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Mus81/Mms4 endonuclease and Sgs1 helicase collaborate to ensure proper recombination intermediate metabolism during meiosis

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SUMMARY

Budding yeast lacking the Sgs1 helicase and the Mus81/Mms4 endonuclease are inviable, and indirect studies implicate homologous recombination gone awry as the cause of death. We show that mutants lacking both enzymes have profound defects in meiotic recombination intermediate metabolism and crossover (CO) formation. Recombination intermediates (joint molecules; JMs) accumulate in these cells, many with structures that are infrequent in wild type cells. These JMs persist, preventing nuclear division. Using an inducible expression system, we restored Mus81 or Sgs1 to *sgs1 mus81* cells at a time when JMs are forming. Mus81 expression did not prevent JM formation, but restored JM resolution, CO formation, and nuclear division. In contrast, Sgs1 expression reduced the extent of JM accumulation. These results indicate that Sgs1 and Mus81/Mms4 collaborate to direct meiotic recombination towards interhomolog interactions that promote proper chromosome segregation, and also indicate that Mus81/Mms4 promotes JM resolution *in vivo*.

INTRODUCTION

Meiosis is the process by which diploid cells produce haploid gametes. At the first meiotic division, homologous parental chromosomes (homologs) segregate, while at the second meiotic division sister chromatids segregate. Interhomolog recombination helps to ensure proper homolog alignment at meiosis I metaphase and thereby proper segregation at the first division (reviewed in Cromie and Smith, 2007). Recombination is initiated by DNA double strand breaks (DSBs) that are formed by the Spo11 nuclease (Keeney et al., 1997). Repair of these DSBs produces heteroduplex DNA-containing recombinant products: crossovers (COs), with flanking marker exchange; and non-crossovers (NCOs), with flanking markers in parental configuration.

Joint molecules (JMs) that contain DNA molecules linked by Holliday junctions are a central intermediate in CO formation. In wild-type budding yeast meiosis, most JMs contain two Holliday junctions (Schwacha and Kleckner, 1995) and are called double-Holliday junction (dHJ) intermediates. The proteins that resolve JMs as COs are not yet known. Cells lacking the mid-meiosis transcription factor Ndt80 or the polo-like kinase Cdc5 show JM accumulation, reduced COs and normal NCO levels, indicating that meiotic JM-resolution,

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with COs as the primary product is regulated by these proteins (Allers and Lichten, 2001b; Clyne et al., 2003). A second set of proteins, the ZMM proteins, are required for normal levels of both JMs and CO products, but not for NCO recombination ([reviewed in Lynn et al., 2007). Some ZMM proteins have biochemical activities that could stabilize nascent JMs, and all are associated with the synaptonemal complex, a structure that assembles between fully co-aligned homologous chromosomes during meiotic prophase.

One important ZMM protein function is to protect early pre-CO intermediates from Sgs1 (Jessop et al., 2006; Oh et al., 2007). Sgs1 is a RecQ-family DNA helicase, a homolog of the mammalian BLM helicase, and both are found in complex with a heterodimer of topoisomerase III (Top3) and Rmi1 (BLAP75 in mammals; Chang et al., 2005; Mullen et al., 2005; Yin et al., 2005). Loss of BLM activity in Bloom's syndrome results in cancer predisposition and a marked increase in sister chromatid exchange (reviewed in Mankouri and Hickson, 2007). It has been suggested that the BLM complex suppresses exchange by disassembling dHJs, and *in vitro* studies have shown that reconstituted BLM/TopIIIα/Rmi1 can resolve synthetic dHJs to NCOs (Mankouri and Hickson, 2007). Loss of Sgs1 in *S. cerevisiae* also causes increased chromosome instability, elevated recombination, and DNA-damage sensitivity, suggesting a role for the Sgs1/Top3/Rmi1 complex in regulating mitotic recombination (Gangloff et al., 1994; Mullen et al., 2000; Watt et al., 1996). During meiosis, *sgs1* mutants show a modest increase in COs and transient accumulation of inter-homolog, inter-sister, and multichromatid JMs (JMs with more than two chromatids), but NCO levels are unaltered (Jessop et al., 2006; Oh et al., 2007; Rockmill et al., 2003). Loss of Sgs1 partially restores COs to *zmm* mutants, indicating that the Sgs1 complex disassembles early CO-specific intermediates that are normally stabilized by the ZMM proteins (Jessop et al., 2006; Oh et al., 2007).

Mus81 is an XPF-family nuclease, and functions in complex with a non-catalytic partner, Mms4 (Eme1 in *S. pombe* and mammals; Kaliraman et al., 2001). Purified Mus81/Mms4 and Mus81/Eme1 resolve HJs *in vitro*, although they are more active on nicked HJs (Gaskell et al., 2007 and references therein). During vegetative growth, *sgs1Δ* is synthetically lethal with *mus81Δ* or *mms4Δ*, and this lethality is suppressed by loss of the RecA homolog Rad51 (Bastin-Shanower et al., 2003; Fabre et al., 2002). This suggests that Sgs1 and Mus81 complexes act in alternate processes that keep lethal recombination intermediates from accumulating, either by preventing their formation or by catalyzing their resolution. During *S. pombe* meiosis, CO recombinant recovery is largely dependent upon Mus81/Eme1 (Smith et al., 2003), and *mus81* cells fail to undergo meiotic divisions and accumulate JMs with single HJs (Cromie et al., 2006). In *S. cerevisiae*, meiotic COs are much less dependent upon Mus81/Mms4, and viable progeny from single mutants show only a modest CO reduction (Argueso et al., 2004; de los Santos et al., 2003; de los Santos et al., 2001). Thus, uncertainty remains regarding the *in vivo* activity and targets of Mus81/Mms4 during budding yeast meiosis.

To learn more about the roles that Sgs1 and Mus81/Mms4 play during meiotic recombination, we examined the meiotic defects of double mutants, using meiotic null (*mn*) alleles of *SGS1* and *MMS4* to bypass the vegetative inviability of *sgs1 mus81* and *sgs1 mms4* mutants. We also created inducible (*IN*) alleles of both *SGS1* and *MUS81*, allowing restoration of either protein to *sgs1-mn mus81Δ* double mutants late in meiosis I prophase.

We find that Sgs1 and Mus81 play significant roles in JM formation and disassembly during meiosis. In cells lacking both Sgs1 and Mus81/Mms4, COs are reduced, JMs accumulate and cells are unable to divide nuclei; similar observations are reported by Oh *et al*. (S. D. Oh, J. Lao, A. F. Taylor, G. R. Smith and N. Hunter, personal communication). Inducing expression of *MUS81* in these cells causes JMs to be resolved, while *SGS1* induction prevents further JM accumulation. In both cases, COs and meiotic divisions are partially restored. These findings illuminate the complementary functions of Sgs1 and Mus81/Mms4 in meiotic recombination

intermediate metabolism, and in particular indicate that Mus81/Mms4 can resolve Holliday junctions *in vivo*.

RESULTS

Meiotic nuclear division requires Sgs1 or Mus81/Mms4

We used a meiotic null allele of *SGS1 (sgs1-mn)*, where the normal *SGS1* promoter is replace by the *CLB2* promoter, which is inactive during meiosis. The *sgs1-mn* strain shows normal meiotic nuclear divisions (Jessop et al., 2006; Figure 1A). Using the same method, we constructed *mms4-mnΔ* Single *mus81Δ* or *mms4-mn* mutants show only modest nuclear division defects at 30°C. In contrast, *sgs1-mn mus81Δ* and *sgs1-mn mms4-mn* cells fail to accomplish even a single nuclear division by 8 hours, the time at which nuclear divisions are complete in wild type and single mutants (Figure 1A and Supplementary Figure 1A).

