*DDC2-rad53kd-3XFLAG::URA3*] |
| YLL2027 | [*DDC2-RAD53-3XFLAG::URA3*] |
| YLL2032 | *dmc1Δ::KANMX4/dmc1Δ::KANMX4* [*DDC2-rad53kd-3XFLAG::URA3*] |
| YLL2033 | *dmc1Δ::KANMX4/dmc1Δ::KANMX4* [*DDC2-RAD53-3XFLAG::URA3*] |
| YLL2082/3B | *sae2Δ::HPHMX4/sae2Δ::HPHMX4 rad9Δ::KANMX4/rad9Δ::KANMX4* |
| YLL2082/12B | *rad9Δ::KANMX4/rad9Δ::KANMX4* |
| YLL2107/4C | *spo11Δ::NATMX4/spo11Δ::NATMX4 rad9Δ::HPHMX4/Rad9Δ::HPHMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL2111/8C | *dmc1Δ::KANMX4/dmc1Δ::KANMX4 rad54Δ::NATMX4/rad54Δ::NATMX4* |
| YLL2111/13C | *dmc1Δ::KANMX4/dmc1Δ::KANMX4 rad54Δ::NATMX4/rad54Δ::NATMX4 mek1Δ::HPHMX4/mek1Δ::HPHMX4* |
| YLL 1772/1D | *sae2Δ::NATMX4::SAE2::LEU2/sae2Δ::NATMX4::SAE2::LEU2 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL 2341/17D | *sae2Δ::NATMX4::sae2-S267A::LEU2/sae2Δ::NATMX4::sae2-S267A::LEU2 MEK1-HA3::URA3/MEK1-HA3::URA3* |

| | |
|---|---|
| YLL 2340/6C | *sae2Δ::NATMX4::sae2-S267D::LEU2/sae2Δ::NATMX4::sae2-S267D::LEU2 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL 1600/1A | *dmc1Δ::KANMX4/dmc1Δ::KANMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL 1990.45/7B | *sae2Δ::NATMX4/sae2Δ::NATMX4 dmc1Δ::KANMX4/dmc1Δ::KANMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL 2438.91/20A | *sae2Δ::NATMX4::sae2-S267A::LEU2/sae2Δ::NATMX4::sae2-S267A::LEU2 dmc1Δ::KANMX4/dmc1Δ::KANMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL 2720.10/5B | *sae2Δ::NATMX4::sae2-S267D::LEU2/sae2Δ::NATMX4::sae2-S267D::LEU2 dmc1Δ::KANMX4/dmc1Δ::KANMX4 MEK1-HA3::URA3/MEK1-HA3::URA3* |
| YLL 1772.5/3C-16 | *sae2Δ::NATMX4::SAE2::LEU2/sae2Δ::NATMX4::SAE2::LEU2 SPO11-MYC18::URA3/SPO11* |
| YLL 2679.27/9C-18 | *sae2Δ::NATMX4/sae2Δ::NATMX4 SPO11-MYC18::URA3/SPO11* |
| YLL 2341/16A-28 | *sae2Δ::NATMX4::sae2-S267A::LEU2/sae2Δ::NATMX4::sae2-S267A::LEU2 SPO11-MYC18::URA3/SPO11* |
| YLL 2340/5D-10 | *sae2Δ::NATMX4::sae2-S267D::LEU2/sae2Δ::NATMX4::sae2-S267D::LEU2 SPO11-MYC18::URA3/SPO11* |
| YLL 2516.60/1D-8 | *exo1Δ::HPH/exo1Δ::HPH pCLB2-SGS1::KANMX6/pCLB2-SGS1::KANMX6 SPO11-MYC18::URA3/SPO11* |
| YLL 2484.22/12D | *dmc1Δ::KANMX4/dmc1Δ::KANMX4* |
| YLL 2485.62/5D | *dmc1Δ::KANMX4/dmc1Δ::KANMX4 exo1Δ::HPH/ exo1Δ::HPH* |
| YLL 2528.21/1D | *dmc1Δ::NATMX4/dmc1Δ::NATMX4 exo1Δ::HPH/exo1Δ::HPH pCLB2-SGS1::KANMX6/pCLB2-SGS1::KANMX6* |
| YLL 2528.21/6D | *dmc1Δ::NATMX4/dmc1Δ::NATMX4 pCLB2-SGS1::KANMX6/pCLB2-SGS1::KANMX6* |
| YLL 2656.2/15C | *dmc1Δ::NATMX4/dmc1Δ::NATMX4 pif1-M2::URA3/pif1-M2::URA3* |
| YLL 2656.2/13D | *dmc1Δ::NATMX4/dmc1Δ::NATMX4 pif1-M2::URA3/pif1-M2::URA3 dna2Δ::HPH/dna2Δ::HPH* |
| YLL 1772/7D | *SAE2-HA3::URA3/SAE2* |
| YLL 2400 | *sae2Δ::NATMX4::sae2-S267A-HA3::URA3::LEU2/sae2Δ::NATMX4::sae2-S267A::LEU2* |
| SKY 615.4-2 | *cdc28-as/cdc28-as SAE2-HA3::URA3/SAE2* |
| YLL 1772.5/3C | *sae2Δ::NATMX4::SAE2::LEU2/sae2Δ::NATMX4::SAE2::LEU2* |
| YLL 2438.91/2A | *sae2Δ::NATMX4::sae2-S267A::LEU2/sae2Δ::NATMX4::sae2-S267A::LEU2* |
| YLL 2340/5D | *sae2Δ::NATMX4::sae2-S267D::LEU2/sae2Δ::NATMX4::sae2-S267D::LEU2* |
| YLL 2679.27/9C | *sae2Δ::NATMX4/sae2Δ::NATMX4* |
| YLL 2547.6/2A | *sae2Δ::NATMX4::sae2-S134A::LEU2/sae2Δ::NATMX4::sae2-S134A::LEU2* |
| YLL 2659.15/9C | *sae2Δ::NATMX4::sae2-S134D::LEU2/sae2Δ::NATMX4::sae2-S134D::LEU2* |
| YLL 2522.71/25A | *sae2Δ::NATMX4::sae2-S267A S134A::LEU2/sae2Δ::NATMX4::sae2-S267A S134A::LEU2* |
| YLL 2644.1/2B | *sae2Δ::NATMX4::sae2-S267A S134D::LEU2/sae2Δ::NATMX4::sae2-S267A S134D::LEU2* |
| YLL 2519.50/8D | *sae2Δ::NATMX4::sae2-S267A S179A::LEU2/sae2Δ::NATMX4::sae2-S267A S179A::LEU2* |

* Plasmids are indicated by brackets

All the strains were SK1 derivatives that were isogenic with the NKY3000 (*MAT*a/*MAT*α *HO/HO lys2/lys2 ura3*::*hisG/ura3*::*hisG leu2*::*hisG/leu2*::*hisG*) strain, kindly provided by N. Kleckner (Harvard University, Cambridge, MA) and R. Cha (Medical Research Council, London, United Kingdom).

Heterozygous diploid strains carrying deletions of the *MEC1*, *TEL1*, *SML1*, *DMC1*, *SAE2*, *MEK1*, *RAD9*, *NDT80*, *RAD54*, *SPO11*, *EXO1*, and *DNA2* genes were obtained by onestep PCR disruption (Wach et al. 1994). The diploid strain carrying the *cdc28-as* allele was kindly provided by S. Keeney (New York, NY). The *pif1-M2* mutation was introduced into an SK1 derivative strain as previously described (Schulz and Zakian, 1994). The *SGS1* promoter was replaced with the *CLB2* promoter using the pFA6a-KANMX6-pCLB2 cassette as described in Lee and Amon, 2003. The *sae2-S267A*, *sae2-S267D*, *sae2-S134A*, *sae2-S134D*, and *sae2-S179A* alleles were constructed by site-directed mutagenesis (Stratagene). ApaI digestion of the integrative plasmids pML469, pML674, pML673, pML692, pML703, pML691.3, pML691.5, and pML704 was used to direct the integration of these plasmids to the *SAE2* promoter region of a SK1-derivative *sae2Δ* strain, giving rise to heterozygous diploid strains carrying single copies of the *SAE2*, *sae2-S267A*, *sae2-S267D*, *sae2-S134A*, *sae2-S134D*, *sae2-S267AS179A*, *sae2-S267A-S134A*, and *sae2-S267A-S134D* alleles, respectively, at the *SAE2* chromosomal locus. Diploid strains homozygous for the above deletions or mutations were obtained after tetrad dissection of the corresponding heterozygous strains and self-diploidization of the spore carrying the desired alleles.

PCR one-step tagging was used to obtain strains carrying *myc*-tagged *SPO11* and HA-tagged *SAE2*, *sae2-S267A*, or *MEK1* alleles. The *SAE2-HA3*, *MEK1-HA3*, and *SPO11-MYC9* alleles were shown to be fully functional, since diploid strains homozygous for *MEK1-HA3*, *SAE2-HA3*, or *SPO11-MYC9* alleles were undistinguishable from the isogenic untagged strains with respect to meiotic progression and meiotic DSB repair.

pRS316 *DDC2-RAD53-3FLAG* (*DDC2-RAD53*) and pRS316 *DDC2-rad53K227A D339A-3FLAG* (*DDC2-rad53kd*) plasmids, used to

transform wild-type (NKY3000) and *dmc1Δ* strains, were kindly provided by D. Stern (University of California, San Francisco) (Lee at al., 2004).
The accuracy of all gene replacements and integrations was verified by Southern blot analysis or PCR.

### *E. coli strains*

***E. coli* DH5α™** strain *(F⁻, Φ80 dlacZDM15, D(lacZYA-argF) U169, deoR, recA1, endA1, hsdR17, (rK-,mK+) supE44, λ⁻, thi-1, gyrA96, relA1)* is used as bacterial host for plasmid manipulation and amplification. ***E. coli* DH5α™** Competent cells are purchased from Invitrogen.

