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Partner Choice during Meiosis Is Regulated by Hop1-promoted Dimerization of Mek1

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Meiotic recombination differs from mitotic recombination in that DSBs are repaired using homologous chromosomes, rather than sister chromatids. This change in partner choice is due in part to a barrier to sister chromatid repair (BSCR) created by the meiosis-specific kinase, Mek1, in a complex with two other meiosis-specific proteins, Hop1 and Red1. *HOP1* contains two functional domains, called the N and C domains. Analysis of a point mutation that specifically inactivates the C domain (*hop1-K593A*) reveals that the N domain is sufficient for Hop1 localization to chromosomes and for Red1 and Hop1 interactions. The C domain is needed for spore viability, for chromosome synapsis, and for preventing *DMC1*-independent DSB repair, indicating it plays a role in the BSCR. All of the *hop1-K593A* phenotypes can be bypassed by fusion of ectopic dimerization domains to Mek1, suggesting that the function of the C domain is to promote Mek1 dimerization. Hop1 is a DSB-dependent phosphoprotein, whose phosphorylation requires the presence of the C domain, but is independent of *MEK1*. These results suggest a model in which Hop1 phosphorylation in response to DSBs triggers dimerization of Mek1 via the Hop1 C domain, thereby enabling Mek1 to phosphorylate target proteins that prevent repair of DSBs by sister chromatids.

INTRODUCTION

In mitotically dividing cells, recombination is used to repair lesions in DNA resulting from problems in replication or exogenous DNA damage. Sister chromatids are the preferred templates for DNA repair in these cells and homologous recombination is mediated primarily by the recombinase, Rad51 (Kadyk and Hartwell, 1992; Symington, 2002). In contrast, recombination during meiosis is initiated by the deliberate introduction of meiosis-specific double-strand breaks (DSBs). The resulting crossovers occur preferentially between nonsister chromatids and are mediated not only by Rad51 but also by the meiosis-specific recombinase, Dmc1 (Bishop *et al.*, 1992; Schwacha and Kleckner, 1997; Keeney, 2001). Crossovers between homologues, in combination with sister chromatid cohesion, physically connect homologous chromosomes, thereby allowing them to align properly at Metaphase I (Petronczki *et al.*, 2003). Failure to cross over leads to high levels of missegregation and aneuploid gametes.

Many of the molecular details of meiotic recombination have been elucidated in the budding yeast, *Saccharomyces cerevisiae* (Hollingsworth and Brill, 2004). Recombination begins by the introduction of DSBs catalyzed by the highly conserved, topoisomerase-like protein, Spo11. The 5′ ends on either side of the break are resected to produce 3′ single-stranded (ss) tails. Resection requires both the trimeric complex *MRE11*/*XRS2*/*RAD50* as well as *SAE2* (also known as *COM1*). After 3′ ss tails are bound by Rad51 and Dmc1, they invade nonsister chromatids to produce D-loops. DNA synthesis extends the D-loops until the displaced strands anneal to the 3′ ss tails on the other side of the breaks. Further DNA synthesis and ligation result in double Holliday junction structures (observed in physical analyses as joint molecules) that may then be resolved to create crossover chromosomes. In addition to this canonical pathway, it has recently been discovered that budding yeast has an additional minor pathway for generating crossovers, mediated by the Mus81/Mms4 structure specific endonuclease, that may not utilize double Holliday junction intermediates (de los Santos *et al.*, 2003).

Relatively little is known about the mechanism by which the change in partner choice from sister chromatids in vegetative cells to nonsister chromatids in meiotic cells is accomplished. An important question is whether the interhomologue bias observed in meiosis is due to the active promotion of interhomologue recombination or because sister chromatid recombination is suppressed (or both). Originally, the discovery of a meiosis-specific recombinase, *DMC1*, seemed to support the former idea. In the absence of *DMC1*, meiotic DSBs are resected but fail to invade the homologue and the DSBs remain unrepaired (Bishop *et al.*, 1992; Hunter and Kleckner, 2001). These persisting DSBs trigger the meiotic recombination checkpoint and the cells arrest in prophase I (Lydall *et al.*, 1996; Xu *et al.*, 1997). Therefore the difference between mitotic and meiotic partner choice could be explained by the use of a different strand

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Abbreviations used: BSCR, barrier to sister chromatid repair; DSB, double-strand break; AE, axial element; SC, synaptonemal complex.

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transfer enzyme, with some property of Dmc1 conferring the ability to discriminate between sister and nonsister chromatids. Recent experiments, however, have indicated that Dmc1 does not itself supply specificity to the strand invasion reaction. Overexpression of *RAD51* largely suppresses the interhomologue recombination defect of *dmc1*, resulting in viable spores (Tsubouchi and Roeder, 2003). Therefore while *DMC1* is required for interhomologue recombination in budding yeast, its function may be simply to increase the level of recombinase, rather than to promote strand invasion specifically of nonsister chromatids. Consistent with this idea, organisms such as worms and fruit flies, which, like yeast, undergo *SPO11*-dependent meiotic recombination, contain Rad51, but no Dmc1, orthologues (Villeneuve and Hillers, 2001).

The fact that strand invasion occurs preferentially between nonsister chromatids, even when Rad51 is the only recombinase available, suggests that there is a barrier to sister chromatid repair (BSCR) in meiotic cells. One potential component of this barrier is *RED1*, a gene encoding a meiosis-specific component of the chromosome cores formed between sister chromatids (Thompson and Roeder, 1989; Smith and Roeder, 1997). Mutation of *RED1* in a *dmc1* diploid results in the disappearance of DSBs, progression through the meiotic divisions, and the generation of dead spores (Schwacha and Kleckner, 1997; Xu *et al.*, 1997; Bishop *et al.*, 1999). That the disappearance of the breaks is due to repair, as opposed to extensive resection, was demonstrated by the finding that mutation of *spo13* suppresses the spore inviability of *red1 dmc1* diploids (Bishop *et al.*, 1999). *spo13* mutants undergo just a single meiotic division, thereby eliminating the requirement for interhomologue crossovers to produce viable spores, (Malone and Esposito, 1981). Furthermore, the disappearance of the DSBs in *red1 dmc1* strains, as well as the spore viability in *red1 dmc1 spo13* diploids, is dependent upon *RAD54*, a gene required primarily for sister chromatid recombination during meiosis (Arbel *et al.*, 1999; Bishop *et al.*, 1999). *red1* mutants are pleiotropic, displaying a number of mutant phenotypes including defects in chromosome structure and synapsis, as well as DSB formation (Rockmill and Roeder, 1990; Xu *et al.*, 1997). The wide range of processes affected by *red1* has made it difficult to determine the specific function that *RED1* has in establishing interhomologue bias.

During meiosis, sister chromatids condense to form structures called axial elements (AEs). AEs of homologous chromosomes are then connected to form a tripartite structure called the synaptonemal complex (SC) *RED1* localizes to AEs in budding yeast and is required for their formation (Rockmill and Roeder, 1990; Smith and Roeder, 1997). It was therefore possible that the defect in the BSCR observed in *red1 dmc1* mutants was due to the failure to create the correct chromosome structure. However studies involving the meiosis-specific kinase, *MEK1*, suggest that the absence of the BSCR in *red1* cells may be best explained by a failure to localize Mek1 to chromosomes (Wan *et al.*, 2004). Like *red1*, *mek1* mutants allow *dmc1* diploids to sporulate and produce inviable spores (Xu *et al.*, 1997; Wan *et al.*, 2004). Unlike *red1*, *mek1* mutants allow AE and even some SC formation, depending on strain background (Rockmill and Roeder, 1990, 1991). A conditional allele called *mek1-as1* allows inactivation of the Mek1 kinase by addition of a chemical inhibitor to sporulating cells (Wan *et al.*, 2004). Inactivation of Mek1 in a *mek1-as1 dmc1* diploid after DSB formation and cell cycle arrest results in the disappearance of DSBs and the production of inviable spores. Given that DSBs were created under wild-type conditions in the presence of Red1, this experiment shows that Mek1 kinase activity acts after *RED1* to prevent DSB repair in *dmc1* strains. Although it was proposed that the disappearance of the DSBs observed in this situation was due to sister chromatid repair, similar to what has been proposed for *red1*, this idea was not proven (Wan *et al.*, 2004).

RED1 and *MEK1* are part of a genetic epistasis group that includes a third meiosis-specific gene, *HOP1* (Rockmill and Roeder, 1990, 1991). Mutations in *hop1*, *red1*, and *mek1* specifically reduce interhomologue recombination and produce inviable spores (Hollingsworth *et al.*, 1995), suggesting that *HOP1* may play a role in the BSCR along with *RED1* and *MEK1*. The genetic data suggesting that these proteins work in a common pathway are supported by biochemical experiments showing that Red1/Hop1 and Red1/Mek1 form complexes in meiotic cells (Bailis and Roeder, 1998; de los Santos and Hollingsworth, 1999; Wan *et al.*, 2004). Two-hybrid experiments indicate Red1 acts as a bridge to bring Hop1 and Mek1 together (Bailis and Roeder, 1998). Determining whether *HOP1* plays a role in the BSCR has been complicated, however, because *hop1* mutants have a more severe DSB phenotype than either *red1* or *mek1* in the SK1 background where the *dmc1* arrest is most pronounced (Woltering *et al.*, 2000; Pecina *et al.*, 2002). Therefore should *hop1*Δ suppress the *dmc1* arrest, it could be an indirect effect due to an insufficient number of DSBs to trigger the meiotic recombination checkpoint.

In this article we present experiments to demonstrate that Mek1 kinase activity is required in *dmc1* diploids to prevent DSB repair using sister chromatids. Furthermore we describe the characterization of a novel allele of *HOP1*, *hop1-K593A*, that is mutated in a domain specifically required for the BSCR called the C domain. The discovery that the *hop1-K593A* mutant can be suppressed by versions of Mek1 containing ectopic dimerization domains suggests that the function of the C domain is to promote dimerization of Mek1 during meiosis. Finally, we show that Hop1 is phosphorylated in a DSB- and C domain-dependent manner, but is independent of *MEK1*. On the basis of these results, we propose that the interhomologue bias observed during meiosis is created by the suppression of intersister recombination mediated by Hop1/Red1/Mek1 complexes. This suppression is most likely achieved by the phosphorylation of as yet unidentified proteins by Mek1 that prevent strand invasion. Activation of Mek1 function by dimerization may be coordinated with DSB formation via phosphorylation of the Hop1 C domain.

MATERIALS AND METHODS

Plasmids

Plasmid names, genotypes, and sources can be found in Table 1. Plasmids for this study were constructed by standard procedures using the *Escherichia coli* strain BSJ72 (Maniatis *et al.*, 1982). Details of plasmid constructions are available upon request. All *MEK1* fusions are expressed under the control of the *MEK1* promoter. The TAP tag was cloned from plasmid pBS1761, obtained from EUROSCARF. Mutations were introduced directly into pLT11 by site-directed mutagenesis (QuikChange kit, Stratagene, La Jolla, CA). The presence of the mutations was confirmed by DNA sequencing (Research Genetics, Huntsville, AL; Center for the Analysis and Synthesis of Macromolecules at SUNY Stony Brook). For *hop1-K593A*, *hop1-K590A*, *hop1-564*Δ, and *hop1-585*Δ, the entire sequence of each allele was determined to ensure that no additional mutations were created during the mutagenesis.