During meiosis, *sgs1-mn* cells lack the entire Sgs1 protein (Jessop et al., 2006). Many *sgs1Δ* phenotypes are moderated by expressing *sgs1-ΔC795*, a mutant that produces the N-terminal Top3/Rmi1-interaction domain of Sgs1 but not the C-terminal helicase and Holliday junction-binding domains (Mullen et al., 2000). However, expressing the N-terminal domain in *sgs1-mn/sgs1-ΔC795 mms4-mn* cells suppresses neither the nuclear division defect nor any of the other defects described below (Supplementary Figure 1), suggesting that the absence of Sgs1 helicase and/or Holliday junction-binding activities are the main cause of these meiotic defects.

sgs1-mn mus81Δ cells fail to divide, but do not arrest in pachytene

If cells fail to repair DSBs or complete meiotic recombination, they arrest at the pachytene stage of meiosis I prophase, a stage that in wild-type is characterized by intact synaptonemal complex (SC), unseparated spindle pole bodies, and unresolved JMs (Roeder and Bailis, 2000). This arrest is due to a failure to activate Ndt80, a transcription factor required for exit from pachytene (Chu and Herskowitz, 1998; Xu et al., 1995). The nuclear division failure of *sgs1-mn mus81Δ* and *sgs1-mn mms4-mn* cells is not due to a DSB repair defect, as DSB formation and disappearance are similar in wild-type, single, and double mutants (Figure 1B, Supplementary Figure 1B). In addition, *sgs1-mn mus81Δ* cells accumulate and degrade the SC component Zip1 with normal kinetics (Figure 1C). Thus, the nuclear division defect in *sgs1-mn mus81Δ* or *sgs1-mn mms4-mn* mutants does not reflect a pachytene arrest. Consistent with this conclusion, *sgs1-mn mus81Δ* cells form normal meiotic spindles. Immunocytology, using anti-beta tubulin sera, showed that linear meiosis I spindles form to about the same extent and with similar timing in wild type and *sgs1-mn mus81Δ* (Figure 1D). Meiosis II spindles can be seen at later time points, and spore walls form in some cells, though the spores do not contain DNA (data not shown). In contrast, pachytene-arrested *ndt80*. cells fail to form meiotic spindles, and contain beta tubulin as a single focus in all cells [(Xu et al., 1995), Figure 1D].

Unresolved recombination intermediates accumulate in *sgs1-mn mus81Δ*

Meiotic nuclear division might fail in *sgs1-mn mus81Δ* cells if chromosomes are held together by unresolved recombination intermediates. To test this suggestion, we examined meiotic recombination at the molecular level, using a 3.5 kb recombination reporter insert that contains divergently transcribed *URA3* and *ARG4* genes, along with a 65 nt telomere-repeat insert that creates a single strong DSB hotspot (Jessop et al., 2005; White et al., 1993). This reporter is inserted at *HIS4* on one parental chromosome *III* and at *LEU2* on the other, and one insert contains a mismatch correction-resistant palindromic mutation. Different restriction enzyme and probe combinations allow detection of DSBs, CO and NCO recombinants, and both interhomolog and intersister recombination intermediates on Southern blots (Jessop et al., 2005, Figure 2A, Supplementary Figure 2).

As described above, DSB repair is completed in all mutants examined (Fig 1B, Supplementary Figure 1B), and NCO recombinants form at levels similar to or slightly greater than those seen in wild-type (Fig 1E, Supplementary Figure 1D). In *sgs1-mn*, *mus81Δ* and *mms4-mn* single mutants, CO recombinants form with near wild-type levels and timing. In contrast, COs are present in *sgs1-mn mus81Δ* and *sgs1-mn mms4-mn* at about 45% of wild type levels (Fig 1E and Supplementary Figure 1D), indicating that Mus81/Mms4 and Sgs1 are redundantly required for normal CO formation.

To determine why COs are reduced, we examined JM formation and resolution. Single *sgs1-mn*, *mus81Δ* or *mms4-mn* mutants formed and resolved JMs. In contrast, unresolved JMs persisted in *sgs1-mn mus81Δ* and *sgs1-mn mms4-mn* (Figure 2B, 2C and Supplementary Figure 1C), reaching a peak at ~6hr before slightly declining as cells attempted nuclear divisions. In *sgs1-mn mus81Δ*, the maximum JM level (6.5% of chromosomes) is about 4-fold greater than the transient maximum seen in wild type (1.5%) and about 80% of the cumulative level in *ndt80Δ*. (8%; Figure 2B and data not shown). A similar JM accumulation occurs in *sgs1-mn mms4-mn* at *URA3-ARG4* (Supplementary Figure 1C), and is also observed at an independent recombination reporter locus (S. Oh, J. Lao, A. F. Taylor, G. R. Smith and N. Hunter, personal communication). JMs also accumulate in *sgs1-mn sgs1-ΔC795 mms4-mn* (Supplementary Figure 1C), showing that Sgs1 helicase and/or HJ-binding domains are needed to prevent JM accumulation. JMs also accumulate in *sgs1-mn mus81Δ* at a native DSB hotspot, (*YGR175C*, Supplementary Figure 3a), and do not accumulate in DSB-defective *sgs1-mn mus81Δ spo11-Y135F* cells (Supplementary Figure 3B and C). Thus, the JMs that accumulate in the absence of Sgs1 and Mus81/Mms4 are products of Spo11-initiated meiotic recombination, as opposed to recombination initiated by other processes (such as pre-meiotic replication).

While interhomolog JMs normally predominate in wild-type, Oh et al. have shown that *sgs1-ΔC795* mutants accumulate substantial amounts of intersister and multichromatid JMs, which are subsequently resolved in an Ndt80-dependent manner (Oh et al., 2007). We observe a similar increase in intersister and multichromatid JMs among the intermediates that form transiently in *sgs1-mn* and that accumulate in *sgs1-mn mus81Δ* and *sgs1-mn mms4-mn* (Figure 2 and Supplementary Figure 1C; similar observations by S. D. Oh, J. Lao, A. F. Taylor, G. R. Smith and N. Hunter, personal communication). While *ndt80Δ* mutants show an interhomolog/intersister JM ratio of ~7.5, consistent with the ratio seen in wild-type (Oh et al., 2007; Schwacha and Kleckner, 1994), intersister JMs are formed at greater levels in *sgs1-mn mus81Δ*, (interhomolog/intersister JM ratio of ~1.7; Figure 2D and Supplementary Figure 4B; similar differences also reported in Oh et al., 2007). In addition, a substantial fraction of the JMs that form transiently in *sgs1-mn* and that accumulate in *sgs1-mn mus81Δ* cells contain 3 and 4 parental-length molecules, identified on the basis of electrophoretic migration and hybridization patterns with *HIS4*- and *LEU2*-specific probes [Figure 2C and D, Supplementary Figure 4B, (Oh et al., 2007) and data not shown]. These multichromatid JMs do form in wild-type, but are considerably more frequent in *sgs1-mn* and *sgs1-mn mus81Δ*. 25–30% of JMs formed in *sgs1-mn* and *sgs1-mn mus81Δ* are multi-chromatid, as opposed to 9–10% of JMs formed in wild-type and *ndt80*. [Figure 2c and data not shown; similar differences reported by (Oh et al., 2007)].

In summary, cells lacking both the Sgs1 helicase and the Mus81/Mms4 endonuclease form NCOs at near-normal levels, COs at substantially reduced levels, and accumulate unresolved JMs, even though these cells continue to progress through other stages of meiosis. The JMs that form contain reduced levels of interhomolog JMs and elevated levels of intersister and multichromatid JMs relative to wild-type.

