Meiotic DNA joint molecule resolution depends on Nse5–Nse6 of the Smc5–Smc6 holocomplex

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ABSTRACT

Faithful chromosome segregation in meiosis is crucial to form viable, healthy offspring and in most species, it requires programmed recombination between homologous chromosomes. In fission yeast, meiotic recombination is initiated by Rec12 (Spo11 homolog) and generates single Holliday junction (HJ) intermediates, which are resolved by the Mus81–Eme1 endonuclease to generate crossovers and thereby allow proper chromosome segregation. Although Mus81 contains the active site for HJ resolution, the regulation of Mus81–Eme1 is unclear. In cells lacking Nse5–Nse6 of the Smc5–Smc6 genome stability complex, we observe persistent meiotic recombination intermediates (DNA joint molecules) resembling HJs that accumulate in mus81Δ cells. Elimination of Rec12 nearly completely rescues the meiotic defects of nse6Δ and mus81Δ cells, indicating that these factors act after DNA double-strand break formation. Likewise, expression of the bacterial HJ resolvase RusA partially rescues the defects of nse6Δ and mus81Δ mitotic cells, as well as the meiotic defects of nse6Δ and mus81Δ cells. Partial rescue likely reflects the accumulation of structures other than HJs, such as hemicatenanes, and an additional role for Nse5–Nse6 most prominent during mitotic growth. Our results indicate a regulatory role for the Smc5–Smc6 complex in HJ resolution via Mus81–Eme1.

INTRODUCTION

The repair of DNA damage by homologous recombination (HR) is central to the faithful propagation of chromosomes during both the mitotic and meiotic cycles. During mitotic growth, exogenous or endogenous genotoxic agents can induce DNA double-strand breaks (DSBs), which may also arise from replication fork mishaps [reviewed in (1)]. The HR-based repair of these insidious forms of DNA damage is executed by a relatively well-defined and largely overlapping set of proteins. Broken DNA ends are first resected to generate a single-stranded (ss) 3'-overhang, which acts as a platform for the subsequent homology search and invasion steps of HR [reviewed in (2)]. The ss DNA-binding protein complex replication protein A initially coats the 3'-overhang, as well as Dmc1 during meiosis (3–5). The Rad51 nucleoprotein filament invades a homologous duplex, forming a displacement loop (D-loop) that is used to prime repair synthesis and, at a broken fork, to restart replication. Meiotically induced Rec12 (Spo11 homolog) and several partner proteins form DSBs during fission yeast meiosis, to form crossovers important for the proper segregation of homologs at the first meiotic division (6,7). Genomic regions that are hotspots for meiotic DSB formation by Rec12 require Rad51 for DSB repair and recombination; in regions with lower DSB levels, both Rad51 and Dmc1, a meiosis-specific paralog, are required (3–5).

Following D-loop formation, there are various postulated pathways for the completion of DSB repair. A major pathway, thought to be essential for crossover formation, requires the processing of four-way DNA junctions called Holliday junctions (HJs (8–11)). The nature of the initiating lesion and whether the cell is in mitosis or

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The authors wish it to be known that in their opinion the first two authors should be regarded as the joint First Authors.

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meiosis influence the mode of HJ processing. Closely spaced double HJs may be dissolved by the concerted action of a RecQ-related helicase and a topoisomerase partner (e.g. Sgs1–Top3 in budding yeast (12)). Notably, in fission yeast, the restart of broken replication forks critically depends on Mus81–Eme1 but not on the dissolution activity of Rqh1 (Sgs1 homolog)–Top3 (13). This likely reflects the formation of single, not double, HJs (or an HJ precursor) during restart of replication (12), because single HJs cannot be dissolved by a helicase and topoisomerase. Endonucleolytic HJ resolution facilitates crossing over between homologous chromosomes (homologs) and dominates during meiosis (8,14,15), when single HJs accumulate in mus81 mutants (8,15). Dissolution of double HJs without associated crossover seems to be the major pathway in mitotic cells of some species (12) and seems to play a role during meiosis in budding yeast (16).