## GROWTH MEDIA

### *S. cerevisiae* media

**YEP** (Yeast-Extract Peptone) is the standard rich media for *S. cerevisiae* and contains 10 g/L yeast extract, 20 g/L peptone and 50 mg/L adenine. YEP must be supplemented with 2% glucose (YEPD), 2% raffinose (YEP+raf) or 2% raffinose and 1% galactose (YEP+raf+gal) as carbon source. YEP-based selective media are obtained including 400 μg/mL G418, 300 μg/mL hygromicin-B or 100 μg/mL nourseotricin. Solid media are obtained including 2% agar. Stock solutions are 50% glucose, 30% raffinose, 30% galactose, 80 mg/mL G418, 50 mg/mL Hygromicin-B and 50 mg/mL Nourseotricin. YEP and glucose stock solution are autoclave-sterilized and stored at RT. Sugars and antibiotics stock solutions are sterilized by micro-filtration and stored at RT and 4°C respectively.
**S.C.** (Synthetic Complete) is the minimal growth media for *S. cerevisiae* and contains 1.7 g/L YNB (without aminoacids), 5 g/L ammonium sulphate, 200μM inositol, 25 mg/L uracil, 25 mg/L

adenine, 25 mg/L hystidine, 25 mg/L leucine, 25 mg/L tryptophan. S.C. can be supplemented with drop-out solution (20 mg/L arginine, 60 mg/L isoleucine, 40 mg/L lysine, 10 mg/L methionine, 60 mg/L phenylalanine, 50 mg/L tyrosine) based on ueast strains requirements. Different carbon sources can be used as in rich media (2% glucose, 2% raffinose or 2% raffinose and 1% galactose). One ore more aminoacid/base can be omitted to have S.C.-based selective media (e.g. S.C.-ura is S.C. lacking uracil). To obtain G418 or NAT S.C. selective medium the 5 g/L ammonium sulphate are replaced with 1 g/L monosodic glutamic acid. Solid media are obtained by including 2% agar. Stock solutions are 17 g/L YNB + 50 g/L ammonium sulphate (or 10g/L monosodic glutamic acid), 5 g/L uracil, 5 g/L adenine, 5 g/L hystidine, 5 g/L leucine, 5 g/L tryptophan, 100X drop out solution (2 g/L arginine, 6 g/L isoleucine, 4 g/L lysine, 1 g/L methionine, 6 g/L phenylalanine, 5 g/L tyrosine), 20mM inositol. All of these solutions are sterilized by micro-filtration and stored at 4°C.

**VB** sporulation medium contains 13.6 g/L sodium acetate, 1.9 g/L KCl, 0.35 g/L $MgSO_4$, 1.2 g/L NaCl. pH is adjusted to 7.0. To obtain solid medium include 2% agar. Sterilization by autoclavation.

**YPA** is the liquid pre-sporulation medium, it contains 1% yeast extract, 2% bactopeptone, 50 mg/liter adenine (YEP) and 1% potassium acetate.

**SPM** (liquid sporulation medium) contains 0,3% potassium acetate and 0,02% raffinose diluted in distilled $H_2O$.

### *E. coli* media

**LD** is the standard growth medium for *E. coli*. LD medium contains 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl. Solid medium is obtained by including 1% agar. LD+Amp selective medium is obtained including 50 μg/mL ampicillin. LD is autoclave-sterilized and stored at RT. Ampicillin stock solution (2.5 g/L) is sterilized by micro-filtration and stored at 4°C.

## CONSERVATION AND STORAGE OF *S. cerevisiae* AND *E. coli* STRAINS

Yeast cells are grown 2-3 days at 30°C on YEPD plates, resuspended in 15% glycerol and stored at -80°C. Bacteria are grown o/n at 37°C on LD+Amp plates, resuspended in 50% glycerol and stored at -80°C. Yeast and bacteria cells can be stored for years in these conditions.

## SYNCHRONOUS MEIOTIC TIME COURSE

The strains of interest were patched on YEPD (1% yeast extract, 2% Bacto peptone, 50 mg/liter adenine, 2% glucose) plates from a -80°C glycerol stock and incubated overnight at 30°C. To obtain synchronous G1/G0 cell population, overnight liquid YEPD cell cultures from these patches were diluted to a final concentration of 1 x $10^7$ cells/ml in 200 ml YPA (1% yeast extract, 2% Bacto peptone, 1% potassium acetate) in a 2-liter flask and grown with vigorous shaking for 13 h at 30°C. Cells were then washed and transferred into the same volume of SPM (0.3% potassium acetate, 0.02% raffinose) to induce meiosis.

## DETECTION OF MEIOTIC DSB FORMATION AND PROCESSING

Genomic DNA was purified from cells collected from synchronized meiotic cultures, and digested with EcoRI, and separated on native agarose gels. DSBs at the *THR4* hot spot were detected with a $^{32}$P-labeled 1.6-kb fragment spanning the 5' region of *THR4* as described in Kee et al., 2006. This probe was obtained by PCR using oligonucleotides PRP686 (5'-GGG GTA CCC CCA AGG TAA AAT TTC ACC GCG-3') and PRP687 (5'-GGG GTA CCC CGG CGT GCA ATA ATT GCA GAA-3') as primers and genomic DNA as the template. DSB end resection at the *THR4* hot spot was analyzed on alkaline agarose gels. The single-stranded probe used to detect DSB resection was obtained by in vitro transcription using Promega

Riboprobe System-T7 and plasmid pML601.11 as template. The latter was constructed by inserting in the pGEM-7Zf EcoRI site a 700-bp fragment containing part of the *THR4* locus (coordinates 212503 to 213199 on chromosome III), obtained by PCR by using yeast genomic DNA as template and PRP924 (5'-CGG AAT TCC ATG GAT GTT CTT GGG CTG GAT-3') and PRP925 (5'-CGG AAT TCT GCA TGA AGA ACT GTG CCG TGA-3') as primers. Quantitative analysis of DSB processing was performed by calculating the ratio of band intensities for ssDNA and parental DNA.

## IN SITU RENATURATION ASSAY

Yeast protein extracts were prepared from TCA-treated cells (see below). Equal amounts of proteins (25μg/sample), mixed with 1X Laemmli buffer, were loaded onto 10% SDS-polyacrilamide gels and run according to standard procedures (see below). After electrophoresis, gels were blotted onto PVDF (Immobilon-P, Millipore). The membranes were then subjected to a denaturation/renaturation protocol according to the procedure described by Ferrel and Martin (1991) with the following modifications.

The denaturating step was for 1 hour at room temperature in 7 M guanidine-HCl, 50 mM Dithiothreitol (DTT; freshly prepared), 2 mM EDTA, 50 mM Tris-HCl pH 8. The membranes were then washed twice in 1X Tris-buffered saline (TBS) buffer for 10 minutes at room temperature. Renaturation was carried out at $4^{o}$C for 12-18 hours with weak shacking in 2 mM DTT (freshly prepared), 2mM EDTA, 0,04% Tween-20, 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 1% bovine serum albumine (BSA). The buffer was changed at least 4 times. The membranes were then washed for 60 minutes in 30 mM Tris-HCl pH 7.5 and equilibrated in kinase buffer (1 mM DTT, 0.1 mM EGTA, 20mM $MgCl_2$, 20 mM $MnCl_2$, 40 mM HEPES-NaOH pH 8, 100 μM sodium orthovanadate) for 30 minutes at room temperature. The autophosphorylation reaction was performed by incubating the membranes in the kinase buffer in the presence of 10μCi/ml of

[$\gamma$ –$^{32}$P]ATP for 1 hour at room temperature. Membranes were then washed as follows: twice for 10 minutes in 30 mM Tris-HCl pH 7.5, once for 10 minutes in 30 mM Tris-HCl pH 7.5 + NP-40 (freshly prepared), 10 minutes in 30 mM Tris-HCl pH 7.5, 10 minutes in 1 M KOH, 10 minutes in water, 10 minutes in 10% TCA and 10 minutes in water. The filters were then dried and exposed.

## ChIP (CHROMATIN IMMUNOPRECIPITATION) ANALYSIS AND REAL-TIME PCR

ChIP analysis was performed as previously described (Viscardi et al., 2007).
Exponentially growing cells (50 mL of $8x10^6$-$1x10^7$ cells/mL) are treated or not (non cross-linked control) with 1.4 mL of 37% formaldehyde for 5 minutes while shacking, in order to create DNA-protein and protein-protein covalent bounds (cross-link). Then 2.5mL of 2.5M glycine are added for other 5 minutes while shacking. Treated cells are kept in ice until centrifugation at 1800 rpm for 5' at 4$^o$C. Cell pellet is then washed first with HBS buffer (50mM HEPES pH 7.5, 140mM NaCl) and then with ChIP buffer (50mM HEPES pH 7.5, 140mM NaCl, 1mM EDTA pH 8, 1% IGEPAL CA-630, 0.1% Sodium deoxycholate, 1mM PMSF). Before each wash, cells are pelletted by centrifugation at 1800 rpm for 5' at 4$^o$C. After the wash with ChIP buffer and subsequent centrifugation, the supernatant is carefully and completely removed. Then additional 0.4mL of ChIP buffer + complete anti-proteolitic tablets (Roche) is adeed. (Store at -80 $^o$C). After breaking cells for 30' at 4$^o$C with glass beads , the latter are eliminated. This passage is followed by centrifugation at 4$^o$C for 30'. Pellet is resuspended in 1.5 mL of ChIP buffer + anti-proteolitics and then sonicated, in order to divide DNA in 500-1000 bp fragments (4 times for 25''). At this point 5μL as "input DNA" for PCR reactions and 20 μL as "input" for western blot analysis are taken. Then 400 μL of the remaining solution is immunoprecipitated with specific Dynabeads-coated antibodies. After proper incubation with desired antibodies, Dynabeads can be washed at RT as follows: 2X