Restoration of Mus81 to *sgs1 mus81* mutants causes JM resolution

The persistence of unresolved JMs in *sgs1-mn mus81Δ* and *sgs1-mn mms4-mn*, and not in *sgs1-mn* alone, implies that Mus81/Mms4 can disassemble these structures *in vivo*. To test this, we constructed strains where *MUS81* expression is rapidly induced by the addition of estradiol (*MUS81-IN*). While meiotic divisions are completely blocked in *sgs1-mn mus81Δ*, basal *MUS81* expression in *sgs1-mn mus81Δ/MUS81-IN* (~25% of wild-type) allows some cells to complete meiotic divisions (12%; Figure 3C). When *MUS81* expression is induced at 4 hours (to at least 4 times wild type, figure 3A), many cells (64%) complete at least one meiotic nuclear division (Figure 3C). This restoration of nuclear division is associated with increased and accelerated JM resolution. In the absence of induced *MUS81* expression, about 60% of accumulated JMs are resolved; when *MUS81* expression is induced at 4 hours, almost all JMs disappear (Figure 3B). JMs are not resolved when *MUS81* expression is induced in *sgs1-mn mms4-mn mus81Δ/MUS81-IN* (data not shown), indicating that JM resolution in *sgs1-mn mus81Δ/MUS81-IN* cells is due to Mus81/Mms4 endonuclease activity, and not due to Mus81 acting in the absence of Mms4.

Mus81 induction has no effect on NCO levels, indicating that Mus81/Mms4 does not resolve inter-homolog JMs to NCOs (Figure 3D). In contrast, *MUS81* expression causes a 1.4-fold increase in COs, which is approximately that expected from the increased JM resolution that occurs. At the end of meiosis, 5.5% of chromosomes are present in JMs in *sgs1-mn mus81Δ*, in contrast to only 0.6% when *MUS81* is induced (Figure 3B). Based on the ratio of interhomolog JMs to intersister JMs in *sgs1-mn mus81Δ* (Figure 2D), we estimate about 60% of JMs, or about 3.3% of chromosomes, could potentially be resolved to form inter-homolog COs. This agrees well with the increase in COs (3% of chromosomes) observed upon *MUS81* induction. While the final level of COs (5.7%) is less than in wild type (8%), these results indicate that Mus81/Mms4 can resolve most of the JMs that are present in *sgs1-mn mus81Δ* mutants, with COs being the predominant product when interhomolog JMs are resolved.

Induction of Sgs1 prevents JM accumulation

Although *in vitro* studies show that human BLM/TopIIIα/Rmi1 resolves synthetic dHJs to NCOs (Wu et al., 2006), studies of Sgs1 meiotic function have not revealed a role in NCO formation (Jessop et al., 2006). To further address this issue, we constructed a strain where *SGS1* expression was estradiol-inducible (*SGS1-IN*; Experimental Procedures). When estradiol is absent, Sgs1 cannot be detected (Figure 4A), less than 5% of cells divide nuclei (Figure 4C), and JMs accumulate to levels (5.5% at 8 hr) similar to those seen in *sgs1-mn mus81Δ* (7%; Figure 4B). When *SGS1* expression is induced at 4 hours (4-fold greater than the endogenous level; figure 4A), 40% of cells complete at least one division, (Figure 4C) and JM accumulation is prevented (figure 4B). Maximum JM levels upon *SGS1* induction at 4 hours (1.4%) are only slightly greater than the maximum in wild type (1.5%), and are substantially less than the maximum seen without *SGS1* induction (5.5%). Although *SGS1* expression in *sgs1-mn mus81Δ* cells led to substantially fewer JMs accumulating, it did not cause extensive NCO or CO production, with modest increases in both products upon *SGS1* induction (induced vs. uninduced: NCOs, 2.5% vs. 1.9%; COs, 4.7% vs. 2.9%). These reduced JM levels suggest that Sgs1, either alone or in complex with Top3 and Rmi1, prevents JMs from forming or promotes their rapid disassembly *in vivo*. If the latter occurs, it does not appear to produce exclusively NCOs or COs.

JMs in *sgs1-mn mus81Δ* can contain either even or odd numbers of Holliday junctions

During meiosis, *S. pombe mus81*$^{-}$ mutants accumulate unresolved JMs containing single HJs (Cromie et al., 2006). We used two-dimensional native/denaturing electrophoresis (Allers and Lichten, 2001b) to ask if this occurs in *S. cerevisiae sgs1-mn mus81Δ* mutants (Supplementary

Figure 4A). In this assay, interhomolog JMs with an even number of HJs will produce only parental-length single strands, while interhomolog JMs with an odd number of HJs will produce parental- and crossover-length strands in equal amounts. Interhomolog JMs from *ndt80Δ* cells contain primarily parental length strands, but ~13% of component strands are CO length. CO-length strands are present at similar levels (15%) in JMs from *sgs1-mn mus81Δ* cells, suggesting that approximately ¼ of JMs in these mutants contain single HJs. Consistent with this suggestion, four-arm JMs with junction structures expected for single HJ intermediates are frequently observed in electron micrographs of meiotic DNA from *sgs1-mn mms4-mn* mutant cells (S. D. Oh, J. Lao, A. F. Taylor, G. R. Smith and N. Hunter, personal communication). Because *MUS81* expression results in the resolution of more than 90% of the JMs that form in *sgs1-mn mus81Δ*, these results indicate that Mus81/Mms4 resolves HJs in intermediates that contain either even or odd numbers of HJs.

DISCUSSION

Sgs1 and Mus81/Mms4 act late in meiotic recombination

In this paper, we show that the mitotic lethality seen in cells lacking Sgs1 and Mus81 or Mms4 (hereafter referred to as *sgs1 mus81/mms4* mutants) also occurs in meiosis. During meiosis, *sgs1 mus81/mms4* mutants accumulate unresolved JMs and fail to divide nuclei, although they form MI and MII spindles on schedule and even form spore walls; similar observations have been made by S. D. Oh, J. Lao, A. F. Taylor, G. R. Smith and N. Hunter (personal communication). *sgs1 mus81/mms4* mutants do not arrest at a meiotic checkpoint prior to JM resolution and nuclear division. Instead, as in *mus81* or *eme1* single mutants of *S. pombe*, the failure to undergo meiotic nuclear division appears to be to the result of persistent inter-homolog links, caused by unresolved recombination intermediates (Boddy et al., 2001; Cromie et al., 2006). This mutant phenotype is suppressed to a substantial extent by restoration of Mus81/Mms4 at a time after JM formation, and to a lesser extent by restoration of Sgs1. As will be discussed below, these results are consistent with the suggestion of distinct roles for the Sgs1/Top3/Rmi1 complex (hereafter called the Sgs1 complex) and for Mus81/Mms4 in preventing the accumulation of unresolved (and thus lethal) recombination intermediates.

During the mitotic cell cycle, *sgs1 mus81* mutants die with a G2 DNA content, consistent with a nuclear division failure caused by unresolved JMs (Ii et al., 2007). This lethality is suppressed by preventing homologous recombination, a finding that is generally taken to indicate that Sgs1 and Mus81/Mms4 act after the initial steps of strand invasion. However, it remains possible that they act earlier, to prevent formation of lesions or abnormal structures that are then processed by homologous recombination to create lethal intermediates. We show here that, in *sgs1 mus81*, meiotic JM accumulation/persistence and blocked nuclear division both require Spo11 activity. This indicates that the primary lethal defect in the *sgs1 mus81/mms4* mutants is a failure to eliminate JMs formed by Spo11-initiated meiotic recombination. It remains possible, and even likely, that recombinogenic lesions form in *sgs1 mus81/mms4* during premeiotic S phase, as they do during mitosis (Ii et al., 2007). However, these lesions must be sufficiently rare to allow nuclear division in the majority of cells and to escape detection on Southern blots in the absence of meiotic recombination initiation. In summary, our data indicate that Sgs1 and Mus81/Mms4 have critical meiotic functions that involve action during late steps in recombination.