HJs arising through HR-mediated DSB repair can be resolved by endonucleolytic activities such as Mus81–Eme1 ((8,15–19)), Slx1–Slx4 (20,21) and Yen1 (19,22). Recently, it was shown in the budding yeast Sacccharomyces cerevisiae that Mus81–Mms4 and Yen1 endonucleases collaborate during meiosis to resolve HJs and that their activity is carefully regulated during the cell cycle (19). Mms4 (Emel homolog) is phosphorylated and then hyperactivated during meiosis I, whereas Yen1 activity is inhibited until meiosis II.

Mus81–Eme1 is critical in the fission yeast Schizosaccharomyces pombe where it is the only known complex involved in meiotic HJ resolution (8,15,23). A Yen1 ortholog has not been identified in the S. pombe genome, and Slx1–Slx4 has no detectable defect in meiotic recombination (our unpublished data). The Mus81–Eme1 heterodimer is required for HR repair at stalled or broken replication forks and is also critical during mitotic (13,24,25) and meiotic HR (15,26).

In vitro, Mus81–Eme1 can cleave various substrates that mimic stalled replication forks and nicked and intact HJs (15,27,28). Mus81–Eme1 not only has a binding and cleavage preference for nicked HJs but also has a robust cleavage activity on intact HJs (29). S. pombe meiotic cells deleted for mus81 accumulate single HJs, as shown by 2D gel analysis and electron microscopy (8). The accumulation of HJs in mus81Δ cells results in severe meiotic defects (15) and sensitivity to the topoisomerase I poison camptothecin (CPT (30)); these phenotypes can be partially suppressed by expression of the Escherichia coli HJ resolvase RusA. Therefore, RusA has been used extensively, and is widely accepted, to identify the structures accumulated in various DNA repair mutants (15,26,30–32).

As described above, the last decade has seen major advances in the identification of activities most proximally involved in the processing of joint molecules (JMs) formed during HR. However, currently, little is known about how JM processing enzymes are recruited to their substrates, and whether their activities are facilitated by additional chromatin-associated factors. Interestingly, in this regard, others and we have identified and characterized the Smc5–Smc6 holocomplex, which seems to play multiple roles in HR (33). The octameric Smc5–Smc6 complex is structurally related to the cohesin and condensin complexes but, uniquely, contains two subunits, Nse1 and Nse2, which exhibit E3 ligase activity for ubiquitin and SUMO (Small Ubiquitin-like Modifier), respectively (33,34). In addition to Smc5, Smc6, Nse1 and Nse2, the complex contains a melanoma antigen-domain protein Nse3, a kleisin-like protein Nse4, and two armadillo/Huntington, Elongation Factor 3, PR65A, TOR repeat proteins, Nse5 and Nse6. Notably, unlike in budding yeast (33), nse5Δ and nse6Δ mutants are viable and display indistinguishable sensitivities to all tested DNA-damaging agents (35). In addition, Nse5 and Nse6 form a stoichiometric heterodimer when purified from insect cells (35) or bacterial cells (our unpublished data), and furthermore, nse5Δ nse6Δ double mutants are no more sensitive to UV irradiation than either single mutant (35). Thus, Nse5 and Nse6 function as an obligate heterodimer.

Reflecting key HR roles of the complex, Smc5–Smc6 in budding yeast and human cells is loaded near an enzymatically induced DNA DSB and is important for HR-mediated repair of the break (36–38). In addition, smc5–smc6 mutation causes hypersensitivity to ionizing radiation (IR)-induced DSBs, which is not additive when combined with a rad51 deletion; smc5–smc6 mutants, like rad51A mutants, fail to restore chromosome integrity following IR (35,39–43). Intriguingly, the Smc5–Smc6 complex has been implicated in the processing of HR intermediates or suppression of their formation or both (reviewed in (33,44)). For example, smc5–smc6 hypomorphs, including nse2 E3 SUMO ligase-deficient cells, accumulate Rad51-dependent HR intermediates following replication stress, indicating a concerted action of the entire complex in the productive completion of HR (35,45–54).