Yeast Strains and Media

Strain genotypes can be found in Table 2. NH246 and NH270 are derived from a cross between the slow sporulating BR and A364a genetic backgrounds (Woltering *et al.*, 2000). All other strains are derived from SK1. Details of strain constructions are available upon request. All experiments were conducted at 30°C. Liquid and solid media have been described

Table 1. Plasmids

Name	Yeast genotype	Source
pRS402	*ADE2*	Brachmann *et al.* (1998)
YCp50	*URA3 CEN ARS*	Rose *et al.* (1987)
YEp352	*URA3* 2μ	Hill *et al.* (1986)
pRS306	*URA3*	Sikorski and Heiter (1989)
YIp5	*URA3*	Parent *et al.* (1985)
YIp5-hop1R6Δ	*hop1-R6Δ URA3*	Friedman *et al.* (1994)
pLT11	*HOP1 URA3*	This work
pLT11-K593A	*hop1-K593A URA3*	This work
pLT11-K590A	*hop1-K590A URA3*	This work
pLT11–585	*hop1–585Δ URA3*	This work
pLT11–564	*hop1–564Δ URA3*	This work
pDW39	*HOP1 ADE2*	This work
pDT12	*hop1-K593A ADE2*	This work
pSB3	*RED1 URA3*	Woltering *et al.* (2000)
pLW20	*MEK1 ADE2*	This work
pTS30	*GST-MEK1 ADE2*	de los Santos and Hollingsworth (1999)
pTS31	*GST-mek1-K199R ADE2*	de los Santos and Hollingsworth (1999)
pTS30-R72P, D76K	*gst-RD-MEK1 ADE2*	This work
pHN16	*TAP-MEK1 ADE2*	This work
pHN23	*lexA-MEK1 ADE2*	This work
pHN24	*lexA-MEK1 ADE2* 2μ	This work
pLP37	*MEK1 URA3*	de los Santos and Hollingsworth (1999)
pBL12	*GST-MEK1 URA3*	This work
pHN26	*GST-mek1-K199R URA3*	This work
pLW28	*DMC1 URA3*	This work
pRS316-DMC1	*DMC1 URA3 CEN ARS*	J. Engebrecht
pNRB143	*RAD54 URA3* 2μ	K. Runge
pR4C4	*MEK1 URA3 CEN ARS*	Hollingsworth and Ponte (1997)
pNH251	*mek1-as1 ARG4*	This work
pNH255	*HOP1p-RAD51 URA3* 2μ	This work

(Vershon *et al.,* 1992; de los Santos and Hollingsworth, 1999). The inhibitor, 1-NA-PP1, 4-amino-1-*tert*-butyl-3-(1′naphthyl)pyrazolo[3,4-*d*]pyrimidine, was diluted from a 10 mM stock purchased from Cellular Genomics (New Haven, CT).

Antibodies, Immunoprecipitations, and Western Blots

The G/R antibodies, as well as the immunoprecipitation (IP) and Western blot protocols, are described in Wan *et al.* (2004). The Hop1 antibodies and phosphatase experiment protocol are described in de los Santos and Hollingsworth (1999). To detect phosphorylated Hop1 by SDS-polyacrylamide gel electrophoresis, proteins were fractionated on 8% gels (29:1 acrylamide:bis-acrylamide) 20 cm in length at 15 mAmp for 15 h.

Time Courses

Liquid sporulation conditions used were 2% potassium acetate at 30°C. Sporulation was monitored by phase contrast microscopy of at least 200 cells per strain. DSB and cytological analyses were as described in Woltering *et al.* (2000) and Loidl *et al.* (1998). For experiments in which strains were transformed with plasmids, the cells were grown to stationary phase in SD-ura medium instead of rich medium before dilution into YPA. Quantitation of DSB fragments was performed using a Molecular Dynamics PhosphorImager, (Amersham, Piscataway, NJ) and Image Quant 1.11 software. The formula used for quantitation was $(DSB_{t=x} - DSB_{t=0})/(DSB_{t=x} + P_{t=x}) \times 100$, where P represents the parental fragment.

RESULTS

Inhibition of Mek1 Kinase Activity after DSB Formation Allows DSB Repair and Produces Viable Spores in a dmc1 spo13 Diploid

Previously we showed that inhibition of Mek1 kinase activity after DSB formation in a *dmc1* mutant suppressed the arrest triggered by the meiotic recombination checkpoint, caused DSBs to disappear, and produced inviable spores (Wan *et al.,* 2004). This result was interpreted to mean that Mek1 kinase activity was necessary to prevent DSB repair using sister chromatids, but this experiment did not rule out alternative explanations. For example, degradation of the ends of the DSBs would also result in the loss of detectable DSBs and dead spores. In these two scenarios, however, the spores are dead for different reasons: the lethality that occurs in spores that have undergone DSB repair using sister chromatids is a result of nondisjunction occurring because homologues are not physically connected before MI. In contrast, the spore death arising from hyper-resection is due to the irreversible loss of genetic information. These two possibilities can be distinguished assaying spore viability in the absence of *SPO13*. Spore inviability arising due to a lack of interhomologue recombination should be suppressed by the single division meiosis conferred by *spo13* (Malone and Esposito, 1981). If, however, spore death is due to DNA degradation, the spores will be dead even in a *spo13* background.

Mek1 kinase activity can be specifically inhibited by the addition of 1-NA-PP1 to strains containing the *mek1-as1* mutant (note that the mutation in *mek1-as1* is Q241G and not Q247G as originally reported in Wan *et al.* (2004)). A *mek1-as1 dmc1 spo13* diploid was transferred to sporulation medium, a final concentration of 1 μM 1-NA-PP1 was added to 1-ml aliquots at 0, 2, 4, and 6 h, and the cells returned to the 30°C incubator. After a total of 24 h, sporulation was monitored by phase contrast light microscopy. In the absence of inhibitor, the diploid behaved like a *dmc1* mutant, with only 4.5% of the cells forming dyads, all of which were immature. In contrast, all of the time points to which inhibitor was added exhibited >35% sporulation, similar or better than the 36% sporulation observed for *spo13* alone (Table 3). In addition, at the 4-h time point, 1 μM 1-NA-PP1 was added to

Table 2. *S. cerevisiae* strains

Name	Genotype[a]	Source
NH246	*MATa CDC10 leu2 his4 arg4-8 thr1-1 ura3-1 CAN1 trp1-1 cyh10 ade2-1* *MATα cdc10-2 LEU2 HIS4 ARG4 THR1 ura3 can1 trp1 CYH10 ade2-1* *spo13::ura3-1 CYH2 red1Δ::ADE2* *spo13-1 cyh2 red1Δ::ADE2*	Woltering *et al.* (2000)
NH270	Same as NH246 only *RED1 hop1Δ::ADE2*	This work
NH144	*MATa leu2ΔhisG his4-x ARG4 ura3 lys2 hoΔ::LYS2* *MATα leu2-K HIS4 arg4-Nsp ura3 lys2 hoΔ::LYS2*	Hollingsworth *et al.* (1995)
YTS3	Same as NH144 only *red1::LEU2*	de los Santos and Hollingsworth (1999)
DW10	Same as NH144 only *hop1::LEU2*	de los Santos and Hollingsworth (1999)
DW11	Same as NH144 only *rec104Δ::LEU2*	de los Santos *et al.* (2001)
NH311	Same as NH144 only *hop1::LEU2 ade2-bgl sae2Δ::URA3*	Woltering *et al.* (2000)
NH217	Same as NH144 only *red1::LEU2 sae2Δ::URA3*	This work
YTS1ade2::pRS402	Same as NH144 only *mek1Δ::LEU2 ade2::ADE2*	Wan *et al.* (2004)
NH566	*MATα HIS4 lys2 hoΔ:: LYS2 ura3 ade2 arg4 hop1::LEU2 mek1Δ::LEU2* *MATa HIS4 lys2 hoΔ:: LYS2 ura3 ade2 arg4 hop1::LEU2 mek1Δ::LEU2*	This work
NH601	*MATa leu2::hiG his4-X hoΔ::LYS2 lys2 ura3 dmc1Δ::LEU2 hop1Δ::kanMX* *MATα leu2::hiG his4-X hoΔ::LYS2 lys2 ura3 dmc1Δ::LEU2 hop1Δ::kanMX*	This work
NH624	*MATa leu2 his4 dmc1Δ::LEU2 mek1Δ::kanMX rad54Δ::NAT arg4 ADE2* *MATα leu2 HIS4 dmc1Δ::LEU2 mek1Δ::kanMX rad54Δ::NAT arg4 ade2* *hoΔ::LYS2 lys2* *hoΔ::LYS2 lys2*	This work
NH639	*MATa leu2Δ::hisG his4-x mek1Δ::kanMX dmc1Δ::NAT hoΔ::LYS2 lys2 ura3* *MATα leu2Δ::hisG his4-x mek1Δ::kanMX dmc1Δ::NAT hoΔ::LYS2 lys2 ura3* *can1 ade2-bgl cyh2 ARG4* *CAN1 ade2-bgl CYH2 arg4*	This work
NH567:: pNH251	*MATa leu2 SCR::URA3 arg4::mek1-as1::ARG4 ade3Δ::kanMX ade2-bgl* *MATα leu2 arg4 ade3Δ::kanMX ade2-bgl* *mek1Δ::LEU2 spo13::hisG ura3 hoΔ::LYS2 lys2* *mek1Δ::LEU2 spo13::hisG ura3 hoΔ::LYS2 lys2*	This work
NH574:: pNH251	Same as NH567::pNH251 only *dmc1Δ::NAT*	This work

[a] Underlines indicate genes on the same chromosome.

one-half of the sporulating culture, and the cells were returned to the 30°C shaker. Cells were then fixed at 2-h intervals for analysis of DSBs at the naturally occurring *YCR048w* and *HIS2* DSB hotspots (Wu and Lichten, 1994; Bullard *et al.*, 1996). In the absence of inhibitor, DSBs failed to be repaired and became hyper-resected with increasing time in spo medium. Within 2 h after the addition of inhibitor, however, the DSBs were no longer detectable (Figure 1, A and B; unpublished data).

The behavior of the DSBs after addition of inhibitor in the *mek1-as1 dmc1 spo13* diploid is highly similar to what was previously observed for the *mek1-as1 dmc1 SPO13* strain (Wan *et al.*, 2004). A major difference in the two experiments is the spore viability. Although only 3% of the spores were viable in the *SPO13* experiment, 46.7% of the spores were viable in the *mek1-as1 dmc1 spo13* mutant (Table 3; Wan *et al.*, 2004). The fact that 32.6% of the dyads contained two viable spores provides strong support for the argument that DSBs are disappearing as a result of repair rather than degradation.

mek1-as1 dmc1 spo13 Mutants Are Reduced for Interhomologue Recombination and Increased for Intersister Recombination

If Mek1 kinase activity is acting to prevent sister chromatid repair in the *mek1-as1 dmc1 spo13* diploid, then inactivation of Mek1 should result in dyads which exhibit reduced levels of interhomologue recombination and increased levels of intersister recombination. To monitor both interhomologue and intrachromosomal recombination, a sister chromatid recombination reporter, SCR::*URA3*, was introduced between *LEU2* and *HIS4* on one of the chromosome III homologues (Figure 1C; Kadyk and Hartwell, 1992). Interhomologue recombination between *URA3* and *MAT* can be detected by a change in the coupling relationship between the two genes, whereas intersister events can be detected by the generation of a full-length *ADE3* gene (see below).