Mus81/Mms4 promotes joint molecule resolution

The vast majority of JMs formed during wild-type meiosis contain two DNA molecules, with contributions from both homologs (80–90%) rather than from sister chromatids (Oh et al., 2007; Schwacha and Kleckner, 1994). The JMs that we observe in *mus81* single mutants are similar in composition to those formed in wild type and are efficiently resolved. Both COs and

NCOs are produced at near normal levels, and the majority of cells progress through meiotic divisions. A previous study reported altered JM metabolism and reduced COs in *mms4Δ* mutants (de los Santos et al., 2003). The basis for these differences remains to be determined, but it may reflect different sporulation conditions and auxotrophic strain requirements used in the two studies (T. Goldfarb and ML, unpublished data). Nevertheless, our data clearly show that, under the appropriate conditions, *mus81Δ* cells can produce a relatively normal spectrum of recombination intermediates and products.

By contrast, in cells lacking Sgs1, JMs accumulate during meiosis to substantially greater levels than are seen in wild type, and intersister and multichromatid JMs are prominent (Oh et al., 2007). All JMs are eventually resolved, and recombinants are recovered at normal levels (Jessop et al., 2006; Oh et al., 2007). In the current work, we show that *sgs1 mus81/mms4* mutants persistently accumulate a similar JM spectrum as is seen transiently in *sgs1*, and also display normal NCO but reduced CO levels. Similar findings are also reported by S. D. Oh, J. Lao, A. F. Taylor, G. R. Smith and N. Hunter (personal communication). Our finding of JM persistence in *sgs1 mus81/mms4* mutants, and their ultimate resolution upon *MUS81* expression in *sgs1-mn mus81Δ/MUS81-IN*, indicates that the Mus81/Mms4 endonuclease can resolve HJs that form in the absence of Sgs1.

While there is considerable biochemical and indirect genetic evidence suggesting that Mus81/Mms4 (Mus81/Eme1) can resolve HJs (Gaskell et al., 2007 and references within), direct *in vivo* evidence has not been obtained. Our findings are most readily interpreted as showing that Mus81/Mms4 has such an activity. It is of interest to note that about one-quarter to one-third of the interhomolog JMs that accumulate in *sgs1-mn mus81Δ* and in *ndt80Δ* mutants have the strand composition expected for a JM with a single HJ (Supplementary Figure 4; (Allers and Lichten, 2001b). Single-HJ JMs may be primary products of DSB repair (Cromie et al., 2006), or they may result when one of the two HJs in a dHJ intermediate is resolved. The near-complete JM resolution seen upon *MUS81* expression in *sgs1-mn mus81Δ* cells indicates that Mus81/Mms4 can catalyze JM resolution and CO production, regardless of the number of HJs that JMs contain.

While we favor the suggestion that Mus81/Mms4 resolves HJs, other explanations are possible. For example, *MUS81* induction could lead to the degradation of JM-containing structures, rather than their resolution. We believe this to be unlikely for two reasons. First, no increase in DNA molecules of lessthan-parental length is detected upon *MUS81* induction (data not shown). Second, the increase in CO recombinants upon JM disappearance is that expected if most inter-homolog JMs were resolved as COs.

It should be kept in mind that there is at least one other HJ resolving activity in budding yeast meiosis, as JMs are resolved and COs are produced at wild-type levels in *mus81Δ* cells, and *sgs1-mn mus81Δ* cells produce CO recombinants at 45% of the level seen in wild-type. The decline in JM levels seen late in meiosis in *sgs1-mn mus81Δ* cells may also reflect the presence of other HJ-resolving activities. JMs that persist in *sgs1 mus81/mms4* cells may form outside of the normal chromosome context in which other resolvases act, or they may contain structures that are poorly resolved by these enzymes. The same may be true in *S. pombe*, where loss of Mus81/Eme1 causes a 75% CO reduction, the persistence of JMs with single HJs, and blocked nuclear division (Cromie et al., 2006 and references within). While Mus81 nuclease may be more important for JM resolution in fission yeast than in budding yeast, other HJ-resolving proteins may be active in *S. pombe*, and it remains possible that some of the JMs formed in this organism contain two HJs.

Sgs1 prevents JM accumulation

In vitro studies have shown that BLM/TopIIIα/Rmi1 disassemble synthetic dHJs to form NCO products. However, it appears that this is not the primary activity of the Sgs1 complex during meiosis. *sgs1* mutants show a transient JM hyper-accumulation (Oh et al., 2007), which is not accompanied by a decrease or delay in NCO recombinant formation (Jessop et al., 2006). Instead, *sgs1* mutants show altered meiotic CO distributions and partially restore JMs, COs and tight homolog association to mutants lacking ZMM proteins (Jessop et al., 2006; Oh et al., 2007; Rockmill et al., 2003), again without altering NCO levels. This suggests that the primary meiotic function of Sgs1 is to prevent unregulated JM assembly, and that CO-designated intermediates are protected from this activity by SC components (Jessop et al., 2006; Oh et al., 2007).

We show here that *SGS1* expression in *sgs1-mn mus81Δ* mutants substantially reduces JM accumulation, causing only modest increases in both NCOs and COs. This indicates that the Sgs1 complex prevents JM accumulation, perhaps by disassembling early strand invasion intermediates that are precursors to the stable JMs detected here; alternatively Sgs1 might disassemble JMs after they form. Our current data do not distinguish unambiguously between these two possibilities. However, when *SGS1* is expressed late in meiosis I prophase in an *sgs1 ndt80* mutant, JMs remain unresolved (L.J. and M.L., unpublished data), consistent with Sgs1 acting primarily to prevent JM formation.

These results are consistent with the suggestion that, by eliminating precursors to inter-sister and multi-chromatid JMs, Sgs1 redirects recombination events towards a second round of interhomolog recombination. Alternatively, Sgs1 may drive pre-existing JMs into conformations that can be resolved by other structure-specific endonucleases, such as Slx1/Slx4 or Rad1/Rad10, perhaps in a way that is not biased towards CO formation. It is also possible that Sgs1 drives some nascent recombination intermediates away from JM/CO formation and towards NCO formation, but in such a way that heteroduplex tracts in products are less than 600 nt long. These would not be detected as NCO recombinants in our assay, as they would not include the palindromic marker used to score NCO recombinants (Jessop et al., 2005). Finally, it should be noted that Sgs1 is overproduced 4-fold in our induction experiments. Because the Top3/Rmi1 heterodimer is relatively stable in the absence of Sgs1 (Chang et al., 2005; Mullen et al., 2005), it is likely that at least some of the re-expressed Sgs1 protein reconstitutes the normal Sgs1/Top3/Rmi1 complex, but it is also likely substantial Sgs1 is present without its usual partners. Whether the JM reduction we see is due to Sgs1/Top3/Rmi1 or due to free Sgs1 remains to be determined.