What is the physical nature of the recombination structures that form when Smc5–Smc6 function is compromised? In budding yeast, a heterogeneous group of hemicatenane and converged replication fork structures arise during replication stress in Smc5–Smc6-deficient mitotic cells (45,47,48,55). These DNA structures appear similar to those that accumulate in sgs1A mutant cells, and thus, it has been proposed that Sgs1 acts in concert with Smc5–Smc6 and sumoylation in the removal of such linkages during vegetative growth (45).

Here, we identify a critical role for fission yeast Nse5–Nse6 in chromosome disjunction during meiosis. We use both genetic and physical assays to determine the underlying cause of meiotic failure in nse6Δ cells, the stage at which Nse5–Nse6 function is required, and the DNA structure(s) that prevent(s) chromosome segregation at the first meiotic division (MI) in nse6Δ cells. Our analyses demonstrate that Nse5–Nse6 acts after programmed meiotic DSB formation to drive the timely resolution of JMs whose persistence in nse6Δ cells causes the profound MI defects. The JMs observed in nse6Δ cells resemble the HJs that accumulate in mus81Δ cells, providing the first direct evidence for a role of the Smc5–Smc6 complex in HJ resolution. Although Nse5–Nse6 and Mus81 have some distinct roles, both proteins are essential to avoid an accumulation of JMs that impede meiosis. Here, we define Nse5–Nse6 as a novel regulator
of nuclear division, which may act directly in the HJ resolution mechanism or by attracting the Smc5–Smc6 complex to its needed sites of action—mitotic or meiotic HJs.

MATERIALS AND METHODS

Standard Schizosaccharomyces pombe techniques

Standard fission yeast methods and media were used in these studies (56). CPT was obtained from Sigma-Aldrich (St. Louis, MO). Table 1 lists the S. pombe strains, and Table 2 lists the primers used.

Unless otherwise indicated, all NBY strains are ura4-D18 and leu1-32. pREPI-RusA, pREPI-RusA-D70N and pREPI were described in (15). pE119, containing LEU2 and GST, was used as a control vector (57). The minichromosome assay using Ch16-MHG, Ch16-MGH and pREPI81X-HO:LEU2 has been described in (58).

Mutations other than mating type and commonly used auxotrophies are described in previous studies: mus81::kanMX6 (15), nse6::kanMX6 (35), prhl1::hphMX6 (this study), rad51::ura4+ (59), dmc1::hphMX6 (this study), rec12-152::LEU2 (60), mbsl-24 and mbsl-25 (61), bub-1243, vtc4-1104 (9), lacO::lys1 lacI-GFP::arg3+ (62) and nse6::natMX6 (the kanMX6 marker in strain NBY385 (35) was switched to natMX6 as described in (64)). In Table 1 ‘::’ indicates marked by the following gene, and ‘-’ indicates that the preceding gene is replaced by the following marker.

Polymerase chain reaction (PCR) was used to create the hphMX6 cassette flanked by genomic DNA, to enable integration into the middle of the 1.2 kb intergenic region between the prhl1 and SPAC2GI1.10c loci. The hphMX6 cassette from pCR2.1 hphMX6 (63) was amplified in two steps, using a combination of the primers shown in Table 2, as described in (63,64). A stable transformant was obtained with the hphMX6 cassette integrated between prhl1 and SPAC2G11.10c loci, approximately 70 kb to the right of the mbsl1 meiotic DSB hotspot on chromosome I (61,65).

PCR was used to create the hphMX6 cassette flanked by genomic DNA, to replace the dmc1 gene. The hphMX6 cassette from pCR2.1 hphMX6 (63,64) was amplified in two steps, using a combination of the primers shown in Table 2, as described in (63,64).