SDS buffer (50mM HEPES pH 7.5, 1mM DETA pH 8, 140mM NaCl, 0.024 SDS), 1X with High-salt Buffer ( 50mM HEPES pH 7.5, 1mM EDTA pH 8, 1M NaCl), 1X with T/L buffer (20mM Tris-HCl pH 7.5, 250mM LiCl, 1mM EDTA pH 8, 0.05% sodium deoxycholate, 0.5% IGEPAL CA-630) and then with T/E buffer (20mM Tris-HCl pH 7.5, 0.1mM EDTA pH8). All washes are done by pulling down Dynabeads for 1' and then nutating for 4' with the specific buffer. After the last wash Dynabeads are resuspended in 145µL TE + 1% SDS buffer, shaked on a vortex, put at 65°C for 2', shaked on vortex again and then pulled down. Now 120µl of the surnatant should be put at 65°C over-night for reverse cross-linking, while 20µl should be stored as samples for western blot analysis of the immunoprecipitated protein amount. Previously taken input DNA samples should be put at 65°C overnight togheter with 115 µl of TE + 1% SDS buffer. The next day DNA should be purified for PCR analysis (QIAgen columns).
PCR reactions were performed with IQ SUPERMIX containing Cyber Green.
Quantification of immunoprecipitated DNA was achieved by quantitative realtime PCR on a Bio-Rad Mini Opticon using primers located 162 bp (DSB) and 2319 bp (CON) distal to the DSB site of the *YCR048W* hotspot and normalized to input signal for each primer set.
PCR reactions were done as follows:

95°C for 3' → 95°C for 10"
60°C for 30"
fluorescence reading x 40 times

Melting Curve:
60°C → 95°C with 0.5°C increments each 10 seconds → fluorescence reading

Sequences of the oligonucleotides used in this analysis:
DSBFW: 5'-TGAAGAATGACGGAGACTAAGGA-3'
DSBRW: 5'-TGTTGGCTTCTGCGGAAT-3'
CONFW: 5'-ATATATGGGTGGCTGCTTCAG-3'
CONRW: 5'-TGTAGCCGTTAATGTCCTGG-3'

*Nicola Manfrini*

## GENOTOXIC TREATMENT OF YEAST CELLS

Yeast cells can be treated with a variety of genotoxic agents, among which UV, Methylmethane sulphonate (MMS), phleomycin or bleomycin, in order to study the cellular response to different types of DNA damage.
In our study we used phleomycin at a concentration of 5μg/ml adding it directly to SPM media after 210 minutes after meiosis induction.

## MOLECULAR BIOLOGY TECHNIQUES

### AGAROSE GEL ELECTROPHORESIS

Agarose gel elctrophoresis is the most easy and common way of separating and analyzing DNA molecules. This technique aloe the separation of DNA fragments based on their different molecular weight (or length in kb). The purpose of this technique might be to visualize the DNA, to quantify it or to isolate a particular DNA fragment. The DNA is visualized by the addition in the gel of ethidium bromide, which is a fluorescent dye that intercalates between bases of nucleic aicds. Ethidium bromide absorbs UV light and transmits the energy as visible orange light, revealing the DNA molecules to which is bound.
To pour a gel, agarose powder is mixed with TAE (0.04M Tris-Acetate 0.001M EDTA) to the desired concentration, and the solution is microwaved until completely melted. Most gels are made between 0.8% and 2% agarose. A 0.8% gel will show good resolution of large DNA fragments (5-10 Kb) and a 2% gel will show good resolution for small fragments (0.2-1 Kb). Ethidium bromide is added to the gel at a final concentration of 1 μg/mL to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at RT or at 4°C. The comb is then removed and the gel is placed into an electrophoresis chamber and just covered with the buffer (TAE). Sample containing DNA mixed with loading buffer are then pipetted

into the sample wells. The loading buffer contains 0.05% bromophenol blue and 5% glycerol, which give colour and density to the sample. A marker containing DNA fragments of known length and concentration is loaded in parallel to determine size and quantity of DNA fragments in the samples. Then current is applied and DNA will migrate toward the positive electrode. When adequate migration has occurred, DNA fragments are visualized by placing the gel on a UV transilluminator.

### DNA EXTRACTION FROM AGAROSE GELS (PAPER STRIP METHOD)

This method allow to isolate a DNA fragment of interest. Using a scalpel blade cut a slit immediately in front of the band to be extracted. Cut a piece of GF-C filter to size to fit inside the slit. Place the paper strip in the slit and switch on the current for 1-2 minutes at 150 V. The DNA runs onward into the paper and is delayed in the smaller mesh size of the paper. Remove the strip of paper and place it into a 0.5 mL microcentrifuge tube. Make a tiny hole in the bottom of the tube using a syringe needle, place the 0.5 mL tube inside a 1.5 mL tube and spin for 30 seconds. Buffer and DNA are retained in the 1.5 mL tube. Extract the DNA with 1 volume of phenol/chloroform and precipitate the DNA with 100mM sodium acetate and 3 volumes of 100% ethanol. After microcentrifugation re-dissolve DNA in a appropriate volume of water, TRIS (10mM Tris HCl pH 8.5) or TE (10mM Tris HCl 1mM EDTA pH7.4) buffer.

### RESTRICTION ENDONUCLEASES

Type II endonucleases (also known as restriction endonuceases or restriction enzymes) cut DNA molecules at defined positions close to their recognitions sequences in a reaction known as enzymatic digestion. They produce discrete DNA fragments that can separated by agarose gel electrophoresis, generating distinct gel banding patterns. For these reasons they are used for DNA analysis and gene cloning. Restriction enzymes are generally stored at -20°C in a solution containing 50% glycerol, in which they are stable but not active. Glycerol concentration in the reaction mixture must be below 5% in order to allow enzymatic reaction to occur. They generally work at 37°C with some exceptions (e.g. ApaI activity is maximal at

25°C) and they must be supplemented with a reaction buffer provided by the manufacturer, and in some cases with Bovin Serum Albumin. We use restriction endonucleases purchased from NEB and PROMEGA.

LIGATION.

DNA is previously purified from agarose gel with the paper strip method, phenol/chloroform extracted, ethanol precipitated and resuspended in the appropriate volume of water or TE buffer. The ligation reaction is performed in the following conditions: DNA fragment and vector are incubated overnight at 16°C with 1 μl T4 DNA Ligase (NEB) and T4 DNA Ligase Buffer (NEB). The ligation reaction is then used to transform competent *E. coli* cells. Plasmids are recovered from Amp+ transformants and subjected to restriction analysis.

PREPARATION OF YEAST GENOMIC DNA FOR POLYMERASE CHAIN REACTION (PCR)

Resuspend yeast cells in 200 μl Yeast Lysis Buffer (2% TRITON X100, 1% SDS, 100mM NaCl, 10mM Tris HCl pH 8, 1mM EDTA pH 8), add 200 μl glass beads, 200 μl phenol/chloroform and vortex 3 minutes. Ethanol precipitate the aqueous phase obtained after 5 minutes centrifugation. Resuspend DNA in the appropriate volume of water and use 1 μl as a template for PCR.

POLYMERASE CHAIN REACTION (PCR)

PCR allows to obtain high copy number of a specific DNA fragment of interest starting from very low quantity of DNA fragment. The reaction is directed to a specific DNA fragment by using a couple of oligonucleotides flanking the DNA sequence of interest. These oligonucleotides work as primers for the DNA polymerase. The reaction consist of a number of polymerization cycles which are based on 3 main temperature-dependent steps: denaturation of DNA (which occur over 90°C), primer annealing to DNA (typically take place at 45-55°C depending on primer characteristic), synthesis of the DNA sequence of interest by a thermophilic DNA polymerase (which usually works at 68 or 72°C). Different polymerases with different

properties (processivity, fidelity, working temperature, etc) are commercially available and suitable for different purpose. Taq polymerase works at 72°C and is generally used for analytical PCR. Polymerases with higher fidelity like Pfx and VENT polymerases, which work respectively at 68 and 72°C, are generally employed when 100% polymerization accuracy is required.

The typical 50 µl PCR mixture contains 1µl of template DNA, 0.5 µM each primer, 200µM dNTPs, 5 µl of 10X Reaction Buffer, 1mM $MgCl_2$, 1-2 U DNA polymerase and water to 50 µL. The typical cycle-program for a reaction is: 1. 3' denaturation at 94-95°C; 2. 30" denaturation at 94-95°C; 3. 30" annealing at primers $T_m$ (melting temperature); 4. 1' polymerization per Kb at 68 or 72°C (depending on polymerase); 5. repeat 30 times from step 2; 6. 5-10' polymerization at 68-72°C. The choice of primer sequences determines the working $T_m$, which depends on the length (L) and GC% content of the oligonucleotides and can be calculated as follows: $T_m = 59.9 + 0.41(GC\%) – 675/L$.

PLASMID DNA EXTRACTION FROM *E. COLI* (I): MINIPREPS BOILING

*E. coli* cells (2ml overnight culture) are harvested by centrifugation and resuspended in 500 µl STET buffer (8% sucrose, 5% TRITON X-100, 50mM EDTA, 50mM Tris-HCl, pH 8). Bacterial cell wall is digested boiling the sample for 2 minutes with 1 mg/mL lysozyme. Cellular impurities are removed by centrifugation and DNA is precipitated with isopropanol and resuspended in the appropriate volume of water or TE.

PLASMID DNA EXTRACTION FROM *E. COLI*. (II): MINIPREPS WITH QIAGEN COLUMNS

This protocol allows the purification of up to 20 µg high copy plasmid DNA from 1-5 mL overnight *E. coli* culture in LD medium. Cells are pelleted by centrifugation and resuspended in 250 µl buffer P1 (100 µg/mL RNase, 50mM Tris HCl pH 8, 10mM EDTA pH 8). After addition of 250 µl buffer P2 (200mM NaOH, 1% SDS) the solution is mixed thoroughly by inverting the tube 4-6 times, and the lysis reaction occur in 5 minutes at RT. 350 ml N3 buffer (QIAGEN) are

added to the solution, which is then centrifuged for 10 minutes. The supernatant is applied to a QIAprep spin column which is washed once with PB buffer (QIAGEN) and once with PE buffer (QIAGEN). The DNA is eluted with EB buffer (10mM Tris HCl pH 8.5) or water.