In *spo13* meioses, chromosomes may segregate either reductionally (homologues segregate to opposite poles), equationally (sisters segregate to opposite poles), or aberrantly (three chromatids go to one pole and one to the other pole; Klapholz and Esposito, 1980; Hugerat and Simchen, 1993). Defects in interhomologue recombination result in improved spore viability and dyads that display predominantly equational segregation (Wagstaff *et al.*, 1982; Hollingsworth and Byers, 1989). The dyads formed by the *mek1-as1 dmc1 spo13* diploid in the presence of inhibitor displayed both of these properties. The spore viability of the *spo13* strain was only 25.5%, whereas the *mek1-as1 dmc1 spo13* spore viabilities ranged from 38.5 to 61.2%, depending on when the inhibitor was added (Table 3). The highest viability was observed when inhibitor was added at 0 h, suggesting that there is a deleterious effect in allowing DSBs to form in the presence of Mek1 kinase activity when *DMC1* is absent. As for segregation, 72.7% of the *spo13* dyads could be unambiguously defined as equational segregants for chro-

Table 3. Sporulation, spore viability, and recombination in *spo13* and *mek1-as1 dmc1 spo13* diploids in which Mek1 kinase activity is inhibited at different times in meiosis

Two viable spore dyads[a]											
			% Nonrecombinant[b]			% Interhomologue recombinant[b]				% Intersister recombinant[b]
			Equat.	Red.	Aberrant	Crossover			Gene conv.	Gene conv.
dmc1 mek1-as1[d] *spo13*	% spor.	% s.v.[c]	NM[e] + −: NM + −	a + −: α − −	a + −: NM + − or α − −: NM + −	NM + −: NM − −	a + −: α + −	a − −: NM + − or α + −: NM + −	NM − −: a + −	NM + +: NM + −
No I[f]	4.5	NA	NA	NA	NA	NA	NA	NA	NA	
I added at 0 h	44.5	61.2 (183)	93.7 (74)	1.3 (1)	3.8 (3)	0	0	0	0	1.3 (1)
I added at 2 h	43.0	44.2 (182)	87.7 (50)	3.5 (2)	7.0 (4)	0	0	0	0	1.7 (1)
I added at 4 h	52.5	46.7 (610)	85.2 (173)	0	9.4 (19)	0.5 (1)	1.0 (2)	0.5 (1)	0	3.4 (7)
I added at 6 h	38.5	38.5 (183)	77.8 (35)	6.7 (3)	13.3 (6)	0	0	0	0	2.2 (1)
Total			86.5 (332)	1.6 (6)	8.3 (32)	0.3 (1)	0.5 (2)	0.3 (1)	0	2.6 (10)
spo13	36.0	25.5 (646)	24.2 (8)	27.3 (9)	24.2 (8)	9.1 (3)	9.1 (3)	0	6.1 (2)	0

One viable spore dyads							
	% Nonrecombinant[b]			% Interhomologue recombinant[b]			
	NM + −	a + −	α − −	α + −	a − −	NM − −	% Intersister recombinant[b]
dmc1 mek1-as1 spo13 I added at 4 h	88.7 (102)	4.3 (5)	6.1 (7)	0	0	0	0.9 (1)
spo13	41.9 (85)	30.5 (62)	12.8 (26)	2.5 (5)	1.0 (2)	11.3 (23)	0

[a] Phenotypes are indicated in the following order: Mat Ura His (*ade3* mutants are His$^-$).

[b] Values in parentheses are number of dyads.

[c] Values in parentheses are number of asci. s.v. indicates spore viability.

[d] The *mek1-as1dmc1 spo13* diploid NH574::pNH251 is isogenic with the *spo13* diploid (NH567::pNH250).

[e] NM, non-mater.

[f] I indicates 1 μM 1-NA-PP1.

mosome III, increasing to 98.4% for *mek1-as1 dmc1 spo13* plus inhibitor. (To discriminate between reductional and equational segregants in recombinant dyads, a centromere linked marker is necessary but was not available in this strain. Because *MAT* and *URA3* are on different arms of chromosome III, nonrecombinant dyads produce distinctive patterns for reductional and equational segregation; Table 3). These results indirectly support the hypothesis that interhomologue recombination is reduced in *mek1-as1 dmc1 spo13* diploids lacking Mek1 kinase activity.

To directly examine interhomologue recombination, changes in the coupling relationship between *URA3* and *MAT* were examined. Because the poor spore viability of the *spo13* diploid resulted in a low yield of two viable spore asci, the analysis used spores from both one and two viable spore dyads (Table 3). Out of 499 dyads from the *mek1-as1 dmc1 spo13* strain to which inhibitor was added, only 4 (0.8%) were recombinant for *MAT* and *URA3*. In contrast, 35 of the 236 *spo13* dyads were recombinant (14.8%). Therefore interhomologue recombination is significantly reduced in the absence of *DMC1* and Mek1 kinase activity (χ^2 analysis; $p < 0.0001$).

Recombination between truncated *ade3* alleles in the *SCR::URA3* reporter was used to determine whether sister chromatid recombination was elevated under these conditions (Figure 1C; Kadyk and Hartwell, 1992). To prevent ectopic interactions, *ADE3* was deleted from its normal chromosomal position. *ADE3* is required for the biosynthesis of histidine and the diploid is therefore His$^-$. Two types of intersister events are detectable by the formation of His$^+$ recombinants: unequal reciprocal recombination and gene conversion (Kadyk and Hartwell, 1992). The frequency of intersister recombination events for the *mek1-as1 dmc1 spo13* diploid was 2.2% (11/499; Table 3). In two viable spore dyads it is possible to discriminate between gene conversion and reciprocal exchange events. All of the His$^+$ two viable spore dyads resulted from gene conversion—no reciprocal recombinants were detected. In the *spo13* diploid, no His$^+$ spore colonies were detected out of 236 *spo13* dyads, although five would be expected if the frequency on intersister recombination were the same as *mek1-as1 dmc1 spo13* (Table 3). Although the sample size is small, these data suggest there may be a bias toward intersister gene conversion events in the absence of *DMC1* and Mek1 kinase activity.

The DMC1-independent DSB Repair Observed in the Absence of MEK1 Requires RAD54

To further test the hypothesis that sister chromatid recombination is responsible for DSB repair in *mek1*Δ *dmc1*Δ, the dependence of this repair on *RAD54* was analyzed. The Rad54 protein stimulates Rad51 activity in vitro and is involved primarily in intersister recombination during meiosis

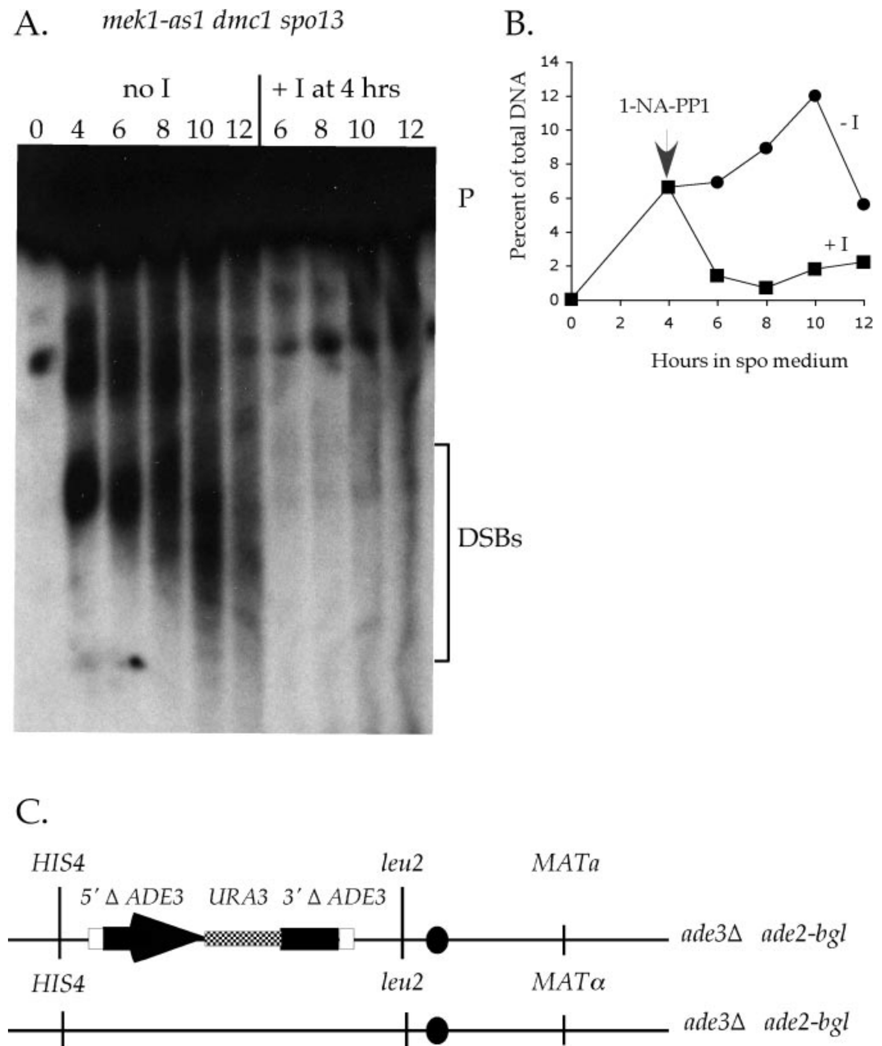

Figure 1. Double-strand breaks in a *mek1-as1 dmc1 spo13* diploid in the absence of inhibitor and after addition of inhibitor after 4 h in spo medium. (A) The *mek1-as1 dmc1 spo13* diploid, NH574::pNH251, was transferred to sporulation medium and incubated at 30°C for 4 h. At that time, 1 μM 1-NA-PP1 was added to one-half of the sporulating cells and returned to the incubator. At 2-h intervals, cells were fixed and analyzed for DSB formation at the *YCR048w* hotspot as described in Woltering *et al.* (2000). Numbers above each lane indicate hours after transfer to sporulation medium. The bracket indicates the DSB fragments. P, parental band. (B) Quantitation of the DSBs shown in A. (C) Configuration of the sister chromatid recombination reporter present on chromosome III in the isogenic *spo13* (NH567::pNH250) and *mek1-as1 dmc1 spo13* (NH574::pNH251) diploids (Kadyk and Hartwell, 1992). The black oval indicates the centromere on chromosome III. The white box indicates the region of shared homology between the 5′Δ *ADE3* and 3′Δ *ADE3* truncations between which recombination can occur.