HO-induced DSB repair assays

Cells were cultured in repressive (+ thiamine; B1) medium (EMM2), and a sample was plated onto non-selective medium (EMM2) for the 0 h time point. Cells were then cultured in repressive or de-repressive (–thiamine) media for 48 h and plated onto non-selective media. Colonies were counted after 3 days growth at 30°C. Replica plating onto media that contained hygromycin (or kanamycin for the rad51A mutant), or lacked either adenine or histidine, allowed calculation of the frequency of marker loss. Data are means and standard error of the mean from three independent assays, as described in (58).

Meiotic crossoves and recombination assays

Cells from equal volumes (10 μl) of each parental haploid culture were mixed, washed, resuspended in water and plated onto supplemented sporulation agar plates (66). After 2 days at 25°C, the cell–ascus mixture was observed by microscopy or processed further for viability assays. Meiotic 4′,6-diamidino-2-phenylindole (DAPI) staining was performed essentially as described in (15).

Zygotnic asci were fixed in methanol (–80°C) for 10 min and then washed three times with phosphate-buffered saline (1× PBS). The fixed asci were treated with Zymolyase 100T (0.1 mg/ml) for 10 min at 37°C. Asci were collected by centrifugation and resuspended in 0.1% Triton X-100 for 2 min at room temperature. Asci were then washed three times (in 1× PBS) and resuspended in a drop of 1× PBS containing 0.5 μg/ml DAPI. Asci were photographed using a Nikon Eclipse E800 microscope equipped with a Photometrics Quantix CCD camera. Images were acquired with IPLab Spectrum software (Signal Analytics Corporation).

For viability assays, the cell–ascus mixture was suspended in 1 ml of H2O and treated with 2% glusulase overnight at room temperature. Addition of ethanol to 30% for 1 h killed remaining vegetative cells, essentially as described in (67). To assess spore viability, spores were counted using a hemocytometer, diluted and plated onto the appropriate media. Spore viability was scored following incubation for 5–6 days at 30°C. For meiotic recombination assays, an appropriate dilution of spores was plated onto non-selective (YES) media and replica plated to appropriate test media: EMM2 (66) ± adenine for ade7-152; ± leucine for leu1-32; ± lysine for lys4-95; ± histidine for his4a-239; ± uracil for ural-61 and YES ± hygromycin (100 μg/ml) for hphMX6.

Analysis of recombination intermediates

pat1-114 strains were thermally induced for meiosis, and DNA was extracted from cells embedded in agarose plugs (68). The DNA was digested with appropriate restriction enzymes and analyzed by gel electrophoresis and Southern blot hybridization (69) and quantification (9).

The sensitivity of DNA to S1 nuclease (Roche) and to RuvC (gift of Ken Marians, Memorial Sloan-Kettering Cancer Center) was assayed by digesting the DNA, in plugs, with PvuII restriction enzyme, followed by washing twice with TE (Tris-EDTA; 10 mM Tris–HCl, pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0) and twice with nuclease S1 buffer (33 mM sodium acetate, 100 mM NaCl, 0.033 mM ZnSO4, pH 4.5 at room temperature) or RuvC buffer (50 mM Tris–acetate pH 7.5, 10 mM Mg(OAc)2, 1 mM DTT, 50 μg of BSA per ml); duration for each wash was 20 min at room temperature. The plugs were incubated in 100 μl of buffer with the indicated amount of S1 for 2 h at 4°C or RuvC for 4 h at 4°C, then for 1 h at 37°C (S1) or for 4 h at 55°C (RuvC) to facilitate branch migration of HJs embedded in agarose; the digestions were stopped by adding 2.5 μl of 500 mM EDTA pH 8.0 and placing the incubation tubes on ice for 10 min. 2D electrophoresis was performed, and
Table 1. Schizosaccharomyces pombe strains and primers