TRANSFORMATION OF *E. COLI* (I): DH5α

DH5α competent cells are thawed on ice. Then, 50-100 μl cells are incubated 30 minutes in ice with 1 μl plasmid DNA. Cells are then subjected to heat shock at 37°C for 30 seconds and then incubated on ice for 2 minutes. Finally, 900 μl LD are added to the tube and cells are incubated 30 minutes at 37°C to allow expression of ampicillin resistance. Cells are then plated on LD+amp and overnight incubated at 37°C.

TRANSFORMATION OF *E. COLI* (II): 6507

*E. coli* 6507 cells competent to transformation are obtained as follows. Inoculate 1 ml of *E. coli* overnight culture into 100 mL LD+amp medium and grow cells at 37°C to 0.5 $OD_{550}$. Cells are pelleted by centrifugation at 4°C, resuspended in 25 mL chilled 0.1M $MgCl_2$ and kept on ice for 30-60 minutes. This step is repeated 2 times with the difference that cells are resuspended first in 25 mL and finally in 5 mL chilled 0.1M $CaCl_2$. *E. coli* 6507 competent cells can be stored at -80°C after the addition of 14% glycerol. Aliquots of cells can be transformed with plasmid DNA with a standard protocol or by electroporation. To perform electroporation, DNA is added to an aliquot of *E. coli* competent cells. After 10 minutes on ice, solution is transferred into an electroporation cuvette and current is applied. Cells are then incubated 60 minute at 37°C with LD media and subsequently plated on LD+amp.

TRANFORMATION OF S. CEREVISIAE SK1 STRAINS

Indicated quantities are sufficient for 4 transformations.

YEPD growing cells are harvested at the concentration of $5x10^6$ - $1x10^7$ cells/mL by centrifugation and washed with 1000 μL of water. Then cells are resuspended in 500 μL of water and then equally distributed (100μL) to 4 2mL-eppendorfs. Each transformation,

containing 100 µL of cells is incubated at 30°C for 50 minutes with 240 µL of PEG (PolyEthylene-Glycol 3350) 50%, 10 µL of carrier DNA (salmon sperm DNA), 36 µL of LiAc 1M and DNA of interest. Then, cells are heat-shocked at 42°C for 20 minutes and, after washing, are plated on non-selective medium. Plates are incubated at 30°C for one night and the next day replicated on the appropriate selective medium.

EXTRACTION OF YEAST GENOMIC DNA (TEENY YEAST DNA PREPS)

Yeast cells are harvested from overnight cultures by centrifugation, washed with 1 mL of 0.9M sorbytol 0.1M EDTA pH 7.5 and resuspended in 0.4 mL of the same solution supplemented with 14mM β-mercaptoethanol. Yeast cell wall is digested by 45 minutes incubation at 37°C with 0.4 mg/mL 100T zimoliase. Spheroplasts are harvested by 30 seconds centrifugation and resuspended in 400 µL TE. After addition of 90 µl of a solution containing EDTA pH 8.5, Tris base and SDS, spheroplasts are incubated 30 minutes at 65°C. Samples are kept on ice for 1 our following addition of 80 µl 5M potassium acetate. Cell residues are eliminated by 15 minutes centrifugation at 4°C. DNA is precipitated with chilled 100% ethanol, resuspended in 500 µL TE and incubated 30 minutes with 25 µL 1 mg/mL RNase to eliminate RNA. DNA is then precipitated with isopropanol and resuspended in the appropriate volume (typically 50 µL) of TE.

SOUTHERN BLOT ANALYSIS

Yeast genomic DNA prepared with standard methods is digested with the appropriate restriction enzyme(s). The resulting DNA fragments are separated by agarose gel electrophoresis in a 0.8% agarose gel. When adequate migration has occurred, gel is washed 40 minutes with a denaturation buffer (0.2N NaOH, 0.6M NaCl), and 40 minutes with a neutralization buffer (1.5M NaCl, 1M Tris HCl, pH 7.4). DNA is blotted onto a positively charged nylon membrane by overnight capillary transfer with 10X SSC buffer (20X SSC: 3M sodium chloride, 0.3M sodium citrate, pH 7.5). Membrane is then washed with 4X SSC and UV-crosslinked.

Hybridization is carried out by incubating membrane for 5 hours at 50°C with pre-hybridization buffer (50% formamide, 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 2% Blocking reagent) following by o/n incubation at 50°C with pre-hybridization buffer + probe. The probe is obtained by random priming method (DECAprime$^{TM}$ kit by Ambion) on a suitable DNA template and with $^{32}$P d-ATP. Filter is then washed (45'+15') at 55°C with a washing solution (0.2M sodium phosphate buffer pH 7.2, SDS 1%, water), air dried and then exposed to an autoradiography film.

DENATURING GEL ELECTROPHORESIS AND SOUTHERN BLOT ANALYSIS TO VISUALIZE SSDNA

A 0.8% agarose gel (in $H_2O$) is submerged in a gel box containing a 50mM NaOH, 1mM EDTA solution for 30 minutes to equilibrate. Ethidium bromide is omitted because it does not efficiently bind to DNA under these conditions. After digestion with the appropriate restriction enzyme(s), DNA samples are prepared by adjusting the solution to 0.3M sodium acetate and 5mM EDTA (pH 8.0) following by addition of 2 volumes of ethanol to precipitate DNA. After chilling (o/n) and centrifuging the samples (15 minutes, possibly at 4°C), pellet is resuspended in alkaline gel loading buffer (1X buffer: 50mM NaOH, 1mM EDTA pH 8.5, 2.5% Ficoll (Type 400) and 0.025% bromophenol blue). After loading the DNA in the gel, a glass plate can be placed on the gel to prevent the dye from diffusing from the agarose during the course of the run. Because of the large currents that can be generated with denaturing gels, gels are usually run slowly at lower voltages (e.g. 30 V o/n). After the DNA has migrated far enough, the gel can be stained with 0.5 μg/ml ethidium bromide in 1X TAE electrophoresis buffer (1 hour). The DNA will be faint because the DNA is single stranded. Gel is then soaked in 0.25N HCl for 7 minutes with gentle agitation, rinsed with water and soaked in 0.5M NaOH, 1.5M NaCl for 30 minutes with gentle agitation. Gel is then rinsed briefly with water and DNA is blotted by capillary transfer onto neutral nylon membrane using 10X SSC. Hybridization is carried out by incubating membrane for 5 hours at 42°C with pre-hybridization buffer (50% formamide, denhardts solution + 4X BSA, 6% destran sulphate, 100 μg/ml salmon sperm DNA, 200 μg/ml tRNA carrier)

following by o/n incubation at 42°C with pre-hybridization buffer + ssRNA probe. The ssRNA probe is obtained by *in vitro* transcription using Promega Riboprobe System-T7 and a pGEM-7Zf-based plasmid as a template. Following hybridization, membrane is washed twice with 5X SSPE (20X SSPE = 3M NaCl, 200μM $NaH_2PO_4$, 20μM EDTA, pH 7.4) at 42°C for 15 minutes, 30 minutes with 1X SSPE 0.1% SDS at 42°C, 30 minutes with 0.1X SSPE 0.1% SDS at 42°C, 15 minutes with 0.2X SSPE 0.1% SDS at 68°C and 5 minutes with 0.2X SSPE at RT. Finally membrane is exposed to a X-ray film.

## Other techniques

### FACS ANALYSIS OF DNA CONTENTS

FACS (Fluorescence-Activated Cell Sorting) analysis allow to determine the DNA content of every single cell of a given population of yeast cells. $6 \cdot 10^6$ cells are harvested by centrifugation, resuspended in 70% ethanol and incubated at RT for 1 hour. Cells are then washed with 1 mL 50mM Tris pH 7.5 and incubated overnight at 37°C in the same solution with 1 mg/mL RNase. Samples are centrifuged and cells are incubated at 37°C for 30 minutes with 5 mg/mL pepsin in 55mM HCl, washed with 1 mL FACS Buffer and stained in 0.5 mL FACS buffer with 50 μg/mL propidium iodide. 100 μL of each sample are diluted in 1 mL 50mM Tris pH 7.5 and analysed with a Becton-Dickinson FACS-Scan. The same samples can also be analysed by fluorescence microscopy to score nuclear division.

### TOTAL PROTEIN EXTRACTS

Total protein extracts were prepared from $10^8$ cells collected from exponentially growing yeast cultures. Cells are harvested by centrifugation and washed with 20% trichloracetic acid (TCA) in order to prevent proteolysis and resuspended in 50 μl 20% TCA. After addition of 200 μL of glass beads, cells are disrupted by vortexing for 8 minutes. Glass beads are washed with 400 μL 5% TCA, and the resulting extract are centrifuged at 3000 rpm for 10 minutes. The

pellet is resuspended in 70 μL Laemmli buffer (0.62M Tris, 2% SDS, 10% glycine, 0.001% Bfb, 100mM DTT), neutralized with 30 μL 1M Tris base, boiled for 3 minutes, and finally clarified by centrifugation.