(Arbel *et al.*, 1999; Bishop *et al.*, 1999; Petukhova *et al.*, 1999). Therefore if the disappearance of DSBs in *mek1*Δ *dmc1*Δ strains is due to recombination between sister chromatids, this repair should not occur in a *rad54*Δ *dmc1*Δ *mek1*Δ diploid. To compare isogenic strains, a *rad54*Δ *mek1*Δ *dmc1*Δ diploid was transformed with *RAD54*, *MEK1*, *DMC1* or vector to generate *mek1*Δ *dmc1*Δ, *rad54*Δ *dmc1*Δ, *mek1*Δ *rad54*Δ and *rad54*Δ *mek1*Δ *dmc1*Δ diploids, respectively. The wild-type strain was also included as a control. DSBs at the naturally occurring *YCR048w* hotspot were monitored by Southern blot analysis (Wu and Lichten, 1994). DSBs appeared in the wild-type diploid by 3 h and the bulk of the breaks were gone by 9 h (Figure 2). The kinetics of DSB appearance and disappearance were similar in the *mek1*Δ *rad54*Δ strain, although there appeared to be significant hyper-resection as indicated by the long smear in the DSB region of the gel (Figure 2A). This repair is presumably being mediated by Dmc1, indicating that *MEK1* is not required to promote Dmc1 function. DSBs persisted in the *rad54*Δ *dmc1*Δ diploid and became hyper-resected (Figure 2). Deletion of *RAD54* from the *mek1*Δ *dmc1*Δ diploid blocked DSB repair, with the DSBs exhibiting even more hyper-resection than the *rad54*Δ *dmc1*Δ strain (Figure 2). There was a delay in the onset of break formation in the triple mutant, raising the possibility that repair might also be delayed. Extending the time course to 15 h gave the identical result, however, making this possibility unlikely (Figure 2B). *RAD54* is therefore necessary for *DMC1*-independent repair in the absence of *MEK1*.

Suppression of dmc1Δ by Overexpression of RAD51 Is Dependent on Mek1 Kinase Activity

Overexpression of *RAD51* largely suppresses the interhomologue recombination and sporulation defects of *dmc1*Δ during meiosis (Tsubouchi and Roeder, 2003). If the ability of Rad51 to promote meiotic interhomologue recombination is due to a BSCR, then *RAD51* suppression of *dmc1*Δ should be dependent on Mek1 kinase activity. This hypothesis was tested by transforming a *dmc1*Δ *mek1-as1* diploid with either vector, *DMC1* or a plasmid overexpressing *RAD51* and testing the transformants for a variety of meiotic phenotypes in the presence or absence of the 1-NA-PP1 inhibitor. To eliminate any possible negative effects arising from the overexpression of *RAD51* in vegetative cells, *RAD51* was expressed under the control of the meiosis-specific *HOP1* promoter (Hollingsworth *et al.*, 1990).

Meiotic time courses were performed on four independent *mek1-as1*, *mek1-as1 dmc1*Δ, and *mek1-as1 dmc1*Δ/2μ *RAD51* cultures. The culture overexpressing *RAD51* was split immediately after transfer to sporulation medium and

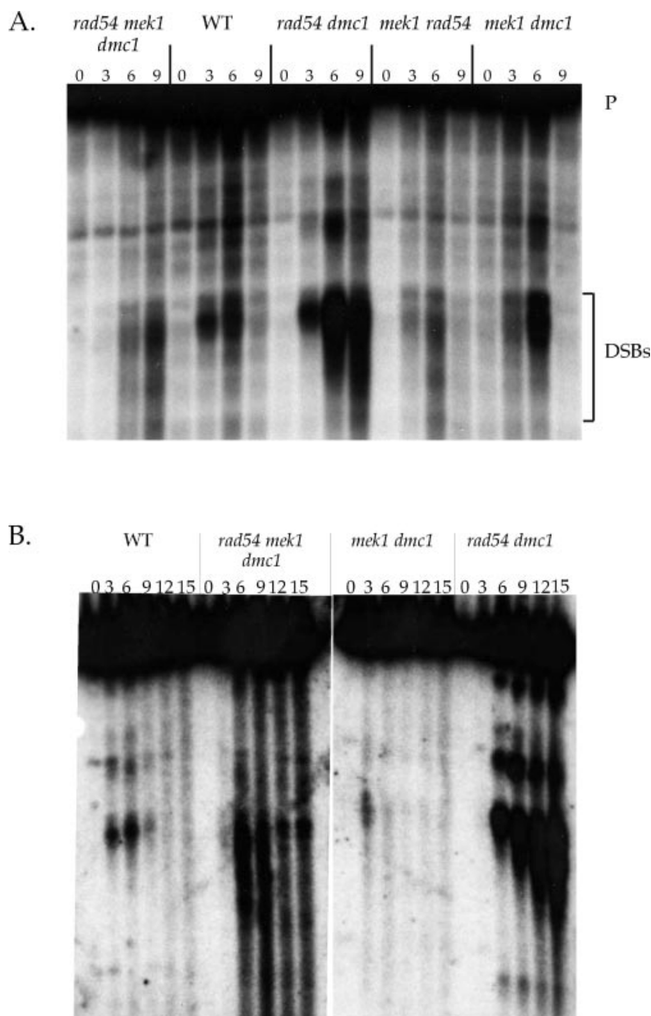

Figure 2. DSBs in strains containing various combinations of *mek1*Δ, *dmc1*Δ and *rad54*Δ. NH624 was transformed with YCp50 (*rad54*Δ *mek1*Δ *dmc1*Δ), pR4C4 (*rad54*Δ *dmc1*Δ), pRS316-DMC1 (*mek1*Δ *rad54*Δ), or pNRB143 (*mek1*Δ *dmc1*Δ). In addition the wild-type diploid, NH144, was transformed with YCp50 so that all of the strains could be grown under selective conditions for the plasmids until they were sporulated at 30°C. DSBs at the *YCR048w* hotspot were analyzed by Southern blot as described in Woltering *et al.* (2000). P, the parental fragment. The bracket indicates the DSBs. (A) Time course carried out for 9 h. (B) Time course carried out for 15 h.

a final concentration of 1 μM 1-NA-PP1 was added to half. The cells were then incubated at 30°C for 24 h. As expected in the absence of inhibitor, the *mek1-as1* diploid sporulated well (96.0 ± 0.9% asci) and produced viable spores (90.0 ± 5.2%, 72 asci dissected), in contrast to the *mek1-as1 dmc1*Δ strain which failed to sporulate. Consistent with the results of Tsubouchi and Roeder (2003), overexpression of *RAD51* partially rescued the sporulation defect of *mek1-as1 dmc1*Δ (36.6 ± 5.1% asci). The high spore viability of the tetrads produced in the absence of inhibitor in this strain (70.0 ± 13.2% viable spores, 102 asci dissected) indicates that interhomologue recombination is occurring. Measurements of plasmid stability at the time of transfer to spo medium showed that ~70% of the *mek1-as1 dmc1*Δ cells contained the *RAD51* plasmid. Therefore the observed suppression of sporulation underestimates the amount of possible suppression. Addition of inhibitor to the *RAD51* overexpressing strain resulted in even higher levels of sporulation (72.6 ± 10.6% asci) but inviable spores (0.8 ± 1.0%, 101 asci dissected).

This experiment provides a unique opportunity to compare the kinetics of meiotic progression in a culture in which Rad51 is being used to repair DSBs either using homologues or sister chromatids as templates. When interhomologue recombination utilizes only Rad51, the onset of MI is delayed, supporting the idea that Dmc1 facilitates this process (Figure 3). In contrast, inhibition of *mek1-as1* in the *RAD51* overexpressing *dmc1* diploid allowed even more rapid meiotic progression than wild type (Figure 3). Comparison of DSBs in the *RAD51* overexpression strain showed that in the presence of inhibitor, DSBs at the *YCR048w* hotspot were repaired by 6 h, whereas in the absence of inhibitor the DSBs did not disappear until 12 h (some of these persisting DSBs are most likely due to those cells that lost the *RAD51* plasmid before the initiation of meiosis). Similar results were obtained looking at the *ARG4* DSB hotspot (unpublished data). These results demonstrate that removal of the BSCR in *dmc1* mutants results in a rapid, efficient repair of DSBs. Furthermore the ability of *RAD51* to mediate interhomologue recombination when overexpressed in a *dmc1* mutant is dependent on the BSCR being present.

Mutagenesis of the C-terminal Tail of Hop1 Identifies Two Lysines, K590A and K593A, Which Are Important for HOP1 Function

Intragenic complementation studies using various mutant alleles of *HOP1* indicated that the 605 amino acid protein contains at least two discrete functional domains (Friedman *et al.*, 1994). One domain consists of the last 20 amino acids of the protein (the C domain), whereas the other domain is comprised of the rest of the Hop1 protein (the N domain). One of the alleles used by Friedman *et al.* (1994), *hop1-R6*Δ, resulted not only in the deletion of the last 41 amino acids of Hop1, but also the addition of 18 amino acids as a result of translation of downstream vector sequences. Therefore it was not clear whether the spore inviability observed for *hop1-R6*Δ was due to the loss of the Hop1 C domain or to the insertion of extra amino acids at the end of the protein. To address this question, stop codons were introduced into *HOP1* immediately after codon 564 (*hop1-564*Δ) or 585 (*hop1-585*Δ), thereby truncating Hop1 by 41 and 20 amino acids, respectively. Diploids carrying either *hop1-564*Δ or *hop1-585*Δ produced 0 viable spores out of 22 tetrads dissected, indicating both mutations create null alleles with regard to spore viability. The truncations do not appear to destabilize the mutant proteins, however, because their protein levels are similar to wild-type Hop1 (measured 3 h after transfer to sporulation medium; Figure 4A). The conclusion therefore, is that the last 20 amino acids of Hop1 are essential for its function.