| Strain No. | Genotype                                      |
|-----------|----------------------------------------------|
| NBY128    | h+ mus81::kanMX6                            |
| NBY185    | h+ pat1-114                                 |
| NBY282    | h+ rec12-152::LEU2                           |
| NBY355A   | h+ mus81::kanMX6 (pREP1::LEU2)               |
| NBY355C   | h+ mus81::kanMX6 (pREP1::LEU2)               |
| NBY356A   | h+ mus81::kanMX6 (pREP1::NSL-RasA::LEU2)     |
| NBY356C   | h+ mus81::kanMX6 (pREP1::NSL-RasA::LEU2)     |
| NBY364    | h+ mus81::kanMX6 (pREP1::NSL-RasA-D70N::LEU2)|
| NBY365    | h+ mus81::kanMX6 (pREP1::NSL-RasA-D70N::LEU2)|
| NBY384    | h+ ade7-152 ura4 (pREP1::LEU2)               |
| NBY420    | h+ ura1-61                                  |
| NBY780    | h+                                          |
| NBY781    | h+                                          |
| NBY835    | h+ nse6::kanMX6                             |
| NBY855    | h+ rad51::ura4+ nse6::kanMX6                |
| NBY871    | h+ nse5::ura4+ nse6::kanMX6                 |
| NBY885C   | h+ rec12-152::LEU2  nse6::kanMX6            |
| NBY896    | h+ nse5::ura4+ nse6::kanMX6                 |
| NBY897    | h+ nse5::ura4+ nse6::kanMX6                 |
| NBY917    | h+ nse5::kanMX6 (pREP1::NSL-RasA-D70N::LEU2)|
| NBY936    | h+ nse5::kanMX6 (pREP1::NSL-RasA-D70N::LEU2)|
| NBY952    | h+ rad51::ura4+ nse6::kanMX6                |
| NBY963    | h+ nse5::kanMX6 ade7-152 leu1 ura4          |
| NBY991    | h+ Chp6-MHH ade6-210 (pREP81X-HO::LEU2)      |
| NBY1000   | h+ nse5::kanMX6 Chp6-MHH ade6-210            |
| NBY1484   | h+ nse5::kanMX6 (pE119::LEU2)                |
| NBY1486   | h+ nse5::kanMX6 (pE119::LEU2)                |
| NBY1753   | h+ rec12-152::LEU2                           |
| NBY2027   | h+ rad51::ura4+ nse6::kanMX6                |
| NBY2051   | h+ rad51::ura4+ nse6::kanMX6 lacO::lys1 lac1-GFP::arg3 |
| NBY2482   | h+ (pREP1::LEU2)                             |
| NBY2551   | h+ dnc1::hphMX6                              |
| NBY2589   | h+ dnc1::hphMX6 nse6::kanMX6                |
| NBY2590   | h+ dnc1::hphMX6 nse6::kanMX6                |
| NBY2610   | h+ dnc1::hphMX6 nse6::kanMX6                |
| NBY2620   | h+ rad51::ura4+ dnc1::hphMX6 nse6::kanMX6   |
| NBY2621   | h+ rad51::ura4+ dnc1::hphMX6 nse6::kanMX6   |
| NBY2622   | h+ rad51::ura4+ dnc1::hphMX6 nse6::kanMX6   |
| NBY2623   | h+ rad51::ura4+ dnc1::hphMX6 nse6::kanMX6   |
| NBY2654   | h+ (pREP1::NSL-RasA::LEU2)                   |
| NBY2660   | h+ nse5::kanMX6 (pREP1::NSL-RasA::LEU2)      |
| NBY2750   | h+ nse5::kanMX6 (pREP1::NSL-RasA::LEU2)      |
| NBY2893   | h+ ura1-61 nse6::kanMX6                     |
| NBY2938   | h+ pat1-114 nse6::natMX6                    |
| NBY2953   | h+ ura4+ LEU2 his4-239 lys4-95 nse6::kanMX6 |
| NBY2962   | h+ ura4+ LEU2 his4-239 lys4-95 nse6::kanMX6 |
| NBY3112   | h+ ura4+ prh1::hphMX6                       |
| NBY3114   | h+ ura4+ nse5::kanMX6 prh1::hphMX6          |
| NBY3304   | h+ nse5::natMX6 mus81::kanMX6               |
| NBY3311   | h+ nse5::natMX6 mus81::kanMX6               |
| NBY4176   | h+ nse5::natMX6 mus81::kanMX6               |
| NBY4178   | h+ nse5::natMX6 mus81::kanMX6 (pREP1::LEU2)  |
| NBY4192   | h+ nse5::ura4+ (pREP1::NSL-RasA::LEU2)       |
| NBY4194   | h+ nse5::ura4+ (pREP1::NSL-RasA::LEU2)       |
| NBY4195   | h+ nse5::ura4+ (pREP1::NSL-RasA::LEU2)       |
| NBY4197   | h+ nse5::ura4+ (pREP1::NSL-RasA::LEU2)       |
| NBY4198   | h+ nse5::ura4+ (pREP1::NSL-RasA::LEU2)       |
| NBY4200   | h+ nse5::natMX6 mus81::kanMX6 (pREP1::LEU2)  |
| NBY4298   | h+ rad51::hphMX6 Chp6-MGH ade6-210 (pREP81X-HO::LEU2) |
| GP1456    | h+ rec12-152::LEU2 ura4-294 ade6-52         |
| GP5082    | h+ ade6-216 ade6-210 pat1-114 pat1-114 +/+ ura1-61 mbs1-24/mbs1-25 his4-239 +/+ lys4-95 mus81::kanMX6/mus81::kanMX6 |