Role of Sgs1 and Mus81/Mms4 in regulating meiotic recombination

Conventional pathway analysis of data from various loss of function mutants has led to a simply-branched unidirectional pathway model for meiotic recombination (reviewed by Cromie and Smith, 2007). In this model, most COs form via a spatially and temporally regulated process, promoted by SC-associated proteins. This process produces single-end invasion and dHJ JMs that are, in turn, resolved in an Ndt80-, Cdc5-regulated manner. The remaining COs, hypothesized to be “unregulated”, are suggested as forming via a ZMM-independent, Mus81/Mms4-dependent process that is otherwise poorly defined. Features of our data and of other recent findings are difficult to reconcile with this view. First, the ZMM-dependence of interhomolog JM and CO formation is partially relieved by loss of Sgs1, which indicates that the Sgs1 complex has a meiotic anti-crossover activity (Jessop et al., 2006; Oh et al., 2007). Second, loss of Mus81/Mms4 has a relatively modest impact on interhomolog JMs and COs, but the further loss of Sgs1 causes reduced COs and the accumulation of unresolved JMs (Figure 1 and Figure 2; S. Oh, J. Lao, A. F. Taylor, G. R. Smith and N. Hunter, personal

communication). This would indicate that Sgs1 has a meiotic pro-crossover activity, at least in the absence of Mus81/Mms4.

To reconcile this apparent contradiction, we suggest that the initial steps of meiotic DSB repair populate multiple sets of early recombination intermediates, and that these intermediates undergo multiple rounds of disassembly and reassembly. A similar suggestion has recently been put forth to explain mitotic recombination by break-induced replication (Smith et al., 2007) and for meiotic NCO recombination (McMahill et al., 2007). The Sgs1 complex, and in particular its helicase activity, prevents all types of meiotic JM formation by disassembling nascent strand invasion structures before they can be stabilized by second end-capture and ligation (Figure 5; Oh et al., 2007). This, combined with the protection of CO-designated precursors by ZMM proteins (Jessop et al., 2006; Oh et al., 2007), has the net effect of inhibiting the formation of JMs that cannot produce crossovers by simple resolution (hereafter called "off-pathway" JMs), by dynamically redirecting early strand exchange intermediates toward interhomolog, bi-chromatid recombination. We further suggest that "off-pathway" intermediates that escape Sgs1-mediated disassembly in wild-type (see signal in wild-type and *ndt80Δ*, Figure 2), or that form in the absence of Sgs1, are frequently resolved by Mus81/Mms4 in a ZMM-independent manner. Whether or not JM resolution is biased towards CO formation, the net effect would be that a minor fraction of COs would be Mus81/Mms4-dependent and ZMM-independent in cells where Sgs1 is active, as has been inferred in several genetic studies (Argueso et al., 2004; de los Santos et al., 2003; de los Santos et al., 2001). It is also possible that, instead of being resolved to full-length products, JMs are cleaved by Mus81/Mms4 and re-enter the recombination intermediate pool. This is unlikely to be the only way that Mus81/Mms4 redirects recombination intermediates, as CO formation by such a process would be expected to remain ZMM-dependent.

In conclusion, we show here that efficient JM metabolism and CO formation during budding yeast meiosis requires the combined activity of the Sgs1 helicase and the Mus81/Mms4 structure-specific endonuclease. We suggest that the former disassembles "off-pathway" precursors, redirecting them towards processes compatible with ordered homolog pairing and regulated crossing-over, while the latter resolves JMs that are formed by events that escape Sgs1 surveillance, and whose structure or context is incompatible with regulated resolution. It will be of considerable interest to determine whether or not Sgs1 and Mus81/Mms4 perform similar functions during the mitotic cell cycle, and to determine the meiotic phenotypes of mutants in genes that display synthetic mitotic lethality with *sgs1* or with *mus81/mms4* mutants (Mullen et al., 2001; St Onge et al., 2007).

EXPERIMENTAL PROCEDURES

Strains and media

Strains are listed in Supplementary Table 1 and are SK1 derivatives (Kane and Roth, 1974). For MJL2984 and derivatives, the recombination interval used contains a single DSB hotspot (Figure 2; Jessop et al., 2006). For MJL2442 and derivatives, the recombination interval contains two DSB hotspots and is less active than the interval in MJL2984 (Allers and Lichten, 2001b; Jessop et al., 2005).

The *sgs1-mn* (Jessop et al., 2006) and *mms4-mn* alleles were created by replacing the relevant promoter regions (+1 to −89 of *SGS1*, +1 to −90 of *MMS4*) with a kanMX-*CLB2* promoter construct (Lee and Amon, 2003). Estrogen-inducible *SGS1-IN* and *MUS81-IN* alleles were made by replacing the same promoter regions with a *GAL1* promoter fragment from pFA6a-kanMX6-PGAL1-3HA (Longtine et al., 1998) in strains that contained a *GPD1* promoter-driven Gal4-estrogen receptor fusion inserted at *URA3* [*ura3::pGPD1-GAL4 (848).ER::URA3* (Benjamin et al., 2003)]. *SGS1-IN* includes a 3xHA tag at the Sgs1 N-

terminus; *MUS81-IN* does not include the 3xHA tag, as it abrogates *MUS81* function (data not shown). Components used in constructing these strains were a gift from A. Amon.

3xFLAG C-terminal epitope-tagged Mus81 constructs, used to measure protein levels, were constructed using a modified p3FLAG-kanMX, (Gelbart et al., 2001) in which the kanMX6 cassette was replaced by a hygromycin-resistance cassette (hphMX, Goldstein and McCusker, 1999), integrated at the C-termini of the *MUS81* or *MUS81-IN* open reading frames.

Details of strain construction and primer sequences are available upon request.

Liquid media, presporulation and sporulation conditions were as described (Jessop et al., 2006).

Molecular analyses

DNA isolation and Southern blot analysis were performed as described (Allers and Lichten, 2000; Allers and Lichten, 2001a). JMs at *YGR174C* were detected using Southern blots of *Xmn*I-digested DNA, probed with the entire *CPB4* open reading (chromosome *VII* coordinates 845915-846396). In graphs, values plotted with error bars reflect the mean value ± standard error of the mean for multiple, independent experiments.

Western blots

Protein extracts from 2 mL of sporulating culture were prepared by TCA precipitation (Foiani et al., 1994). The equivalent of 10μL of a sporulating culture was displayed on 4–12% polyacrylamide gradient Tris-Glycine mini gels (Invitrogen) and electroblotted to PVDF membrane (Invitrogen) using the XCell II Blot module (Invitrogen) as recommended by the manufacturer. Blots were washed for at least 1 hr in 0.2% I-block (Tropix) in 1X PBS, on an orbital shaker at RT. Primary antibody, diluted in blocking buffer, was added to the blot, and incubated on an orbital shaker at RT for at least 1 hour. Blots were then washed 3 × 10 minutes with blocking buffer, and then incubated with secondary antibody for 1 hour with shaking at RT, and wash steps were repeated. Signal was developed using the chemiluminescent CPD-star substrate (Tropix) and detected using a Fuji LAS3000 CCD camera. All blots were stripped by the 2-mercaptoethanol/SDS method in the Tropix Western-Light (Applied Biosystems) technical manual, and reprobed for Arp7 to normalize for loading. Primary antisera were: Zip1 polyclonal (a gift from G.S. Roeder) at 1:1000; Arp7 polyclonal (Santa Cruz Biotechnology, Inc; sc-8961) at 1:500; anti-HA monoclonal (clone 12CA5, Roche; 11583816001) at 1:10000; FLAG M2 monoclonal (Sigma, F1804) at 1:10000. Secondary antibodies were alkaline phosphatase conjugates of goat-anti-mouse (Sigma, A3562) and goat-anti-rabbit (Sigma, A3687), both used at 1:10000.