Assuming that the C domain of Hop1 represents a discrete functional module, it should be possible to isolate point mutations that abolish the function of this domain without affecting activities that are mediated by the rest of the protein. Null alleles of *HOP1* exhibit a number of mutant phenotypes, including low spore viability, reduced levels of interhomologue recombination and DSBs, as well as a defect in chromosome synapsis (Hollingsworth and Byers, 1989; Woltering *et al.*, 2000; Wan *et al.*, 2004). In addition, Hop1 is a DNA binding protein that physically interacts both with itself and with Red1 (Kironmai *et al.*, 1998; de los Santos and Hollingsworth, 1999). To generate separation of function alleles, charged residues located between amino acids 567 and 605 were mutated to alanine and assayed for defects in

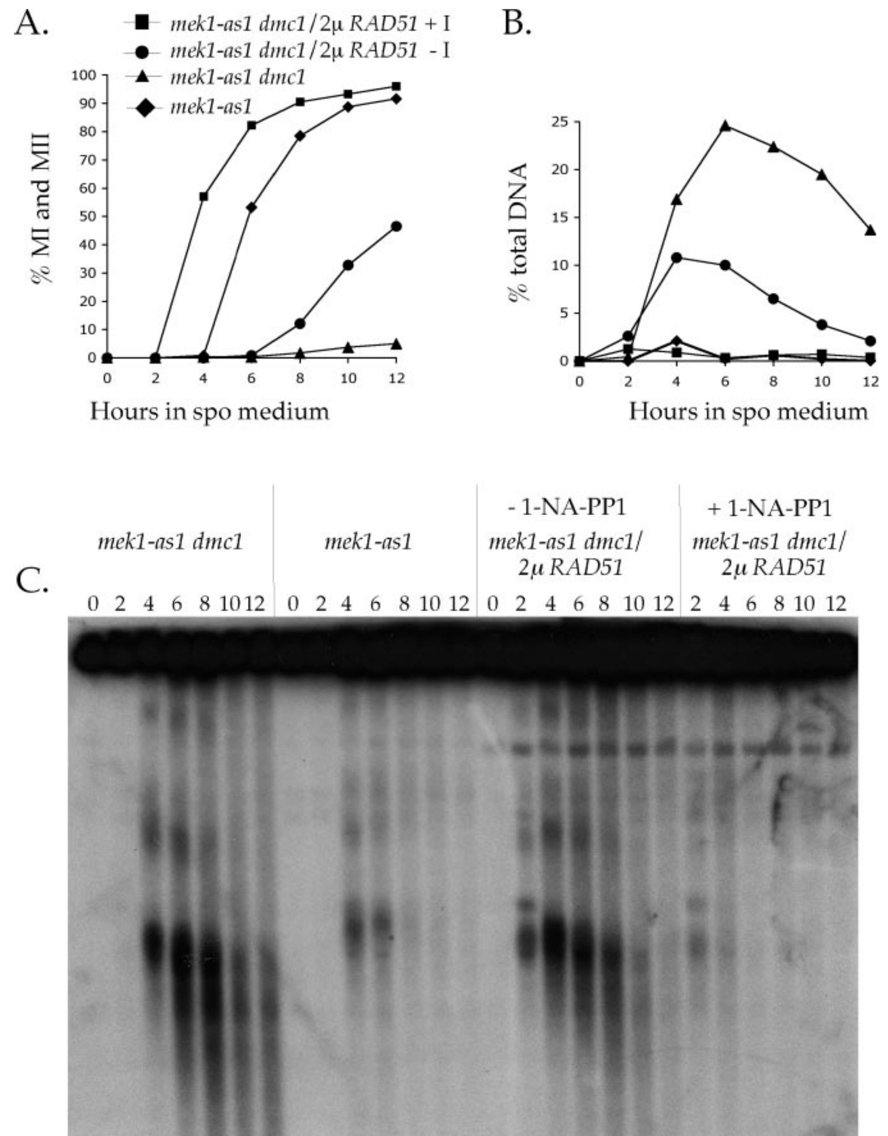

Figure 3. Meiotic progression and DSBs in *mek1-as1 dmc1* diploids overexpressing *RAD51* in the presence or absence of 1-NA-PP1. NH639 was transformed with pRS306 (*mek1-as1 dmc1*Δ), pLW28 (*mek1-as1*), or pNH255 (*mek1-as1 dmc1*Δ/2 μ *RAD51*). A final concentration of 1 μM 1-NA-PP1 was added to half of the pNH255 containing culture immediately after transfer to sporulation medium and the cells were incubated at 30°C. (A) Meiotic progression was measured by DAPI staining to determine the fraction of binucleate (MI) and tetranucleate (MII) cells. The graph represents the averages of four independent cultures for each strain. (B) Quantitation of the percentage of total DNA contained in the DSB fragments shown in C. (C) Southern blot exhibiting DSB fragments generated at the *YCR048w* hotspot on chromosome III.

spore viability. Out of 15 amino acids that were mutated, two resulted in a reduction in spore viability. The most severe mutant, *hop1-K593A*, produced <1% viable spores in the SK1 background (36 asci dissected), equivalent to a deletion of *HOP1*. In addition, changing K590 to alanine or methionine reduced spore viability to 42 and 59%, respectively (*hop1-K590A*, 49 asci; *hop1-D584A K590M*, 49 asci). The amino acid substitutions present in *hop1-K590A* and *hop1-K593A* do not appear to decrease protein stability (Figure 4A).

hop1-K593A Mutants Exhibit Increased Levels of Crossovers and DSBs Compared with a Null Allele of HOP1

*hop1*Δ and *red1*Δ mutants reduce, but do not eliminate, meiotic recombination (Hollingsworth and Byers, 1989; Rockmill and Roeder, 1990; Mao-Draayer *et al.*, 1996). *hop1*Δ displays a more severe recombination phenotype than *red1*Δ, with *hop1*Δ *red1*Δ resembling *hop1*Δ alone, suggesting that *HOP1* has a function in recombination independent of *RED1* (Rockmill and Roeder, 1990). To determine whether the C-terminus is required for this recombination function, the effect of *hop1-K593A* on interhomologue crossing over was measured in *spo13* diploids heterozygous for markers on two different chromosomes. A *hop1*Δ mutation was used as the null control. The *hop1*Δ diploid exhibited a mean 60-fold reduction in crossing over measured in four intervals. In contrast, *hop1-K593A* decreased crossing over on average only 11-fold (Table 4). This phenotype is highly similar to that observed for *hop1-R6*Δ, supporting the idea that the K593A mutation abolishes the function of the C domain. Both *hop1-K593A* and *hop1-R6*Δ are phenotypically similar to an isogenic *red1*Δ diploid, which also reduced crossing over on average 11-fold (Table 4). These results argue that *HOP1* contains a recombination function that is partially intact in the *hop1-K593A* mutant.

*hop1*Δ and *red1*Δ mutants exhibit decreased steady state levels of DSBs (Mao-Draayer *et al.*, 1996; Xu *et al.*, 1997; Woltering *et al.*, 2000). In the SK1 background, *red1*Δ mutants exhibit a higher frequency of DSBs compared with *hop1*Δ, even when resection of the ends is prevented by mutation of *SAE2/COM1* (Figure 5; Woltering *et al.*, 2000; Pecina *et al.*, 2002). *hop1*Δ is epistatic to *red1*Δ with regard to

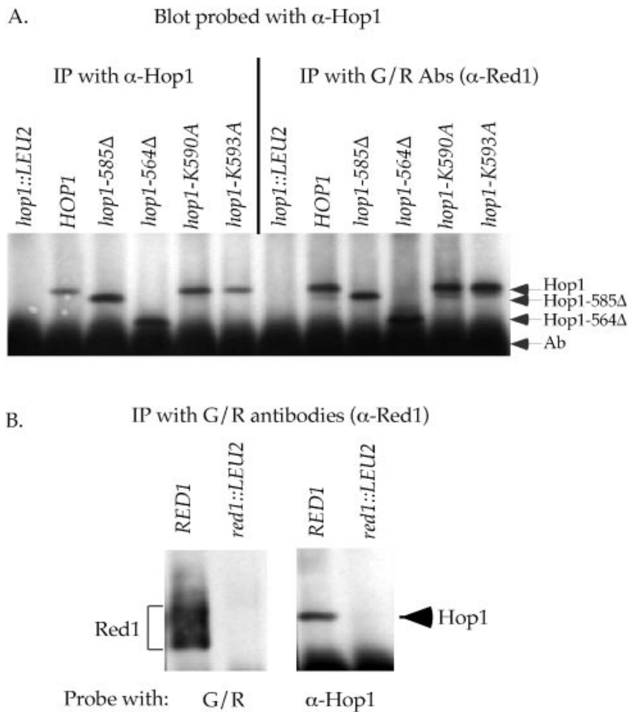

Figure 4. Coimmunoprecipitation of various Hop1 mutant proteins with Red1. A. *hop1::LEU2* (DW10::YIp5), *HOP1* (DW10::pLT11), *hop1-585Δ* (DW10::pLT11-585), *hop1-564Δ* (DW10::pLT11-564), *hop1-K590A* (DW10::pLT11-K590A), and *hop1-K593A* (DW10::pLT11-K593A) diploids were sporulated for 3 h at 30°C and soluble yeast extracts used for immunoprecipitation with either α-Hop1 or G/R (α-Red1) antibodies. The IPs were fractionated by SDS-PAGE using 6% gels and probed with either α-Hop1 or G/R antibodies as indicated. (B) Red1 was IPed from extracts derived from *RED1* (YTS1::pTS30-Q241G) and *red1::LEU2* (YTS3) diploids using G/R antibodies. The Red1 IPs were then probed with either G/R or Hop1 antibodies to detect Red1 or Hop1, respectively.

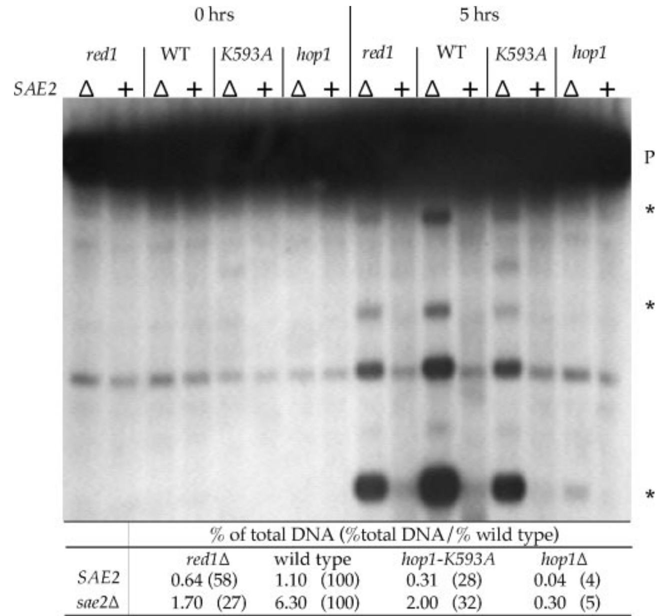

Figure 5. DSB formation at the *YCR048w* hotspot in various *hop1* diploids. Isogenic diploids were transferred to sporulation medium and cells were fixed after 0 and 5 h at 30°C. A Southern blot of digested DNA was probed to detect DSB fragments formed at the *YCR048w* hotspot. P, parental band; asterisks indicate meiosis-specific DSB fragments. *red1::LEU2 sae2Δ* (NH217); *red1::LEU2* (YTS3); *sae2Δ* (NH311::pDW39); wild type (DW10::pLT11); *hop1-K593A sae2Δ* (NH311::pDT12); *hop1-K593A* (DW10::pLT11-K593A); *hop1::LEU2 sae2Δ* (NH311::pRS402); *hop1::LEU2* (DW10::YIp5).

DSBs, as a *red1Δ hop1Δ sae2Δ* diploid produces a level of DSBs equivalent to *hop1Δ sae2Δ* (unpublished data). The amount of DSBs in the *hop1-K593A sae2Δ* diploid resembled that of *red1Δ sae2Δ*, representing a sixfold increase over the *hop1Δ sae2Δ* strain (Figure 5). Similar results were obtained in the *SAE2* strains, although the levels of DSBs in these strains were lower than those in the *sae2Δ* diploids, presumably because DSBs in the *SAE2* diploids are repaired and therefore do not accumulate (Figure 5). These results argue that the N domain either promotes initiation of DSBs or prevents DSB ends from being degraded.