(continued)

Table 2. Primers used to integrate the hphMX6 marker between prh1 and SPAC2G11.10c (primers 1–4) and to replace the dmc1 gene with the hphMX6 marker (primers 5–8)

| Primer   | Sequence                                      |
|----------|-----------------------------------------------|
| Primer 1 | 5'-AATTGAGCCTATTATTTCTGAG-3'                  |
| Primer 2 | 5'-TATTAATACCCGGGAGTCCGTTTACCTTGACTGTTAC-5'   |
| Primer 3 | 5'-GTTTAAAGCAGCTGAAITATTCCATGGAGTAGTTATGGTTG-5' |
| Primer 4 | 5'-CTTTCCTGGGCTTCTCCTACA-3'                   |
| Primer 5 | 5'-GCAGCGGTCATTTGTTAC-3'                      |
| Primer 6 | 5'-TATTAATACCGGAGTCGATCTTCTACCATTTTTATTGTAAC-3' |
| Primer 7 | 5'-GTATATAACAGGATCTGAAATTCATGGAGTAGTTATGGTTG-5' |
| Primer 8 | 5'-AGTGGCTTTTGCGGTTGTTG-3'                    |

RESULTS

Roles of Nse5–Nse6 in mitotic DNA double-strand break repair

The budding yeast and human Smc5–Smc6 complexes have been implicated in the efficient repair of an enzymatically induced DNA DSB (36–38). To determine whether the fission yeast Nse5–Nse6 functionally inter-dependent heteromeric complex (35) plays an analogous role, we tested the DSB repair capacity of nse6Δ cells using a derivative of the non-essential minichromosome Ch16, which contains the MATa target site for the S. cerevisiae HOmational switching (HO) endonuclease (see schematic in Figure 1A, described in (58)). Given the relatively mild IR sensitivity of fission yeast nse6Δ cells versus the extreme sensitivity of rad51Δ cells (35), it was surprising that both nse6Δ and rad51Δ cells were highly defective in the HR-based repair of the HO-induced break. Unlike wild-type cells, which repaired the majority of the HO-induced DSBs by gene conversion, the nse6Δ and rad51Δ mutants instead lost nearly all of the HO cut recombination intermediates were detected at mbs1 as described in (8).
Figure 1. Mitotic roles for the Smc5–Smc6 complex. (A) Schematic of the HO-induced DSB minichromosome system. The minichromosome Ch16 and full-length Ch III with their centromeric regions (black ovals), histidine marker his3\(^+\) (dark grey box) and complementing ade6 heteroalleles (ade6-216 and ade6-210 (86); white boxes) are shown along with a MAT\(a\) site for HO DSB formation (black rectangle) and the adjacent hphMX6 (hygromycin)-resistance marker (light gray box) for wild-type and nse6\(D\) cells. As rad51 was deleted using the hygromycin-resistance marker, a minichromosome bearing a kan (kanamycin)-resistance marker was used in this background. MAT\(a\) is \(24\)\(\text{kb}\) from the ade6 marker. Removal of thiamine (B1) derepresses the HO gene, whose product generates a DSB at the MAT\(a\) target site (vertical arrowhead). HO-induced DSBs can result in (i) loss of Ch16 with diagnostic Ade\(^+\)/Hyg\(^-\) and Hyg\(^+\)/Ade\(^-\) phenotype, (ii) repair of the DSB by