### SDS-PAGE AND WESTERN BLOT ANALYSIS

Protein extracts are loaded in 10% polyacrylamide gels (composition). Proteins are separated based on their molecular weight by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). When adequate migration has occurred proteins are blotted onto nitrocellulose membrane. Membrane is saturated by 1 hour incubation with 4% milk in TBS containing 0.2% TRITON X-100 and incubated for 2 hours with primary antibodies. Membrane is washed three times with TBS for 10 minutes, incubated for 1 hour with secondary antibodies and again washed with TBS. Detection is performed with ECL (Enhanced ChemiLuminescence – GE Healthcare) and X-ray films according to the manufacturer. Primary polyclonal rabbit anti-Rad53 antibodies are kindly provided by John Diffley (Clare Hall Laboratories, London) while anti-Rad9 polyclonal antibodies are kindly provided by N. Lowndes (National University of Ireland, Ireland). Primary monoclonal 12CA5 anti-HA and 9E10 anti-MYC antibodies are purchased at GE Healthcare, as well as peroxidase conjucated IgG anti-rabbit and anti-mouse secondary antibodies.
FLAG-tagged proteins were detected with monoclonal anti-FLAG antibodies purchased from Sigma.

# REFERENCES

**Adams, K.E., A.L. Medhurst, D.A. Dart, N.D. Lakin.** 2006. Recruitment of ATR to sites of ionising radiation-induced DNA damage requires ATM and components of the MRN protein complex, Oncogene 25, 3894–3904.

**Akamatsu, Y., Y. Murayama, T. Yamada, T. Nakazaki, Y. Tsutsui, K. Ohta, H. Iwasaki.** 2008. Molecular characterization of the role of the *Schizosaccharomyces pombe nip1+/ctp1+* gene in DNA double-strand break repair in association with the Mre11–Rad50–Nbs1 complex, Mol. Cell. Biol. 28, 3639– 3651.

**Alani, E., R. Padmore, and N. Kleckner.** 1990. Analysis of wild-type and *rad50* mutants of yeast suggests an intimate relationship between meiotic chromosome synapsis and recombination. Cell 61, 419–436.

**Amon, A., U. Surana, I. Muroff I, K. Nasmyth.** 1992. Regulation of p34CDC28 tyrosine phosphorylation is not required for entry into mitosis in S. cerevisiae. Nature. 355, 368-371.

**Amundsen, S. K., and Smith, G. R**. 2003. Interchangeable parts of the *Escherichia coli* recombination machinery. Cell 112, 741–744

**Aylon, Y., and M. Kupiec.** 2003. The checkpoint protein Rad24 of *Saccharomyces cerevisiae* is involved in processing double-strand break ends and in recombination partner choice. Mol. Cell. Biol. 23, 6585–6596.

**Aylon, Y., B. Liefshitz, M. Kupiec.** 2004. The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle, EMBO J. 23, 4868–4875.

**Bae, S. H., Bae, K. H., Kim, J. A., Seo, Y. S.** 2001. RPA governs endonuclease switching during processing of Okazaki fragments in eukaryotes. Nature 412, 456–461

**Bailis, J. M., and G. S. Roeder.** 1998. Synaptonemal complex morphogenesis and sister-chromatid cohesion require Mek1-dependent phosphorylation of a meiotic chromosomal protein. Genes Dev. 12, 3551–3563.

**Bakkenist, C.J., M.B. Kastan.** 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimmer dissociation, Nature 421, 499–506.

**Barlow, C., M. Liyanage, P. B. Moens, M. Tarsounas, K. Nagashima, K. Brown, S. Rottinghaus, S. P. Jackson, D. Tagle, T. Ried, and A. Wynshaw-Boris.** 1998. Atm deficiency results in severe meiotic disruption as early as leptonema of prophase I. Development 125, 4007–4017.

**Baroni, E., V. Viscardi, H. Cartagena-Lirola, G. Lucchini, M.P. Longhese.** 2004. The functions of budding yeast Sae2 in the DNA damage response require Mec1- and Tel1-dependent phosphorylation, Mol. Cell. Biol. 24, 4151–4165.

**Benjamin, K. R., C. Zhang, K. M. Shokat, I. Herskowitz.** 2003. Control of landmark events in meiosis by the CDK Cdc28 and the meiosis-specific kinase Ime2. Genes Dev. 17, 1524–1539.

**Benjamin, K. R., C. Zhang, K.M. Shokat, I. Herskowitz.** 2003. Control of landmark events in meiosis by the CDK Cdc28 and the meiosis-specific kinase Ime2. Genes Dev. 17, 1524–1539.

**Bishop, A. C., Ubersax, J. A., Petsch, D. T., Matheos, D. P., Gray, N. S., Blethrow, J., Shimizu, E., Tsien, J. Z., Schultz, P. G., Rose, M. D., Wood, J. L., Morgan, D. O., and Shokat, K. M.** 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature 407, 395–401.

**Bishop, D. K., D. Park, L. Xu, and N. Kleckner.** 1992. *DMC1*: a meiosis specific yeast homolog of *E. coli* recA required for recombination, synaptonemal complex formation, and cell cycle progression. Cell 69, 439–456.

**Bishop, D.K. and D. Zickler.** 2004. Early decision; meiotic crossover interference prior to stable strand exchange and synapsis. Cell 117, 9-15.

**Bolte, M., P. Steigemann, G. H. Braus, S. Irniger.** 2002. Inhibition of APC-mediated proteolysis by the meiosis-specific protein kinase Ime2. Proc. Natl Acad. Sci. USA 99, 4385–4390.

**Bonetti, D., Martina, M., Clerici, M., Lucchini, G., and Longhese, M. P.** 2009. Multiple pathways regulate 3' overhang generation at S. cerevisiae telomeres. Mol. Cell 35, 70–81

**Booher R.N., R.J. Deshaies, M.W. Kirschner.** 1993. Properties of Saccharomyces cerevisiae wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins. EMBO J. 12, 3417-3426.

**Borde, V., Goldman, A. S., and Lichten M.** 2000. Direct coupling between meiotic DNA replication and recombination initiation. Science 290, 806–809

**Budd, M. E., Reis, C. C., Smith, S., Myung, K., and Campbell, J. L.** 2006. Evidence suggesting that Pif1 helicase functions in DNA replication with the Dna2 helicase/nuclease and DNA polymerase delta. Mol. Cell. Biol. 26, 2490–2500

**Carballo, J. A., A. L. Johnson, S. G. Sedwick, and R. S. Cha.** 2008. Phosphorylation of the axial element protein Hop1 by Mec1/Tel1 ensures meiotic interhomolog recombination. Cell 132, 758–770.

**Carballo, J. A., and R. S. Cha.** 2007. Meiotic roles of Mec1, a budding yeast homolog of mammalian ATR/ATM. Chromosome Res. 15, 539–550.

**Carlile T.M., A. Amon.** 2008.Meiosis I is established through division-specific translational control of a cyclin. Cell. 133, 280-291.

**Cartagena-Lirola, H., I. Guerini, V. Viscardi, G. Lucchini, and M. P. Longhese.** 2006. Budding yeast Sae2 is an in vivo target of the Mec1 and Tel1 checkpoint kinases during meiosis. Cell Cycle 5, 1549–1559.

**Celerin, M., S.T. Merino, J.E. Stone, A.M. Menzie, M.E. Zolan.** 2000. Multiple roles of Spo11 in meiotic chromosome behavior. EMBO J. 19, 2739-50.

**Chen, Y.T., C.A. Venditti, G. Theiler, B.J. Stevenson, C. Iseli, A.O. Gure, C.V. Jongeneel, L.J. Old, A.J. Simpson.** 2005. Identification of CT46/HORMAD1, an immunogenic cancer/testis antigen encoding a putative meiosis-related protein. Cancer Immun. 7, 5-9.

**Chu, S., and I. Herskowitz.** 1998. Gametogenesis in yeast is regulated by a transcriptional cascade dependent on Ndt80. Mol. Cell 1, 685–696.

**Clerici, M., D. Mantiero, G. Lucchini, and M. P. Longhese.** 2006. The *Saccharomyces cerevisiae* Sae2 protein negatively regulates DNA damage checkpoint signalling. EMBO Rep. 7, 212–218.

**Clerici, M., D. Mantiero, G. Lucchini, M.P. Longhese.** 2005. The *Saccharomyces cerevisiae* Sae2 protein promotes resection and bridging of double strand break ends, J. Biol. Chem. 280, 38631–38638.

**Clerici, M., V. Baldo, D. Mantiero, F. Lottersberger, G. Lucchini, M.P. Longhese.** 2004. A Tel1/MRX-dependent checkpoint inhibits the metaphase-to-anaphase transition after UV irradiation in the absence of Mec1, Mol. Cell. Biol. 24, 10126–10144.

**Clyne, R.K., V.L Katis, L. Jessop, K.R. Benjamin, I. Herskowitz, M. Lichten, K. Nasmyth.** 2003. Polo-like kinase Cdc5 promotes chiasmata formation and cosegregation of sister centromeres at meiosis I. Nature Cell Biol. 5, 480–485.

**Cortez, D., S. Guntuku, J. Qin, S.J. Elledge.** 2001. ATR and ATRIP: partners in checkpoint signalling, Science 294, 1713–1716.

**Cuadrado, M., B. Martinez-Pastor, M. Murga, L.I. Toledo, P. Gutierrez-Martinez, E. Lopez, O. Fernandez-Capetillo. 2006.** ATM regulates ATR chromatin loading in response to DNA double-strand breaks, J. Exp. Med. 203, 297–303.

**Dirick, L., L. Goetsch, G. Ammerer, and B. Byers.** 1998. Regulation of meiotic S phase by Ime2 and a Clb5,6-associated kinase in *Saccharomyces cerevisiae*. Science 281, 1854–1857.

**Durocher, D., J. Henckel, A.R. Fersht, S.P. Jackson.** 1999. The FHA domain is a modular phosphopeptide recognition motif, Mol. Cell 4, 387–394.

**Edwards, R.J., N.J. Bentley, A.M. Carr.** 1999. A Rad3-Rad26 complex responds to DNA damage independently of other checkpoint proteins, Nat. Cell Biol. 1, 393–398.

**Falck, J., J. Coates, S.P. Jackson.** 2005. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage, Nature 434, 605–611.

**Ferrell, J.E. Jr., G.S. Martin.** 1991. Assessing activities of blotted protein kinases. Methods Enzymol. 200, 430-435.