The Hop1-K593A Protein Localizes to Chromosomes but hop1-K593A Mutants Are Defective in Chromosome Synapsis

Null mutants of *HOP1* result in the formation of AEs but no SCs (Hollingsworth *et al.*, 1990; Loidl *et al.*, 1994). Electron microscopic analysis of spread chromosomes from *hop1-K593A* revealed a similar phenotype (Figure 6). The synapsis defect was also manifested by the failure of Zip1, a component of meiotic chromosomes frequently used as an indicator of synapsis (Sym *et al.*, 1993; Sym and Roeder, 1995), to localize along the lengths of chromosomes from both *hop1-*

Table 4. Effect on meiotic interhomologue crossing over in *red1 spo13* and various *hop1 spo13* mutants

Strain::plasmid	Relevant genotype	Map distance (cM)[a] *HIS4-LEU2*	*LEU2-CDC10*	*CDC10-MAT*	*ARG4-THR1*	Mean fold reduction
NH246::pSB3[b]	*RED1 HOP1*	18.3 (254)	13.1 (245)	22.2 (248)	12.1 (286)	1
NH246::pRS306[b]	*red1Δ*	0.7 (284)	1.6 (286)	1.7 (295)	2.0 (318)	11
NH270::YIp5	*hop1Δ*	0.3 (317)	0 (317)	0.6 (317)	0.2 (324)	60
NH270::YIp-hop1R6Δ	*hop1-R6Δ*	0.6 (427)	1.6 (427)	2.8 (427)	0.9 (440)	11
NH270::pLT11-K593A	*hop1-K593A*	0.5 (401)	1.4 (401)	2.3 (401)	1.3 (414)	12

[a] Map distances were calculated as described in Hollingsworth *et al.* (1995). Values in parentheses are the number of dyads analyzed.
[b] Data are taken from Woltering *et al.* (2000).

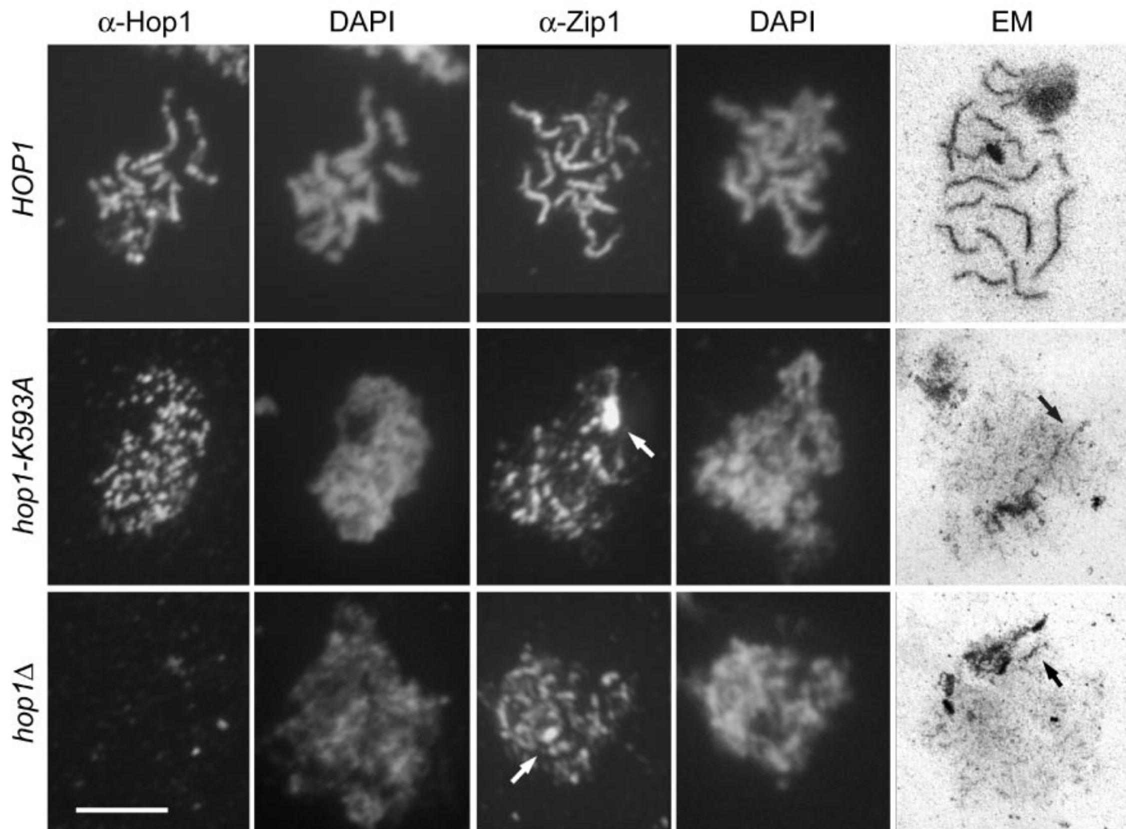

Figure 6. Hop1 localization and chromosome synapsis in *hop1-K593A* diploids. Preparation of nuclei, immunostaining, and silver staining for electron microscopy were done as described by Loidl *et al.* (1998). Hop1 and Zip1 were localized by immunostaining with α-Hop1 and α-Zip1 antibodies. DNA was visualized using DAPI. Silver-stained chromosomes were analyzed by electron microscopy (EM). The few thick structures observed by EM are probably polycomplexes (indicated by arrows) that are prominently decorated by Zip1 as well (arrows). Bar, 5 μm. *HOP1* (DW10::pLT11); *hop1-K593A* (DW10::pLT11-K593A); *hop1::LEU2* (DW10::YIp5).

K593A and *hop1*Δ diploids. Localization of the Hop1-K593A protein to unsynapsed chromosomes was unaffected, however, indicating that although the C domain is necessary for synapsis, it is not required for Hop1 to interact with chromosomes (Figure 6).

The C Domain Is Not Required for Interaction with Red1 or Hop1

The fact that overexpression of *RED1* specifically suppresses a mutation at codon 595 in the *HOP1* C domain had suggested that Red1 might physically interact with the Hop1 C-terminus (Hollingsworth and Johnson, 1993). This idea was tested by examining the ability of Red1 to IP various Hop1 tail mutants. For these experiments a polyclonal antibody generated against a fragment of the Red1 protein was used (Wan *et al.,* 2004). Co-IP of Red1 with Hop1 is readily detectable using these antibodies (Figure 4B). Soluble extracts from isogenic diploids containing various alleles of *HOP1* were used to precipitate either Hop1 or Red1 by addition of the appropriate antibody. After the IPs were fractionated by SDS-PAGE, the filters were probed with α-Hop1 antibodies. As expected, no Hop1 was observed in the strains deleted for *HOP1* (Figure 4A). Hop1, Hop1-K590A, Hop1-K593A, Hop1-564, and Hop1-585 all co-IPed with Red1 (Figure 4A). The C-terminus of Hop1 is therefore dispensable for Red1 binding.

Purified Hop1 protein exists as a dimer in solution (Kironmai *et al.,* 1998). To test whether the C domain affects Hop1 dimerization, Hop1 and Hop1-585 were purified after expression in *E. coli* and analyzed by gel filtration analysis under nondenaturing conditions. Both proteins exhibited the same elution profile, indicating that Hop1 can dimerize in the absence of the C domain (unpublished data). These results are consistent with genetic data showing that mutations in the C domain can intragenically complement mutations in the N domain (Friedman *et al.,* 1994).

C Domain Mutants in HOP1 Can Be Bypassed by Ectopic Dimerization of Mek1

Given that the Hop1 C domain functions downstream of DSBs as well as Red1 and Hop1 binding, we wanted to test whether the C-terminal tail of Hop1 is needed for Mek1 activation. Our standard Mek1 kinase assay uses Gst-Mek1 partially purified from meiotic extracts and monitors Mek1 autophosphorylation (de los Santos and Hollingsworth, 1999). A *hop1-K593A GST-MEK1* diploid was therefore constructed (in these experiments all of the *HOP1* and *MEK1* alleles are integrated into the chromosome in single copy, unless otherwise stated). Given that a *hop1-K593A MEK1* diploid produces inviable spores, the finding that *hop1-K593A GST-MEK1* produces nearly the same high level of viable spores as an isogenic *HOP1 GST-MEK1* diploid was unexpected (Table 5). Suppression of *hop1-K593A* requires Mek1 kinase activity, as a catalytically inactive mutant, *GST-mek1-K199R*, fails to suppress (Table 5). Zip1 staining dem-

Table 5. Spore viabilities of different *hop1* strains containing various alleles of *MEK1*

	HOP1 genotype[a]			
MEK1 genotype[a]	*hop1::LEU2*	*HOP1*	*hop1-K593A*	*hop1–585*
mek1Δ	ND[b]	1.0 (25)	<2.0 (13)	ND
MEK1	<1.0 (26)	96.4 (77)	<1.0 (76)	<1.0 (78)
GST-MEK1	1.0 (26)	93.5 (50)	87.9 (128)	47.6 (103)
GST-mek1-K199R	<2.0 (13)	<1.0 (26)	<1.0 (24)	<2.0 (13)
gst-R72P,D76K-MEK1	ND	91.4 (54)	6.7 (104)	ND
lexA-MEK1	ND	86.8 (76)	23.3 (182)	ND
2μ *lexA-MEK1*	ND	89.4 (26)	46.5 (182)	ND
TAP-MEK1	1.9 (26)	87.0 (77)	1.0 (52)	<1.0 (78)

[a] Values are the percentage of viable spores, with number of asci dissected in parentheses. All strains are derived from the same *hop1::LEU2 mek1Δ::LEU2* SK1diploid, NH566. The *hop1* alleles were introduced by integrating the following plasmids: *hop1::LEU2*, pRS306; *HOP1*, pLT11; *hop1-K593A*, pLT11-K593A; *hop1–585*, pLT11–1–585. The *MEK1* alleles were introduced by integrating the following plasmids into the appropriate *hop1* strain: *mek1Δ*, pRS402; *MEK1*, pLW20; *GST-MEK1*, pTS30; *GST-mek1-K199R*, pTS31; *gst-R72P, D76K-MEK1*, pTS30-R72P, D76K; *lexA-MEK1*, pHN23; 2μ *lexA-MEK1*, pHN24; *TAP-MEK1*, pHN16.

[b]ND, no data.

onstrated that *GST-MEK1* also suppresses the synapsis defect of *hop1-K593A* (unpublished data). These results suggest that the presence of GST in the Mek1 protein is sufficient to bypass the requirement for the Hop1 C domain during meiosis.

It has previously been shown that GST dimerizes in solution (Lim *et al.,* 1994; Vargo *et al.,* 2004). This observation raised the possibility that the function of the Hop1 C domain is to promote dimerization of Mek1. In this case, the presence of a dimerization domain such as GST in Mek1 could bypass the requirement for the Hop1 C domain by providing an alternative means for dimerization. Two experiments were performed to test this hypothesis. In the first experiment, alternative N-terminal fusions to *MEK1* were assayed for their ability to complement the spore inviability of *mek1Δ* in a *HOP1* diploid as well as for their ability to suppress *hop1-K593A*. Similar to GST, the lexA protein has been shown to form dimers in solution (Mohana-Borges *et al.,* 2000). In contrast, the TAP tag contains protein A sequences as well as a calmodulin-binding domain, neither of which is known to dimerize (De *et al.,* 1997; Puig *et al.,* 2001). The *TAP-MEK1* and *lexA-MEK1* fusions were transformed into *mek1Δ HOP1* and *mek1Δ hop1-K593A* diploids and assayed for spore viability. In single copy, both *lexA-MEK1* and *TAP-MEK1* complemented well, producing >85.0% viable spores (Table 5). *TAP-MEK1* failed to improve the spore viability of *hop1-K593A*, but *lexA-MEK1* conferred partial suppression, producing 23.3% viable spores (Table 5). Overexpression of *lexA-MEK1* exhibited better suppression of *hop1-K593A* than single copy *lexA-MEK1*, producing 46.5% viable spores (Table 5), suggesting that the partial suppression exhibited by *lexA-MEK1* may be due to inefficient dimerization within the cell.