interchromosomal gene conversion using Ch III as a template, resulting in the loss of MAT\(a\) and hphMX6 cassette and an Ade\(^-\)/Hyg\(^+\) phenotype or (iii) maintenance of the initial Ade\(^+\)/Hyg\(^-\) phenotype, if the DSB is repaired by either non-homologous end-joining (NHEJ) or sister chromatid repair. The HO system can also assay levels of spontaneous minichromosome Ch16 loss, by scoring marker loss in the absence of HO induction (0 h, and 48 h culturing in thiamine). (B) and (C) Genetic analysis of site-specific DSB repair in wild-type (left panel), nse6\(D\) (middle panel) and rad51\(D\) (right panel) backgrounds. Percentage marker loss is given for cells grown in repressive (0 h and 48 h, + B1) or derepressive media (48 h, –B1). Data are means and standard errors from three independent experiments. \#Loss of heterozygosity (LOH) can occur through various mechanisms, as described in (58). *Sister chromatid repair (SCR) also results in a Hyg\(^+\)/Ade\(^-\) phenotype. (D) RusA expression partially rescues the CPT sensitivity of nse6\(D\), mus81\(D\) and nse6\(D\) mus81\(D\) mutants. (E) and (F) RusA expression partially rescues the CPT sensitivity of nse6\(D\) and nse6\(D\), but does not rescue that of rad51\(D\) cells. (D–F) Five-fold serial dilutions of the indicated strains were plated onto medium that lacked thiamine to derepress plasmid-borne gene expression. Cells were either untreated or treated with the indicated concentrations of CPT and grown at 32°C for 3 days. pVec denotes an empty vector (87).
minichromosomes (Figure 1B and C (58,70)). This result indicates that in fission yeast, Smc5–Smc6, is required for the repair of enzymatically induced DSBs.

To test for an additional role of Nse6 in mitotic DNA break repair, we determined the sensitivity of nse6Δ mutants to CPT, which stabilizes topoisomerase I–DNA covalent complexes and can induce fork breakage during replication (30). The nse6Δ mutant was sensitive to CPT, although not as sensitive as mus81Δ or the mus81Δ nse6Δ double mutant ([71] and Figure 1D). Although the expression of E. coli RusA HJ resolvase is mildly toxic to wild-type cells, as observed previously (72), the CPT sensitivities of both nse6Δ and mus81Δ cells were suppressed by RusA expression (Figure 1D). Notably, the nse5Δ mutant has a CPT sensitivity similar to that of nse6Δ, which is also rescued by RusA expression (Figure 1E). This result indicates a role for Nse5–Nse6, like Mus81, in HJ resolution. The mus81Δ nse6Δ double mutant was also suppressed by expression of RusA, although not as well as the single mutants, indicating that Nse5–Nse6 and Mus81 have distinct and overlapping roles. Although nse6Δ behaves like rad51Δ in the HO-induced DSB assay (Figure 1B and C (58)), the hypersensitivity of rad51Δ cells to CPT was not suppressed by RusA expression (Figure 1F).