**Furuse, M., Y. Nagase, H. Tsubouchi, K. Murakami-Murofushi, T. Shibata, and K. Ohta.** 1998. Distinct roles of two separable in vitro activities of yeast Mre11 in mitotic and meiotic recombination. EMBO J. 17, 6412–6425.

**Fusco, C., A. Reymond, A.S. Zervos.** 1998. Molecular cloning and characterization of a novel retinoblastoma binding protein, Genomics 51, 351–358.

**Garvik, B:, M. Carson, L. Hartwell.** 1995. Single-stranded DNA arising at telomeres in *cdc13* mutants may constitute a specific signal for the *RAD9* checkpoint, Mol. Cell. Biol. 15, 6128–6138.

**Geijsen, N., M. Horoschak, K. Kim, J. Gribnau, K. Eggan, G.Q. Daley.** 2004. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 427, 148–154.

**Gilbert, C.S., C.M. Green, N.F. Lowndes.** 2001. Budding yeast Rad9 is an ATP-dependent Rad53 activating machine, Mol. Cell 8, 129–136.

**Gravel, S., J.R. Chapman, C. Magill, S.P. Jackson.** 2008. DNA helicases Sgs1 and BLM promote DNA double-strand break resection, Genes Dev. 22, 2767– 2772.

**Gruber, S., C. H. Haering, K. Nasmyth. 2003.** Chromosomal cohesin forms a ring. Cell 112, 765–777.

**Grushcow, J. M., T. M. Holzen, K. J. Park, T. Weinert, M. Lichten, and D. K. Bishop.** 1999. *Saccharomyces cerevisiae* checkpoint genes *MEC1*, *RAD17* and *RAD24* are required for normal meiotic recombination partner choice. Genetics 153, 607–620.

**Hassold, T., H. Hall, P. Hunt.** 2007. The origin of human aneuploidy: where we have been, where we are going. Hum Mol Genet. 16 Spec No. 2, 203-208.

**Henderson, K. A., Kee, K., Maleki, S., Santini, P. A., and Keeney, S.** 2006. Cyclin-dependent kinase directly regulates initiation of meiotic recombination. Cell 125, 1321–1332

**Henderson, K.A., S: Keeney.** 2004. Tying synaptonemal complex initiation to the formation and programmed repair of DNA double-strand breaks. Proc Natl Acad Sci USA. 101, 4519-4524.

**Hepworth, S.R., H. Friesen, J. Segall.** 1998. *NDT80* and the meiotic recombination checkpoint regulate expression of middle sporulation-specific genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 18, 5750-5761.

**Hepworth, S.R., L.K. Ebisuzaki, J. Segall.** 1995. A 15-base-pair element activates the SPS4 gene midway through sporulation in Saccharomyces cerevisiae. Mol Cell Biol. 15, 3934-3944.

**Hochwagen, A., and A. Amon.** 2006. Checking your breaks: surveillance mechanisms of meiotic recombination. Curr. Biol. 16, 217–228.

**Hollingsworth, N.M. and B. Byers.** 1989. *HOP1*: a yeast meiotic pairing gene. Genetics 121, 445-462.

**Hollingsworth, N.M., L.Ponte.** 1997. Genetic interactions between *HOP1*, *RED1* and *MEK1* suggest that *MEK1* regulates assembly of axial element components during meiosis in the yeast *Saccharomyces cerevisiae*. Genetics. 147, 33-42.

**Holm, PB.** 1977. The premeiotic DNA replication of euchromatin and heterochromatin in *Lilium longiflorum* (Thunb.). Carlsberg Res. Commun. 42, 249–81

**Honigberg, S. M. and K. Purnapatre.** 2003. Signal pathway integration in the switch from the mitotic cell cycle to meiosis in yeast. J. Cell Sci. 116, 2137–2147.

**Hübner, K., G. Fuhrmann, L.K.Christenson, J. Kehler, R. Reinbold, R. De La Fuente, J. Wood, J.F. 3rd Strauss, M. Boiani M, H.R. Schöler.** 2003**.** Derivation of oocytes from mouse embryonic stem cells. Science 300, 1251–1256.

**Huertas, P., and S. P. Jackson.** 2009. Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. J. Biol. Chem. 284, 9558–9565.

**Huertas, P., F. Corte´s-Ledesma, A. A. Sartori, A. Aguilera, S. P. Jackson.** 2008. CDK targets Sae2 to control DNA-end resection and homologous recombination. Nature 455, 689–692.

**Ira, G., A. Pellicioli, A. Balijja, X. Wang, S. Fiorani, W. Carotenuto, G. Liberi, D. Bressan, L. Wan, N.M. Hollingsworth, J.E. Haber, M. Foiani.** 2004. DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1, Nature 431, 1011–1017.

**Ivanov, E.L., N. Sugawara, C.I. White, F. Fabre, J.E. Haber.** 1994. Mutations in *XRS2* and *RAD50* delay but do not prevent mating-type switching in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 14, 3414–3425.

**Jazayeri, A., J. Falck, C. Lukas, J. Bartek, G.C. Smith, J. Lukas, S.P. Jackson.** 2006. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks, Nat. Cell Biol. 8, 37–45.

**Kadyk, L. C., and L. H. Hartwell.** 1992. Sister chromatids are preferred over homologs as substrates for recombinational repair in *Saccharomyces cerevisiae*. Genetics 132, 387–402.

**Kee, K., and S. Keeney.** 2002. Functional interactions between *SPO11* and *REC102* during initiation of meiotic recombination in *Saccharomyces cerevisiae*. Genetics 160, 111–122.

**Kee, K., J.M. Gonsalves, A.T. Clark, R.A. Pera.** 2006. Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells. Stem Cells Dev. 15, 831-837.

**Keegan, K. S., D. A. Holtzman, A. W. Plug, E. R. Christenson, E. E. Brainerd, G. Flaggs, N. J. Bentley, E. M. Taylor, M. S. Meyn, S. B. Moss, A. M. Carr, T. Ashley, and M. F. Hoekstra.** 1996. The Atr and Atm protein kinases associate with different sites along meiotically pairing chromosomes. Genes Dev. 10, 2423–2437.

**Keeney, S.** 2001. Mechanism and control of meiotic recombination initiation. Curr Top Dev Biol. 52, 1-53.

**Keeney, S. and N. Kleckner.** 1995. Covalent protein-DNA complexes at the 5’ strand termini of meiosis-specific double-strand breaks in yeast, Proc. Natl. Acad. Sci. USA 92, 11274–11278.

**Keeney, S., C. N. Giroux, and N. Kleckner.** 1997. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell 88, 375–384.

**Klein, F., P. Mahr, M. Galova, S.B. Buonomo, C. Michaelis, K. Nairz, K. Nasmyth.** 1999. A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell 98, 91–103.

**Klein, F., P. Mahr, M. Galova, S.B. Buonomo, C. Michaelis, K. Nairz, K. Nasmyth.** 1999. A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell 98, 91–103.

**Kondo, T., T. Wakayama, T. Naiki, K. Matsumoto, K. Sugimoto.** 2001. Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms, Science 294, 867–870.

**Kozlov, S.V., M.E. Graham, C. Peng, P. Chen, P.J. Robinson, M.F. Lavin.** 2006. Involvement of novel autophosphorylation sites in ATM activation, EMBO J. 25, 3504–3514.

**Lee, B. H., and Amon, A.** 2003 Role of Polo-like kinase CDC5 in programming meiosis I chromosome segregation. Science 300, 482–486

**Lee, S. J., J. K. Duong, and D. F. Stern.** 2004. A Ddc2-Rad53 fusion protein can bypass the requirements for *RAD9* and *MRC1* in Rad53 activation. Mol. Biol. Cell 15, 5443–5455.

**Lengsfeld, B. M., Rattray, A. J., Bhaskara, V., Ghirlando, R., and Paull, T. T.** 2007 Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex. Mol. Cell 28, 638–651

**Leu, J. Y. and G.S. Roeder.** 1999. The pachytene checkpoint in *S. cerevisiae* depends on Swe1-mediated phosphorylation of the cyclin-dependent kinase Cdc28. Mol. Cell 4, 805-814.

**Li, S., P.L. Chen, T. Subramanian, G. Chinnadurai, G. Tomlinson, C.K. Osborne, Z.D. Sharp, W.H. Lee.** 1999. Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage, J. Biol. Chem. 274, 11334–11338.

**Limbo, O., C. Chahwan, Y. Yamada, R.A. de Bruin, C. Wittenberg, P. Russell.** 2007. Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination, Mol. Cell 28, 134–146.

**Lindgren, A., D.Bungard, M. Pierce, J. Xie, A. Vershon, E. Winter.** 2000. The pachytene checkpoint in *Saccharomyces cerevisiae* requires the Sum1 transcriptional repressor. EMBO J. 19, 6489–6497.

**Lisby, M., J.H. Barlow, R.C. Burgess, R. Rothstein.** 2004. Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins, Cell 118, 699–713.

**Lobachev, K., D.A. Gordenin, M.A. Resnick.** 2002. The Mre11 complex is required for repair of hairpin-cappeddouble-strand breaks and prevention of chromosome rearrangements, Cell 108, 183–193.

**Loidl, J., F. Klein, H. Scherthan**. 1994. Homologous pairing is reduced but not abolished in asynaptic mutants of yeast. J Cell Biol. 125, 1191-1200.

**Longhese, M. P., Bonetti, D., Guerini, I., Manfrini, N., and Clerici, M.** 2009. DNA double-strand breaks in meiosis: checking their formation, processing and repair. DNA Repair 8, 1127–1138

**Longhese, M.P., D. Mantiero, M. Clerici.** 2006. The cellular response to chromosome breakage, Mol. Microbiol. 60, 1099–1108.

**Longhese, M.P., I. Guerini, V. Baldo, and M. Clerici.** 2008. Surveillance mechanisms monitoring chromosome breaks during mitosis and meiosis. DNA Repair 7, 545–557.