Cytology

For microtubule detection, 4 mL aliquots of sporulating cultures were fixed and stained as described (Nasmyth et al., 1990). Anti-Tub2 (a gift from M. Basrai) was used as a primary antibody at a 1:500 dilution in 1% BSA, 1X PBS, and AlexaFluor 488 goat anti-rabbit IgG antisera (Molecular Probes; A11034) was used as a secondary antibody at a 1:100 dilution. Vectashield with DAPI (Vector Laboratories, Inc, H1200) was used to simultaneously stain DNA.

Nuclear divisions were monitored by staining alcohol-fixed whole cells with DAPI (Goyon and Lichten, 1993).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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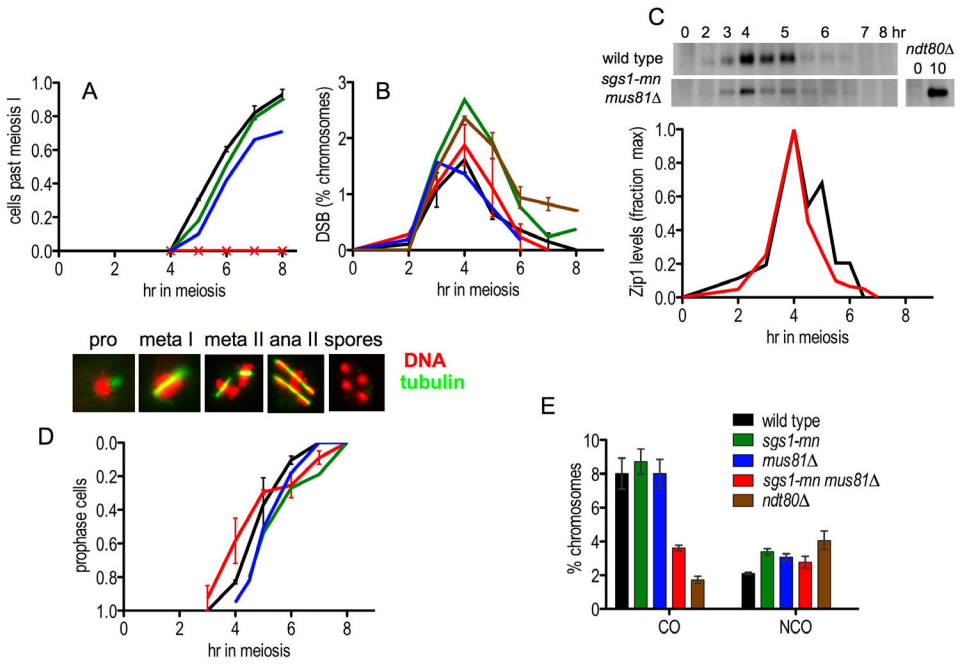

Figure 1. *sgs1-mn mus81Δ* mutants progress through meiosis but fail to undergo nuclear division

A. Meiotic nuclear divisions. The fraction of cells having transited meiosis I are plotted. Cells with 2 or more nuclei (determined by DAPI staining) were scored as having transited meiosis I. Data from 2 independent experiments were used for wild type (MJL2984, black), *sgs1-mn mus81Δ* (MJL3181, blue) and *ndt80* (MJL3164, brown). Data from a single experiment are shown for *sgs1-mn* (MJL3166, green) and *mus81Δ* (MJL3019, blue).

B. DSB formation and repair. DSB frequencies, determined from Southern blots of *Xho*I digested DNA probed with argD (see Supplemental Figure 2), are plotted as a percentage of total lane signal, which reflects the percent of chromosomes III with a DSB in the *URA3-ARG4* insert. Strains and number of experiments analyzed are as in part A.

C. Zip1 protein levels. Western blots were probed with anti-Zip1. Graph--Zip1 protein levels, relative to maximum level, from a single experiment. At 10 hr in pachytene-arrested *ndt80Δ* cells (not plotted), Zip1 levels are about 2-fold greater than peak levels in wild type or *sgs1-mn mus81Δ*.

D. Meiotic spindle formation. Fixed whole cells were stained with anti-Tub2 (tubulin, green) and with DAPI (DNA, red). Images illustrate spindle morphology for (left to right) meiosis I prophase, meiosis I metaphase, meiosis II metaphase, meiosis II anaphase, and having completed sporulation. The meiosis I prophase image is an *ndt80Δ* cell, and illustrates the typical prolonged arrest without spindle formation. The meiosis II anaphase image is from an *sgs1-mn mus81Δ* cell, and illustrates typical nuclear division failure. The fraction of cells remaining in meiosis I prophase (single tubulin focus) is plotted on an inverted y-axis, to illustrate progression through meiosis I.

E. CO and NCO recombinant levels. Recombinants were quantified using Southern blots of *Xho*I/*Eco*RI digested DNA probed with hisU (see Supplemental Figure 2). The average of 7 and 8 hours was calculated from 2 independent experiments for wild type, *sgs1-mn mus81*, and *ndt80Δ* and from a single experiment for *sgs1-mn* and *mus81Δ*.

All plots report mean ± s. e. m.