The second experiment to test the dimerization hypothesis was to introduce amino acid substitutions into GST that are likely to disrupt dimerization. Using the crystal structure of dimerized GST as a guide for mutagenesis (Lim *et al.,* 1994; Vargo *et al.,* 2004), proline was substituted for an arginine at position 72 and an arginine was substituted for an aspartic acid at position 76 to disrupt hydrophobic and hydrophilic interactions required for dimerization (*gst-RD-MEK1*). *gst-RD-MEK1* complemented well in the *mek1Δ HOP1* diploid, indicating that the *MEK1* in this fusion is functional (Table 5). Suppression of the spore viability defect of *hop1-K593A* was greatly reduced in the *gst-RD-MEK1* background, however, dropping from 87.9% for *GST-MEK1 hop1-K593A* to 6.7% (Table 5). Furthermore, Zip1 staining revealed that chromosome synapsis was reduced in the *hop1-K593A gst-RD-MEK1* strain, although some nuclei showed partial SC formation indicating that the phenotype was somewhat better than *hop1-K593A* alone (unpublished data). Attempts to confirm Mek1 dimerization by coimmunoprecipitation of differentially tagged Mek1 proteins have thus far been unsuccessful. Given the strong genetic evidence for dimerization, this negative result seems likely to be due to technical issues. One possibility is that only a fraction of Mek1 is dimerized during meiosis. If this fraction is small relative to the total amount of Mek1 protein, then detecting the interaction by biochemical methods may be difficult.

GST-MEK1 not only suppresses the K593A point mutant in the *HOP1* C-domain, it also partially suppresses a version of Hop1 in which the tail is deleted (*hop1-585Δ*; Table 4). This result argues that the primary role of the Hop1 C domain is to actively promote dimerization of Mek1.

Dimerization of Mek1 Is Necessary for Preventing DMC1-independent Repair of Meiotic DSBs

We infer that the spore inviability of *hop1-K593A* results from missegregation of chromosomes at Meiosis I due to a failure to prevent recombination between sister chromatids. If true, then the absence of the BSCR in *hop1-K593A* should allow DSBs in *dmc1Δ* diploids to be repaired and *dmc1Δ hop1-K593A* diploids should sporulate. A *hop1Δ dmc1Δ* diploid was constructed and various alleles of *HOP1* and *MEK1* introduced by transformation. Addition of both *HOP1* and *DMC1* creates a wild-type diploid that sporulates well and exhibits high spore viability (Table 6). In the *HOP1 dmc1Δ* diploid, sporulation was reduced to <0.2% as previously reported (Bishop *et al.,* 1992). Deletion of *HOP1* in the *dmc1Δ* background allowed the cells to sporulate, consistent with a role for *HOP1* in the BSCR. The *hop1-K593A dmc1Δ* diploid also sporulated well and produced dead spores, indicating that the Hop1 C domain is required for preventing *DMC1*-independent repair (Table 6). The possibility that *hop1-K593A* does not generate sufficient DSBs to trigger the meiotic recombination checkpoint is ruled out by the fact that *GST-MEK1*, while having no effect on sporulation in the *hop1Δ dmc1Δ* strain, restores the meiotic arrest of the *hop1-*

Table 6. Sporulation and spore viability in *dmc1* strains carrying various alleles of *HOP1* and *MEK1*

Relevant genotype[a]	% spo	Viable spores
HOP1 DMC1	78.2	Yes
HOP1 dmc1Δ	<0.2	ND[b]
hop1Δ dmc1Δ	79.8	No
hop1Δ dmc1Δ GST-MEK1	81.7	No
hop1Δ dmc1Δ hop1-K593A	83.0	No
hop1Δ dmc1Δ hop1-K593A GST-MEK1	4.2	ND
hop1Δ dmc1Δ hop1-K593A GST-mek1-K199R	77.8	No

[a] All strains were derived by transformation of the *dmc1Δ::LEU2 hop1Δ::kanMX* diploid, NH601. *HOP1* alleles were integrated at *ura3* in one haploid parent using the following plasmids: *HOP1*, pLT11; *hop1-K593A*, pLT11-K593A. *MEK1* alleles and *DMC1* were integrated at *ura3* into the other haploid parent using the following plasmids: *DMC1*, pLW28; *MEK1*, pLP37; *GST-MEK1*, pBL12; *GST-mek1-K199R*, pHN26. The resulting transformants were then mated in the appropriate combinations to give the indicated genotypes. Spore viability was determined by tetrad dissection.

[b] ND, no data.

K593A dmc1Δ strain (Table 6). As with spore viability, the ability of *GST-MEK1* to restore the arrest to the *hop1-K593A dmc1Δ* diploid requires Mek1 kinase activity (Table 6).

Hop1 Is a DSB-dependent Phosphoprotein

Because of the genetic interactions between the Mek1 kinase and Hop1, we tested to see whether Hop1 is a phosphoprotein. Our previous work had detected only a single band on protein gels for Hop1 (e.g., Figure 4). We found, however, that running the gels for a much longer period of time enabled the detection of slower migrating species (Figure 7A). The slower migrating forms are eliminated by treatment of IPed Hop1 with λ protein phosphatase, demonstrating that Hop1 is a phosphoprotein. The absence of *MEK1* did not affect the Hop1 mobility shift, indicating that Mek1 is unlikely to be the kinase that phosphorylates Hop1 (Figure 7A). Hop1-585, which is deleted for the Hop1 C domain, exhibits only a single, phosphatase-insensitive form, indicating either that the C-domain is phosphorylated directly or that its presence is required for phosphorylation elsewhere on Hop1 (Figure 7A).

To test whether Hop1 phosphorylation is regulated by DSBs, the gel mobility of Hop1 IPed from a *rec104* diploid was examined. *REC104* is one of several meiosis-specific genes required for generating meiotic DSBs (Pecina *et al.*, 2002). Although the phosphorylated form of Hop1 was present at both 5 and 7 h after the induction of meiosis, the bulk of the Hop1 remained unphosphorylated in the *rec104* diploid at both time points (Figure 7B). Similar results have been obtained with *spo11* mutants (unpublished data). Therefore the majority of phosphorylated Hop1 protein present in meiotic cells is dependent on the formation of DSBs.

DISCUSSION

Previously we proposed that the *DMC1*-independent repair observed in the absence of Mek1 kinase activity utilized sister chromatids, based on the assumption that *MEK1* behaves analogously to *RED1* in this process (Wan *et al.*, 2004). Several pieces of evidence demonstrate that this assumption is correct. First, the spore lethality observed in *mek1-as1 dmc1* is partially suppressed by eliminating the need for interhomologue crossovers for proper segregation using *spo13*. Second, the viable spores formed in *mek1-as1 dmc1 spo13* diploids are decreased for interhomologue recombination and increased for intersister recombination compared with wild type. Third, the DSB repair observed in *mek1Δ dmc1Δ* diploids is dependent on *RAD54*, a gene required primarily for sister chromatid recombination in meiosis. Finally, Mek1 kinase activity is necessary for the production of viable spores in a *dmc1* diploid overexpressing *RAD51*. Given that in mitotic cells the preferred substrate of Rad51 is the sister chromatid, this observation supports the idea that overexpression of *RAD51* rescues the interhomologue recombination defect of *dmc1* because of a *MEK1*-dependent BSCR.

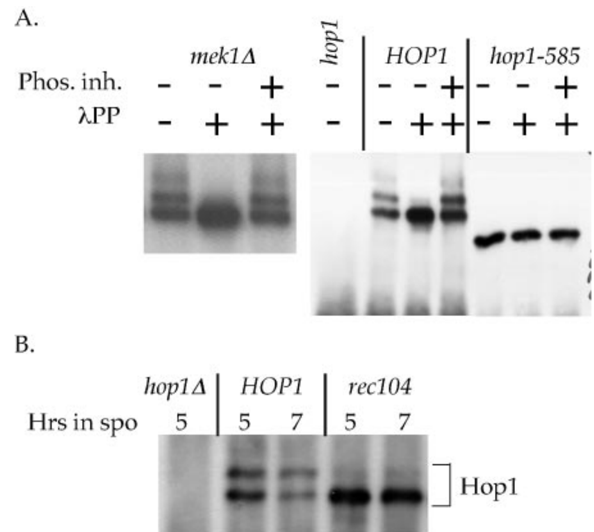

Figure 7. Hop1 phosphorylation in *mek1Δ* and *rec104* diploids. (A) Phosphatase treatment of Hop1 and Hop1-585Δ. Hop1 or Hop1-585Δ was IPed from *mek1Δ* (YTS1ade2::pRS402), *hop1::LEU2* (DW10), *HOP1* (DW10::pLT11), or *hop1-585Δ* (DW10::pLT11-585) extracts using Hop1 antibodies. The proteins were fractionated on an 8% SDS-polyacrylamide gel. The IPs were treated with λ protein phosphatase (λPP) as described in de los Santos and Hollingsworth (1999). The phosphatase inhibitors, NaF and $Na_4P_2O_7$, were used at 10 and 1 mM final concentrations, respectively. The Western blot was then probed with Hop1 antibodies. (B) Hop1 was IPed and detected as described in A using protein extracts from wild-type (NH144) and *rec104* (DW11) diploids 5 or 7 h after transfer to sporulation medium as indicated.

Our results indicate that meiotic interhomologue bias results from the suppression of intersister recombination created by phosphorylation of target proteins by Mek1. This idea is in contrast to a previous proposal that meiotic interhomologue bias is an active process in which a subset of *RED1*-dependent DSBs become destined for interhomologue recombination (Schwacha and Kleckner, 1997). The basis for this idea was the observation that DSBs are reduced in a *red1* mutant and that the frequency of joint molecules between sister chromatids is not increased, as would be predicted if sister chromatid recombination is suppressed by *RED1* (Schwacha and Kleckner, 1997). Consistent with the latter finding, no increase in meiotic unequal sister chromatid exchange was observed for *red1* mutant using a genetic assay (Hollingsworth *et al.*, 1995). However, the interpretation of these experiments is complicated by the pleiotropic phenotypes of *red1*, as well as by the fact that it assumes that sister chromatid recombination utilizes primarily joint mol-

ecule intermediates. Our experiments with the chemically inhibitable *mek1-as1* mutant avoid these complications because DSBs can be allowed to form under wild-type conditions (i.e., the presence of Red1 and Mek1 kinase activity). The failure to observe an increase in intersister recombination in *red1* and *mek1* diploids may be because the physical and genetic assays used by Schwacha and Kleckner (1997) and Hollingsworth *et al.* (1995) measured crossing over. In fact, an increase in intersister recombination by *red1* and *mek1* was observed by Thompson and Stahl (1999) using a genetic assay that produced a positive signal either by exchange or gene conversion, although they did not discriminate between the two. Thompson and Stahl (1999) proposed that one explanation for the difference between their results and those of Schwacha and Kleckner (1997) could be because sister chromatid repair occurring in *mek1* and *red1* diploids utilizes a pathway that does not generate joint molecules. Our finding that intersister gene conversion events are specifically increased in the *mek1-as1 dmc1 spo13* dyads supports the idea that DSB repair in this diploid is occurring by a non-crossover recombination pathway such as synthesis-dependent strand annealing (Paques and Haber, 1999).