**Longhese, M.P., R. Fraschini, P. Plevani, G. Lucchini.** 1996. Yeast *pip3/mec3* mutants fail to delay entry into S phase and to slow DNA replication in response to DNA damage, and they define a functional link between Mec3 and DNA primase, Mol. Cell. Biol. 16, 3235–3244.

**Longhese, M.P., V. Paciotti, R. Fraschini, R. Zaccarini, P. Plevani, G. Lucchini.** 1997. The novel DNA damage checkpoint protein Ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage in budding yeast, EMBO J. 16, 5216–5226.

**Lorenz, A., J.L. Wells, D.W. Pryce, M. Novatchkova, F. Eisenhaber,R.J. McFarlane, J. Loidl.** 2004. *S. pombe* meiotic linear elements contain proteins related to synaptonemal complex components. J Cell Sci. 117, 3343-3351.

**Lukas, C., J. Falck, J. Bartkova, J. Bartek, J. Lukas.** 2003. Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage, Nat. Cell. Biol. 5, 255–260.

**Lydall, D., Y. Nikolsky, D. K. Bishop, and T. Weinert.** 1996. A meiotic recombination checkpoint controlled by mitotic checkpoint genes. Nature 383, 840–843.

**Majka, J. and P.M. Burgers.** 2004. The PCNA-RFC families of DNA clamps and clamp loaders, Prog. Nucleic Acid Res. Mol. Biol. 78, 227–260.

**Majka, J., A. Niedziela-Majka, P.M. Burgers.** 2006. The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint, Mol. Cell 24, 891–901.

**Malkova, A., L. Ross, D. Dawson, M. F. Hoekstra, and J. E. Haber.** 1996. Meiotic recombination initiated by a double-strand break in rad50 delta yeast cells otherwise unable to initiate meiotic recombination. Genetics 143, 741–754.

**Mantiero, D., M. Clerici, G. Lucchini, M.P. Longhese.** 2007. Dual role for *Saccharomyces cerevisiae* Tel1 in the checkpoint response to double-strand breaks, EMBO Rep. 8, 380–387.

**Marston, A. L., and A. Amon.** 2004. Meiosis: cell-cycle controls shuffle and deal. Nat. Rev. Mol. Cell Biol. 5, 983–997.

**McKee, A. H. Z. and N. Kleckner.** 1997. A general method for identifying recessive diploidspecific mutations in *Saccharomyces cerevisiae*, its application to the isolation of mutants blocked at intermediate stages ofmeiotic prophase and characterization of a new gene *SAE2*, Genetics 146, 797–816.

**McKim, K.S., B.L. Green-Marroquin, J.J. Sekelsky, G. Chin, C. Steinberg, R. Khodosh, R.S. Hawley.** 1998. Meiotic synapsis in the absence of recombination. Science. 279, 876-878.

**Melo, J.A., J. Cohen, D.P. Toczyski.** 2001. Two checkpoint complexes are independently recruited to sites of DNA damage in vivo, Genes Dev. 15, 2809–2821.

**Mimitou, E.P. and L.S. Symington.** 2008. Sae2, Exo1 and Sgs1 collaborate in DNA doublestrand break processing, Nature 455, 770–774.

**Morales, M., J.W. Theunissen, C.F. Kim, R. Kitagawa, M.B. Kastan, J.H. Petrini.** 2005. The *Rad50S* allele promotes ATM-dependent DNA damage responses and suppresses ATM deficiency: implications for the Mre11 complex as a DNA damage sensor, Genes Dev. 19, 3043–3054.

**Moreau, S., J. R. Ferguson, and L. S. Symington.** 1999. The nuclease activity of Mre11 is required for meiosis but not for mating type switching, end joining, or telomere maintenance. Mol. Cell. Biol. 19, 556–566.

**Moreno-Herrero, F., M. de Jager, N.H. Dekker, R. Kanaar, C. Wyman, C. Dekker.** 2005. Mesoscale conformational changes in the DNA-repair complex Rad50/Mre11/Nbs1 upon binding DNA, Nature 437, 440–443.

**Murakami, H., Borde, V., Shibata, T., Lichten, M., and Ohta, K.** 2003 Correlation between premeiotic DNA replication and chromatin transition at yeast recombination initiation sites. Nucleic Acids Res. 31, 4085–4090

**Myers, J.S. and D. Cortez.** 2006. Rapid activation of ATR by ionising radiation requires ATM and Mre11, J. Biol. Chem. 281, 9346–9350.

**Nairz, K. and F. Klein.** 1997. *mre11S*-a yeast mutation that blocks double-strand-break processing and permits nonhomologous synapsis in meiosis, Genes Dev. 11, 2272–2290.

**Nakada, D., K. Matsumoto, K. Sugimoto.** 2003. ATM-related Tel1 associates with double-strand breaks through an Xrs2-dependent mechanism, Genes Dev. 16, 1957–1962.

**Neale, M. J. and S. Keeney.** 2006. Clarifying the mechanics of DNA strand exchange in meiotic recombination. Nature 442, 153–158.

**Neale, M. J., J. Pan, and S. Keeney.** 2005. Endonucleolytic processing of covalent protein-linked DNA double strand breaks. Nature 436, 1053–1057.

**Neale, M.J., M. Ramachandran, E. Trelles-Sticken, H. Scherthan, A.S. Goldman.** 2002. Wild-type levels of Spo11-induced DSBs are required for normal single-strand resection during meiosis, Mol. Cell 9, 835–846.

**Newlon, C. S.** 1988. Yeast chromosome replication and segregation. Microbiol. Rev. 52, 568–601.

**Niu, H., L. Wan, B. Baumgartner, D. Schaefer, J. Loidl, and N. M. Hollingsworth.** 2005. Partner choice during meiosis is regulated by Hop1-promoted dimerization of Mek1. Mol. Biol. Cell 16, 5804–5818.

**Niu, H., L. Wan, V. Busygina, Y. Kwon, J. A. Allen, X. Li, R. C. Kunz, K. Kubota, B. Wang, P. Sung, K. M. Shokat, S. P. Gygi, N. M. Hollingsworth.** 2009. Regulation of Meiotic Recombination via Mek1-Mediated Rad54 Phosphorylation. Molecular Cell 36, 393-404.

**Niu, H., X. Li, E. Job, C. Park, D. Moazed, S. P. Gygi, and N. Hollingsworth.** 2007. Mek1 kinase is regulated to suppress double-strand break repair between sister chromatids during budding yeast meiosis. Mol. Cell. Biol. 27, 5456–5467.

**Paciotti, V., G. Lucchini, P. Plevani, M.P. Longhese.** 1998. Mec1p is essential for phosphorylation of the yeast DNA damage checkpoint protein Ddc1p, which physically interacts with Mec3p, EMBO J. 17, 4199–4209.

**Paciotti, V., M. Clerici, G. Lucchini, M.P. Longhese.** 2000. The checkpoint protein Ddc2, functionally related to *S. pombe* Rad26, interacts with Mec1 and is regulated by Mec1-dependent phosphorylation in budding yeast, Genes Dev. 14, 2046–2059.

**Page, S. L., and R. S. Hawley.** 2004. The genetics and molecular biology of the synaptonemal complex. Annu. Rev. Cell Dev. Biol. 20, 525–558.

**Pak, J. and J. Segall.** 2002. Role of Ndt80, Sum1, and Swe1 as targets of the meiotic recombination checkpoint that control exit from pachytene and spore formation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 22, 6430–6440.

**Pellicioli, A., C. Lucca, G. Liberi, F. Marini, M. Lopes, P. Plevani, A. Romano, P. P. Di Fiore, and M. Foiani.** 1999. Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. EMBO J. 18, 6561–6572.

Penkner, A., Z. Portik-Dobos, L. Tang, R. Schnabel, M. Novatchkova, V. Jantsch, J. Loidl. 2007. A conserved function for a *Caenorhabditis elegans* Com1/Sae2/CtIP protein homolog in meiotic recombination, EMBO J. 26, 5071–5082.

**Petronczki, M., Siomos, M. F., and Nasmyth, K**. 2003. Un ménage à quatre: the molecular biology of chromosome segregation in meiosis. Cell 112, 423–440

**Prieler, S., Penkner, A., Borde, V., and Klein, F.** 2005. The control of Spo11's interaction with meiotic recombination hotspots. Genes Dev. 19, 255–269

**Primig, M., R.M. Williams, E.A. Winzeler, G.G. Tevzadze, A.R. Conway, S.Y. Hwang, R.W. Davis, R.E.** 2000. The core meiotic transcriptome in budding yeasts. Nature Genet. 26, 415-423.

**Prinz, S. and A. Amon, F. Klein.** 1997. Isolation of *COM1*, a new gene required to complete meiotic double-strand break-induced recombination in *Saccharomyces cerevisiae*, Genetics 146, 781–795.

**Rattray, A.J., C.B. McGill, B.K. Shafer, J.N. Strathern**. 2001. Fidelity of mitotic doublestrand- break repair in *Saccharomyces cerevisiae*: a role for *SAE2*/*COM1*, Genetics 158, 109–122.

**Rhoades, M.M.** 1961. Meiosis. In The Cell: Biochemistry, Physiology, Morphology. Meiosis and Mitosis, ed. J Brachet, AE Mirsky, New York: Academic 3, 1–75.

**Rockmill, B., Roeder G.S.** 1990. Meiosis in asynaptic yeast. Genetics. 126, 563-574.

**Romanienko, P.J., R.D. Camerini-Otero.** 2000. The mouse Spo11 gene is required for meiotic chromosome synapsis. Mol Cell. 6, 975-87.

**Rouse, J. and S.P. Jackson.** 2000. *LCD1*: an essential gene involved in checkpoint control and regulation of the MEC1 signalling pathway in *Saccharomyces cerevisiae*, EMBO J. 19, 5801–5812.

**Sanchez, Y., J. Bachant, H. Wang, F. Hu, D. Liu, M. Tetzlaff, S.J. Elledge.** 1999. Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms, Science 286, 1166–1171.