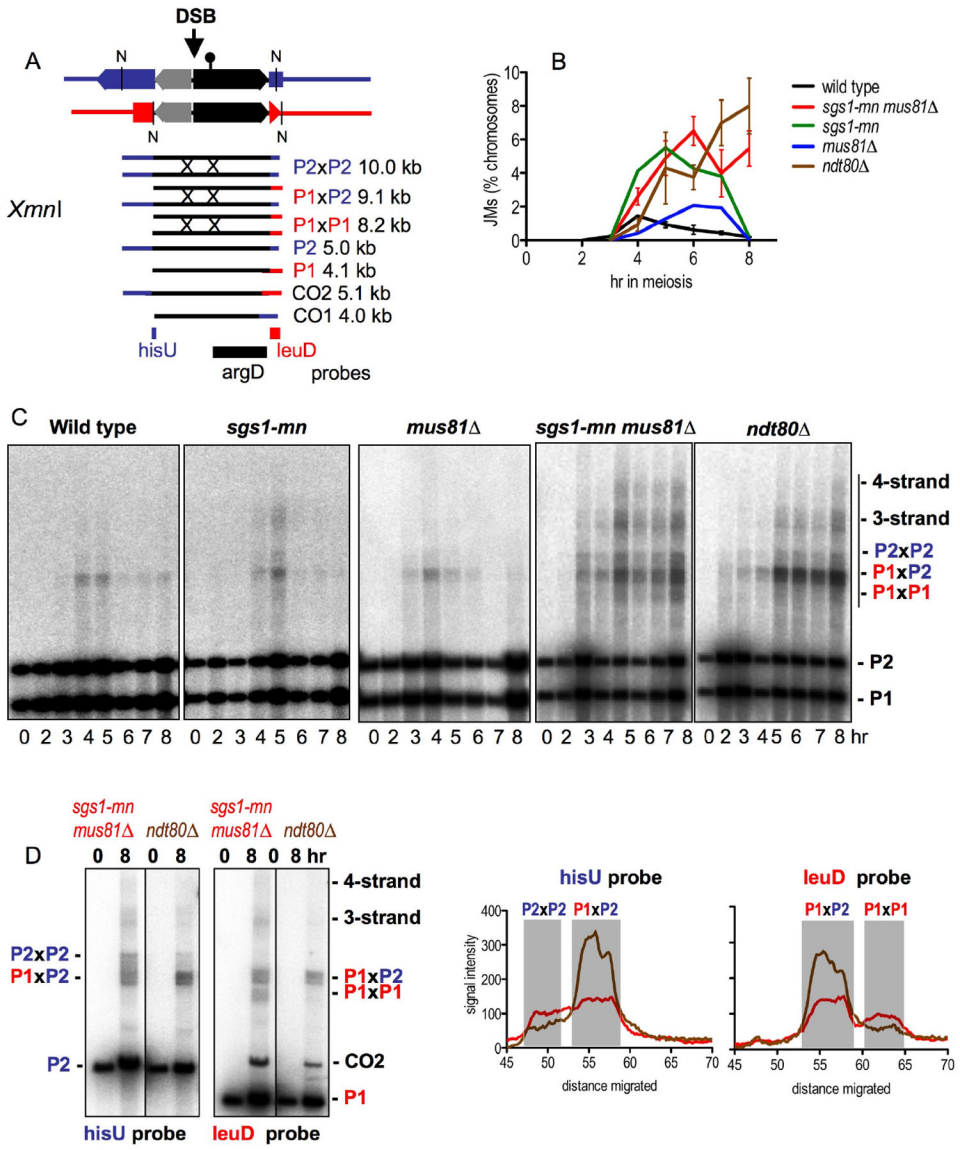

Figure 2. *sgs1 mus81* mutants accumulate meiotic recombination intermediates

A. Recombination interval used to score recombination. A 3.5 kb fragment with *URA3* (gray) and *ARG4* (black) arranged head to head is inserted at *HIS4* (blue) on one chromosome III homolog and at *LEU2* (red) on the other. 65 nucleotides of yeast telomere DNA, inserted at the junction of the *URA3* and *ARG4* promoters, creates a strong meiotic DSB (Jessop et al., 2005). A short palindrome containing an *Eco*RI site (lollipop) is inserted in *ARG4*, ~ 600 bp from the DSB, in the copy at *HIS4*. Arrows denote the direction of transcription. *HIS4* is 16.7 kb from *LEU2*. Restriction fragments produced from an *Xmn*I (N) digest are probed with DNA from *ARG4* (black bar, argD), *HIS4* (blue bar, hisU) and *LEU2* (red bar, leuD) to detect interhomolog (P1 × P2) and intersister (P1 × P1, P2 × P2) JMs. Digests and probes used to detect DSBs, COs and NCOs are shown in Supplementary Figure 2.

B. Quantification of total JM frequencies from southern blots in panel C, plotted as percent of total insert sequences. 2 independent experiments were used for wild type and *sgs1-mn mus81Δ*, and 3 independent experiments for *ndt80Δ*. Strains used and other details are as in Figure 1.

C. Southern blots for JMs as described in part A. Vertical bar on right denotes region of blot used for JM frequency measurements.
D. Increased intersister JMs and decreased interhomolog JMs in *sgs1-mn mus81Δ* mutants. Southern blots of *Xmn*I-digested DNA from 0 and 8 hour samples of *sgs1-mn mus81Δ* (red line) and *ndt80Δ* (brown line) were probed with either hisU (left) or leuD (right). Signal intensities (arbitrary units) from 8 hour samples are plotted.
All plots report mean ± s. e. m.

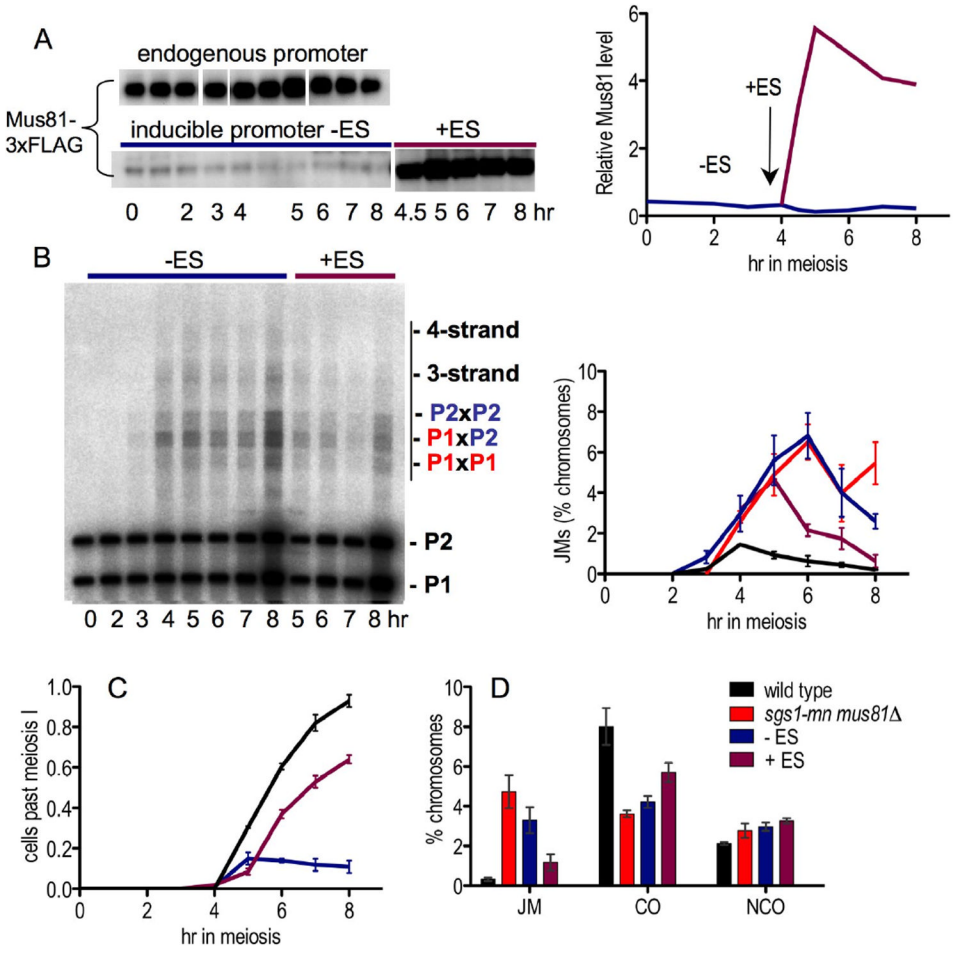

Figure 3. Mus81 induction in *sgs1-mn mus81Δ* cells

A. Mus81 protein expressed from its own promoter (*MUS81-3xFLAG*, MJL3269, top) and from the estrogen-inducible promoter (*MUS81-IN-3XFLAG*, MJL3270, bottom) were displayed on Western blots and probed with anti-FLAG. *MUS81-IN*, uninduced (−ES, blue line); *MUS81-IN* with estradiol added at 4 hours (+ES, maroon line). Mus81 protein levels are plotted relative to endogenous Mus81 levels at 0 hour.

B. Mus81 induction causes JM resolution. DNA was isolated from *sgs1-mn mus81Δ/MUS81-IN* (MJL3243) sporulated without or with estradiol added at 4 hr and JMs were detected as in Figure 2A. Average JM levels (% of total signal) are calculated from 2 independent experiments. Color code—same as in part D.

C. Mus81 induction promotes nuclear division. The fraction of cells completing at least one meiotic division were determined by DAPI staining of wild type and of *sgs1-mn mus81Δ/MUS81-IN* sporulated without or with estradiol addition at 4 hr.

D. Mus81 induction partially restores COs but does not increase NCOs. Recombinants were quantified as in Figure 1E (also see Supplemental Figure 2). JMs, COs and NCO levels (in terms of % of total lane signal) are an average of 7 and 8 hour samples from 2 independent experiments.

All plots report mean ± s. e. m.