The critical step in determining partner choice is at the time of strand invasion. A BSCR is therefore only necessary after a DSB has been generated on one of the two chromatids to ensure that invasion of the homologue occurs. How might the BSCR be regulated so that it is established after DSB formation? This problem could be solved by activating Mek1 function in a DSB-dependent manner. Our work indicates that Hop1 may be the bridge that connects the creation of a DSB on the DNA with the activation of Mek1 function.

Previous work has indicated that Mek1 must be in a complex with Hop1 and Red1 for the kinase to become activated (Wan *et al.*, 2004). Mek1 binds to phosphorylated Red1 via a conserved protein-protein interaction module in its N-terminus called the FHA domain (Wan *et al.*, 2004). Purified Hop1 exists as a dimer in solution and binds to DNA in vitro (Kironmai *et al.*, 1998). Given that *hop1*Δ has more severe recombination and DSB phenotypes than *red1*Δ, we propose that Hop1 dimers bind directly to the DNA in chromosomes, although this disagrees with cytological studies that indicate that *RED1* is required for Hop1 localization to chromosomes (Smith and Roeder, 1997). Red1 acts as a bridge between Hop1 and Mek1 in two-hybrid experiments and Hop1 and Red1 are bound to chromosomes in the absence of DSBs (Smith and Roeder, 1997; Bailis and Roeder, 1998). These results lead us to propose that inactive complexes of Hop1/Red1/Mek1 are assembled onto DNA before DSB formation (Figure 8).

Inactivation of the *HOP1* C domain by the point mutation, *hop1-K593A*, creates a protein that is still able to bind Red1, localize to chromosomes and produce higher levels of detectable DSBs than the *hop1*Δ, presumably because the mutant protein is still able to bind DNA. *hop1-K593A* mutants exhibit defects in spore viability and synapsis and allow *dmc1* mutants to sporulate, indicating that the Hop1 C domain has a role in creating the BSCR. This role appears to be enabling Mek1 to dimerize. The need for the C domain can be completely bypassed by providing Mek1 with an alternative means to dimerize such as Gst. Changes in amino acids in Gst-Mek1 that are predicted to disrupt Gst dimerization abolish suppression of *hop1-K593A*, further supporting the idea that the function of the C domain is to mediate Mek1 dimerization.

What role does Mek1 dimerization play in creating the BSCR? One possibility is that dimerization allows two Mek1 proteins to phosphorylate each other at threonine 327, a conserved residue in the activation loop whose phosphorylation is required for kinase activation (Wan *et al.*, 2004; Figure 8). In this model, the absence of a functional C domain prevents kinase activation in the *hop1-K593A* mutant, thereby preventing phosphorylation of Mek1 target proteins to create the BSCR. Ectopically dimerizing Mek1 would allow kinase activation and formation of the barrier. Gst-Mek1 exhibits higher levels of kinase activity in *hop1-K593A* mutants compared with *hop1*Δ (H. Niu and N. M. Hollingsworth, unpublished results), indicating that dimerization is not sufficient to activate the kinase but that it must also be localized to

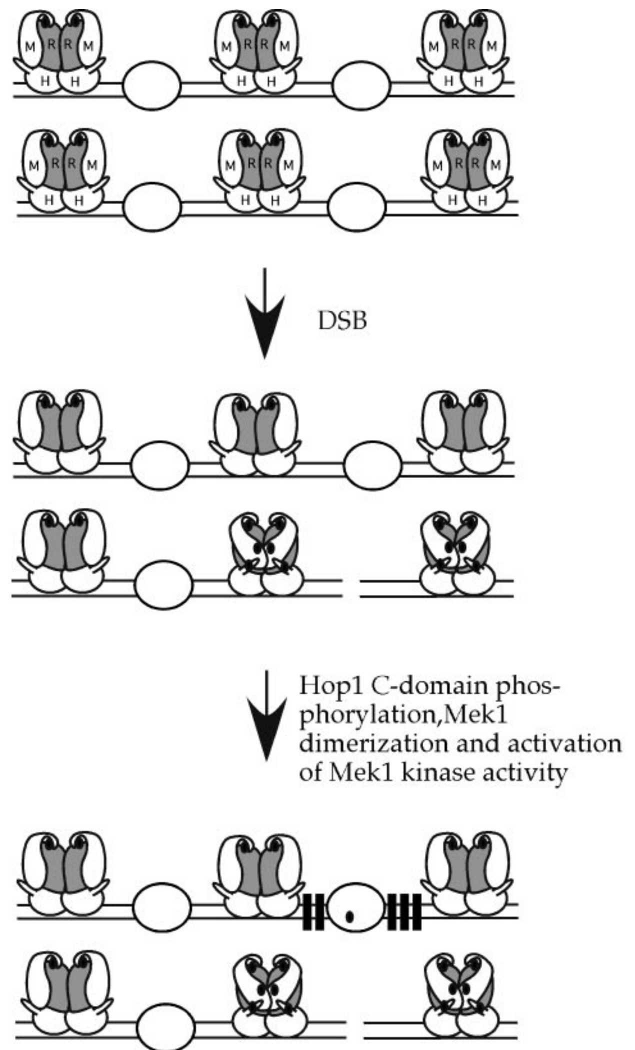

Figure 8. Model coordinating DSB formation, Mek1 dimerization and the creation of a BSCR. Hop1 (H), Red1 (R), and Mek1 (M) complexes are bound to chromosomes in regions where DSBs are likely to form. A single pair of sister chromatids is shown. Hop1 binding to DNA is mediated by the N domain. Hop1 binds to Red1 and Mek1 binds to phosphorylated Red1 via its FHA domain. Phosphate groups are indicated by black ovals. Large white ovals indicate target proteins for the Mek1 kinase that, when phosphorylated, prevent strand invasion. In this model, introduction of a DSB results in phosphorylation of Hop1 molecules adjacent to the DSB. Phosphorylation of the Hop1 C domain triggers dimerization of Mek1. Dimers of Mek1 become activated by auto-phosphorylation of threonine 327 in *trans*. Activated Mek1 then phosphorylates target proteins that act to prevent strand invasion on the sister chromatid. The BSCR is indicated by black rectangles.

chromosomes. A similar conclusion was drawn from the fact that Gst-Mek1 kinase activity is reduced by mutants in *red1* and the Mek1 FHA domain (Wan *et al.*, 2004). Experiments to test the requirement of the C domain for Mek1 activation require a kinase assay that uses a form of Mek1 that is not ectopically dimerized. Thus far efforts to develop a reproducible kinase assay using a tagged, nondimerized form of Mek1 have been unsuccessful. A second possible role for Mek1 dimerization may be to facilitate binding to target proteins. In this model, Mek1 is active even in the undimerized state, but is unable to interact with its substrates in the *hop1-K593A* mutant.

Hop1 is a phosphoprotein whose phosphorylation is dependent on both DSB formation and the presence of the C domain, but is independent of *MEK1*. Deletion of the C domain produces an unphosphorylated, truncated Hop1 protein that is still capable of being suppressed by *GST-MEK1*. Therefore, providing an alternative means for Mek1 to dimerize bypasses not only the requirement for the C domain but also the requirement for Hop1 phosphorylation. It may be that phosphorylation of Hop1 has no functional role in meiosis, an idea that seems unlikely given its DSB dependence. Alternatively, Hop1 phosphorylation could be directly tied to C domain function. Our model proposes that phosphorylation of the Hop1 C domain in response to DSBs triggers Hop1 to promote dimerization of Mek1, which in turn allows kinase activation or binding to target proteins (Figure 8). In this way, barriers to sister chromatid repair can be regulated to occur only after a DSB has occurred on one of the two sisters. Furthermore, by controlling the extent of Hop1 phosphorylation, for example, by modifying only those Hop1 molecules adjacent to DSBs, the BSCR could be localized to the part of the sister chromatid opposite a DSB. Such local control would reduce the risk of overly inhibiting strand invasion, which in excess could inhibit DSB repair even between homologues.

An important question is whether the mechanism for interhomologue bias proposed here for budding yeast is evolutionarily conserved. Comparison of *hop1* mutants in other organisms suggests the answer is yes. In plants, nematodes, and fission yeast, meiotic mutants have been found in genes that encode chromosome core components analogous to Hop1 (Zetka *et al.*, 1999; Caryl *et al.*, 2000; Lorenz *et al.*, 2004). These proteins, HIM-3, ASY1, and SpHop1, respectively, all contain a HORMA domain but lack the C domain of Hop1. In addition, the CT46/HORMAD1 gene from humans encodes a HORMA domain protein that is preferentially expressed in the testis and may represent a mammalian ortholog of Hop1 (Chen *et al.*, 2005). In *him-3* worms, homologues are unsynapsed and fail to undergo interhomologue recombination, yet Rad51 foci, which are presumed to mark the sites of recombination intermediates, disappear with kinetics similar to wild-type. This observation led the authors to propose that "HIM-3's presence at chromosome axes inhibits the use of sister chromatids as templates for repair" (Couteau *et al.*, 2004). Fission yeast *hop1* mutants exhibit an increase in meiotic sister chromatid recombination, consistent with a role for Hop1 in creating a BSCR in this organism (V. Latypov and J. Kohli, personal communication). Mek1 and Red1 orthologues have been described in *S. pombe*, but not in any non-yeast species (Lorenz *et al.*, 2004). Therefore, although Hop1 may have a conserved role in the formation of a BSCR during meiosis, whether it regulates a kinase to generate a barrier in these organisms is not yet known.

In summary, this work suggests a specific molecular pathway by which the single-stranded ends generated by DSBs may be prevented from invading sister chromatids during meiosis, thereby ensuring that crossovers occur between homologues. A number of important questions remain to be answered. For example, is Mek1 activated only in regions adjacent to a DSB, thereby preventing strand invasion in part of the sister opposite the break, or is there a global effect on recombination? What is the kinase that phosphorylates Hop1 and is this phosphorylation biologically relevant? Is Mek1 dimerization needed for kinase activation or for substrate recognition? Finally, what is the target of Mek1 phosphorylation and how does its phosphorylation prevent strand invasion? It has recently been shown that the meiotic cohesin, Rec8, may be involved in preventing sister chromatid repair during meiosis (Zierhut *et al.*, 2004). Is Rec8 a target of Mek1 or do Rec8 and Mek1 act independently to suppress recombination between sister chromatids? Having a molecular model for how the BSCR is generated will greatly facilitate finding the answers to these interesting questions.

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