**Sartori, A.A., C. Lukas, J. Coates, M. Mistrik, S. Fu, J. Bartek, R. Baer, J. Lukas, S.P. Jackson.** 2007. Human CtIP promotes DNA end resection, Nature 450, 509–514.

**Schaeper, U., T. Subramanian, L. Lim, J.M. Boyd, G. Chinnadurai.** 1998. Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A(CtBP) and anovel cellular protein is disruptedbyE1Athrougha conserved PLDLS motif, J. Biol. Chem. 273, 8549–8552.

**Schulz, V. P., and Zakian, V. A.** 1994. The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. Cell 76, 145–155

**Schwacha, A., and N. Kleckner.** 1994. Identification of joint molecules that form frequently between homologs but rarely between sister chromatids during yeast meiosis. Cell 76, 51–63.

**Schwacha, A., and N. Kleckner.** 1997. Interhomolog bias during meiotic recombination: meiotic functions promote a highly differentiated interhomolog-only pathway. Cell 90, 1123–1135.

**Shiloh, Y.** 2006. The ATM-mediated DNA-damage response: taking shape, Trends Biochem. Sci. 31, 402-410.

**Shinohara, M., K. Sakai, T. Ogawa, and A. Shinohara.** 2003. The mitotic DNA damage checkpoint proteins Rad17 and Rad24 are required for repair of double-strand breaks during meiosis in yeast. Genetics 164, 855–865.

**Simchen, G.** 1973. Are mitotic functions required in meiosis? Genetics 76, 745–753.

**Smith, A.V. and G.S. Roeder.** 1997. The yeast Red1 protein localizes to the cores of meiotic chromosomes. J. Cell Biol. 136, 957-967.

**Sorger, P.K. and A.W. Murray.** 1992. S-phase feedback control in budding yeast independent of tyrosine phosphorylation of p34cdc28. Nature. 355, 365-368.

**Sourirajan, A., M. Lichten.** 2008. Polo-like kinase Cdc5 drives exit from pachytene during budding yeast meiosis. Genes Dev. 22, 2627-2632.

**Strom, L. and C. Sjogren** 2007. Chromosome segregation and double-strand break repair – a complex connection, Curr. Opin. Cell Biol. 19, 344–349.

**Stuart, D. and C. Wittenberg.** 1998. *CLB5* and *CLB6* are required for premeiotic DNA replication and activation of the meiotic S/M checkpoint. Genes Dev. 12, 2698–2710.

**Sun, Z., J. Hsiao, D.S. Fay, D.F. Stern.** 1998. Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint, Science 281, 272–274.

**Sweeney, F.D., F. Yang, A. Chi, J. Shabanowitz, D.F. Hunt, D. Durocher.** 2005. *Saccharomyces cerevisiae* Rad9 acts as a Mec1 daptor to allow Rad53 activation, Curr. Biol. 15, 1364–1375.

**Sym, M., J.A. Engebrecht, G.S. Roeder.** 1993. ZIP1 is a synaptonemal complex protein required for meiotic chromosome synapsis. Cell. 72, 365-378.

**Tessé, S, A. Storlazzi, N. Kleckner, S. Gargano, D. Zickler.** 2003. Localization and roles of Ski8p protein in Sordaria meiosis and delineation of three mechanistically distinct steps of meiotic homolog juxtaposition. Proc Natl Acad Sci USA. 100, 12865-12870.

**Thompson, D. A., and F. W. Stahl.** 1999. Genetic control of recombination partner preference in yeast meiosis. Isolation and characterization of mutants elevated for meiotic unequal sister-chromatid recombination. Genetics 153, 621–641.

**Toyooka, Y., N. Tsunekawa, R. Akasu, T. Noce.** 2003. Embryonic stem cells can form germ cells in vitro. Proc. Natl Acad. Sci. USA 100, 11457–11462.

**Tsubouchi, H., and Ogawa, H.** 1998. A novel mre11 mutation impairs processing of double-strand breaks of DNA during both mitosis and meiosis. Mol. Cell. Biol. 18, 260–268.

**Tsubouchi, H., and Ogawa, H.** 2000. Exo1 roles for repair of DNA double-strand breaks and meiotic crossing over in Saccharomyces cerevisiae. Mol. Biol. Cell 11, 2221–2233.

**Tung, K.S., E.J. Hong, G.S. Roeder.** 2000. The pachytene checkpoint prevents accumulation and phosphorylation of the meiosis-specific transcription factor Ndt80. Proc. Natl. Acad. Sci. USA. 97, 12187-12192.

**Uanschou, C., T. Siwiec, A. Pedrosa-Harand, C. Kerzendorfer, E. Sanchez-Moran, M. Novatchkova, S. Akimcheva, A. Woglar, F. Klein, P. Schlögelhofer.** 2007. A novel plant gene essential for meiosis is related to the human CtIP and the yeast COM1/SAE2 gene, EMBO J. 26, 5061–5070.

**Ubersax, J.A., E.L. Woodbury, P.N. Quang, M. Paraz, J.D. Blethrow, K. Shah, K.M. Shokat, D.O. Morgan.** 2003. Targets of the cyclin-dependent kinase Cdk1, Nature 425, 859–864.

**Usui, T., H. Ogawa, J.H.J. Petrini.** 2001. A DNA damage response pathway controlled by Tel1 and the Mre11 complex, Mol. Cell 7, 1255–1266.

**Usui, T., Ohta, T., Oshiumi, H., Tomizawa, J., Ogawa, H., and Ogawa, T.** 1998. Complex formation and functional versatility of Mre11 of budding yeast in recombination. Cell 95, 705–716

**Vialard, J. E., C. S. Gilbert, C. M. Green, and N. F. Lowndes.** 1998. The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. EMBO J. 17, 5679–5688.

**Viscardi, V., Bonetti, D., Cartagena-Lirola, H., Lucchini, G., and Longhese M. P.** 2007. MRX-dependent DNA damage response to short telomeres. Mol. Biol. Cell 18, 3047–3058

**Wach, A., A. Brachat, R. Pöhlmann, P. Philippsen.** 1994. New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast. 10, 1793-1808.

**Wakayama, T., T. Kondo, S. Ando, K. Matsumoto, K. Sugimoto.** 2001. Pie1, a protein interacting with Mec1, controls cell growth and checkpoint responses in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 21, 755–764.

**Wan, L., Niu, H., Futcher, B., Zhang, C., Shokat, K. M., Boulton, S. J., and Hollingsworth, N. M.** 2008. Cdc28-Clb5 (CDK-S) and Cdc7-Dbf4 (DDK) collaborate to initiate meiotic recombination in yeast. Genes Dev. 22, 386–397

**Wan, L., T. de los Santos, C. Zhang, K. Shokat, and N. M. Hollingsworth.** 2004. Mek1 kinase activity functions downstream of *RED1* in the regulation of meiotic double strand break repair in budding yeast. Mol. Biol. Cell 15, 11–23.

**Watanabe, Y., S. Yokobayashi, M. Yamamoto, P. Nurse.** 2001. Pre-meiotic S phase is linked to reductional chromosome segregation and recombination. Nature 409, 359–363.

**Weber, L., B. Byers.** 1992. A *RAD9*-dependent checkpoint blocks meiosis of *cdc13* yeast cells, Genetics 131, 55–63.

**Weiner, B.M., N. Kleckner.** 1994. Chromosome pairing via multiple interstitial interactions before and during meiosis in yeast. Cell 77, 977-991.

**Weinert, T.A., G.L. Kiser, L.H. Hartwell. 1994.** Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair, Genes Dev. 8, 652–665.

**Woltering, D., B. Baumgartner, S. Bagchi, B. Larkin, J. Loidl, T. de los Santos, and N. M. Hollingsworth.** 2000. Meiotic segregation, synapsis, and recombination checkpoint functions require physical interaction between the chromosomal proteins Red1p and Hop1p. Mol. Cell. Biol. 20, 6646–6658.

**Xie, J., M. Pierce, V. Gailus-Durner, M. Wagner, E. Winter, A.K. Vershon.** 1999. Sum1 and Hst1 repress middle sporulation-specific gene expression during mitosis in Saccharomyces cerevisiae. EMBO J. 18, 6448-6454.

**Xu, L., B. M. Weiner, and N. Kleckner.** 1997. Meiotic cells monitor the status of the interhomolog recombination complex. Genes Dev. 11, 106–118.

**Xu, L., M. Ajimura, R. Padmore, C. Klein, and N. Kleckner.** 1995. *NDT80*, a meiosis-specific gene required for exit from pachytene in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 15, 6572–6581.

**Xu, Y., T. Ashley, E. E. Brainerd, R. T. Bronson, M. S. Meyn, and D. Baltimore.** 1996. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. Genes Dev. 10, 2411–2422.

**Yamamoto, M.** 1996. Regulation of meiosis in fission yeast. Cell Struct. Funct. 21, 431–436.

**You, Z., C. Chahwan, J. Bailis, T. Hunter, P. Russell.** 2005. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1, Mol. Cell. Biol. 25, 5363–5379.

**Yu, X., L.C. Wu, A.M. Bowcock, A. Aronheim, R. Baer.** 1998. The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression, J. Biol. Chem. 273, 25388–25392.

**Yun, M. H. and Hiom, K.** 2009. CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. Nature 459, 460–463.

**Zhao, X., E. G. D. Muller, and R. Rothstein.** 1998. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol. Cell 2, 329–340.

**Zhu, Z., W.H. Chung, E.Y. Shim, S.E. Lee, G. Ira.** 2008. Sgs1 helicase and two nucleases Dna2and Exo1 resectDNAdouble-strand break ends, Cell 134, 981–994.

**Zou, L. and S.J. Elledge.** 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes, Science 300, 1542–1548.

**Zou, L., D. Cortez, S.J. Elledge.** 2002. Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin, Genes Dev. 16, 198–208.