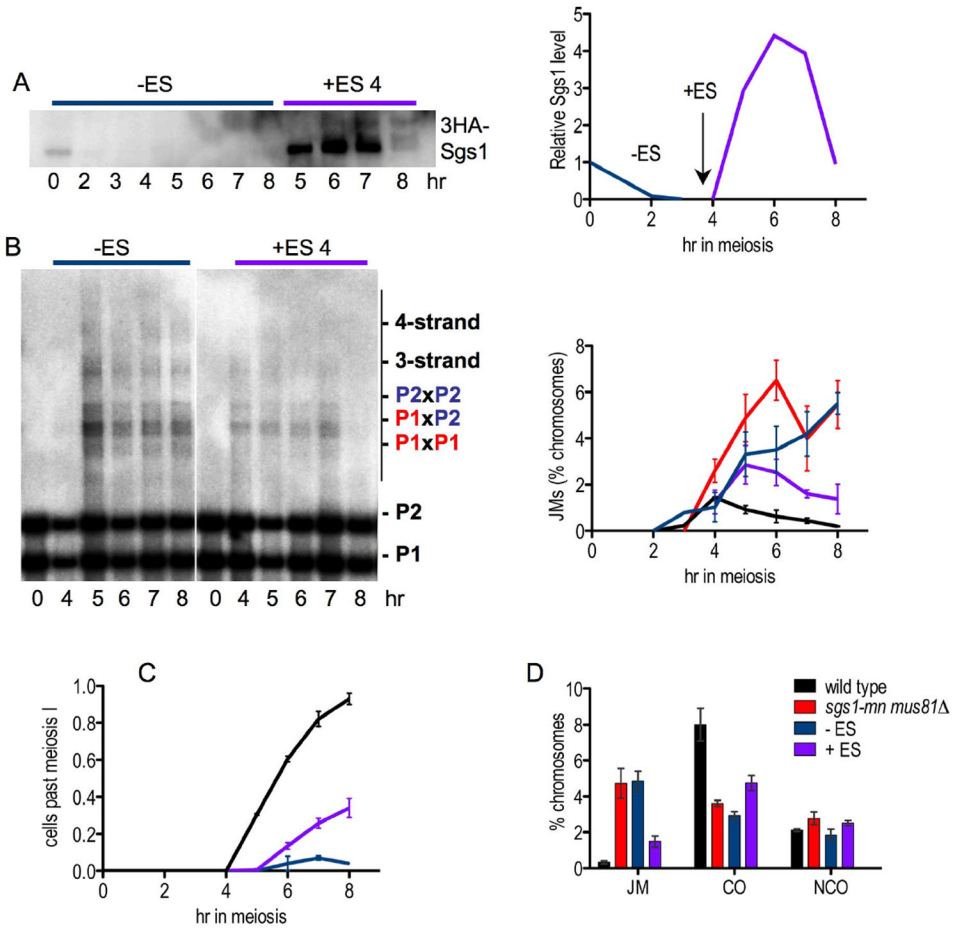

Figure 4. Sgs1 induction in *sgs1-mn mus81Δ* cells

A. Sgs1 protein expression from the estrogen-inducible promoter. Samples from *3XHA-sgs1-mn/3XHA-SGS1-IN mus81Δ* (MJL3244) were displayed on Western blots and probed with anti-HA antisera. Uninduced (−ES; blue line); estradiol added at 4 hours (+ES; purple line). Sgs1 protein levels are plotted relative to levels at 0 hr, reflecting protein expressed from *sgs1-mn*, which is roughly equal to endogenous Sgs1 levels in wild type (Jessop et al., 2006).

B. Sgs1 induction prevents JM accumulation. JMs were detected as in Figure 2A in DNA from *3XHA-sgs1-mn/3XHA-SGS1-IN mus81Δ* (MJL3244) sporulated without estradiol (−ES), or with estradiol added at 4 (+ES4). Total JM levels are plotted in comparison to those seen in wild type and *sgs1-mn mus81Δ* (MJL3181). Data from 4 independent experiments were used.

C. Sgs1 induction partially restores nuclear division. The fraction of cells completing at least one meiotic division were determined by DAPI staining of wild type and of *3XHA-sgs1-mn/3XHA-SGS1-IN mus81Δ* sporulated without estradiol or with estradiol added at 4 hours.

D. Effect of Sgs1 induction on COs and NCOs. Recombinants were detected and quantified as in Figure 1E and Supplemental Figure 2. Frequencies (% of total lane signal) are averages of 7 and 8 hour samples from 4 independent experiments.

All plots report mean ± s. e. m.

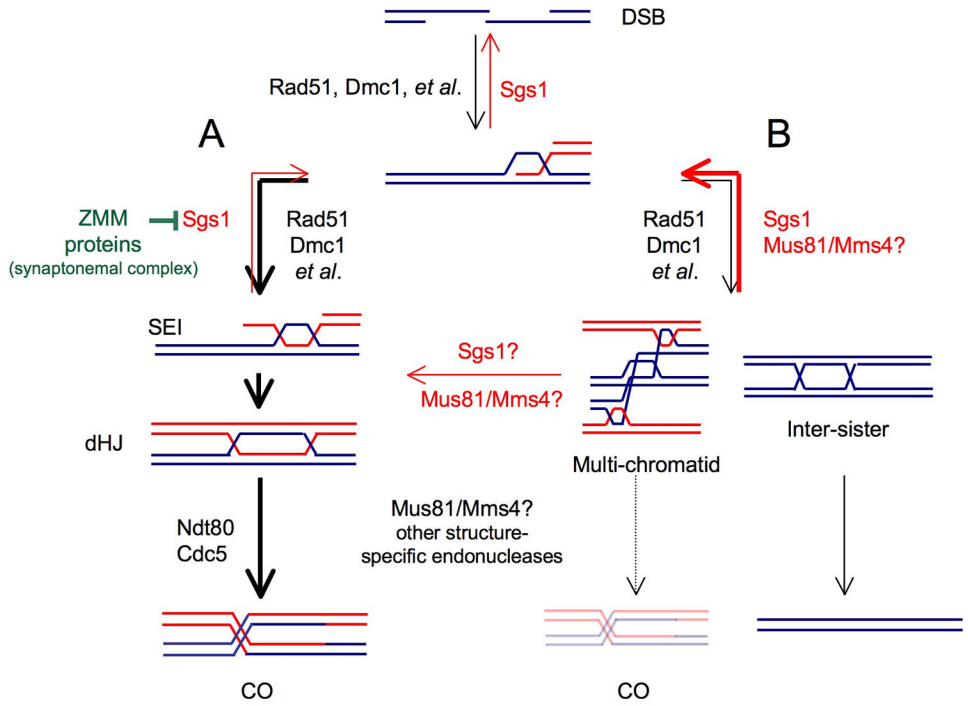

Figure 5. Regulation of interhomolog recombination by Sgs1 and Mus81. DSB ends invade homologous sequences to form JM precursors that can be disassembled by the Sgs1 complex

A. CO-designated intermediates form in the context of synaptonemal complex-associated proteins (ZMMs), which protect them from Sgs1. Inter-homolog JMs are resolved to produce COs by a process, regulated by Ndt80 and/or Cdc5, involving either Mus81/Mms4 or other structure-specific endonucleases.

B. JM precursors can also undergo unregulated events, including invading sister-chromatids or multiple chromatids to form off-pathway JMs. Because this occurs outside of the context of ZMM proteins, the Sgs1 complex readily disassembles these structures, providing a second opportunity to form CO-productive inter-homolog JMs. Mus81/Mms4 can resolve the unregulated JMs that escape Sgs1 surveillance, either returning events to the recombination precursor/intermediate pool, or resolving them directly to COs.