Nse5–Nse6 executes an essential function during meiosis

Due to the similarity of the mitotic phenotypes of nse5Δ, nse6Δ and mus81Δ cells, we tested the role of Nse5–Nse6 in meiosis, an HJ-generating process that is heavily dependent on Mus81–Eme1 resolution activity (8,15,23). Wild-type meiosis produces ascus with four haploid spores, with DNA present in each spore (Figure 2A). Ascii from nse5Δ and nse6Δ mutant crosses were largely devoid of well-defined spores but infrequently contained one single large spore (Figure 2A), much like mus81Δ asci (15). This result shows that Nse5–Nse6 plays an important role in meiosis.

If Nse5–Nse6, like Mus81–Eme1, promotes JM resolution, then preventing the early stages of meiotic recombination should render Nse5–Nse6 unnecessary for meiotic nuclear division. For these tests, we first eliminated Rec12, which is essential for meiotic DSB formation and recombination (68). Ascii from rec12Δ crosses were very heterogeneous: Some ascii had four equal-sized spores, which are not observed for the nse6Δ single mutant (Figure 2A). As expected, spore viability from homoygous nse5Δ or nse6Δ crosses was extremely low: Only ~0.5% of spores germinated and produced colonies, compared to ~80% for wild-type (Figure 2B (15)). Notably, both rec12Δ and rec12Δ nse6Δ crosses yielded similar spore viabilities of ~20%, and, furthermore, their ascii morphologies were indistinguishable (Figure 2B). The same was true for rec12Δ and rec12Δ mus81Δ crosses. The rec12Δ nse6Δ and rec12Δ mus81Δ double mutants had indistinguishable defects from those observed in a rec12Δ deletion, indicating that rec12Δ is epistatic to both nse6Δ and mus81Δ in meiosis.

The morphology of ascii produced by crosses of nse6Δ, mus81Δ and mus81Δ nse6Δ double mutants were indistinguishable (Figure 2B). However, the viability of spores from mus81Δ nse6Δ crosses was ~8-fold lower than that of spores from crosses of the single mutants (Figure 2B). Deletion of rec12 rescued the mus81Δ nse6Δ meiotic ascus phenotype, but only partially rescued the low spore viability phenotype, between 1 and 2% spore viability.

Next, we exploited the aberrant ascii morphology of nse6Δ cells to determine whether Nse5–Nse6 acts before or after strand invasion promoted by the RecA homologs Rad51 and Dmc1 (4,5). Cells lacking Rad51 fail to repair many meiotic DSBs (73) but can produce ascii containing four discrete, although mostly inviable, spores (15), presumably because there are no physical linkages, such as HJs, between the chromosomes to prevent their segregation (Figure 2C). Although not essential for meiotic progression and abundant formation of viable spores, Dmc1 is required for normal levels of meiotic recombination in most genetic intervals (4,9). In contrast to rad51Δ or dmc1Δ single mutants, four-spore ascii were not observed in the nse6Δ single or rad51Δ nse6Δ or dmc1Δ nse6Δ double mutants (Figure 2B and C). However, the dmc1Δ rad51Δ double and nse6Δ dmc1Δ rad51Δ triple mutants produced similar ascii that sometimes contained four spores, which are not observed for the nse6Δ single mutant (Figure 2B and C). Thus, suppression of the nse6Δ phenotype (to a level equivalent to that of the dmc1Δ rad51Δ double mutant) requires elimination of both strand-exchange proteins. These results indicate that Nse6 acts after strand exchange by Rad51 and Dmc1, perhaps during JM recombination.

Genetic and physical analyses of meiotic crossing over in nse6Δ cells

Next, we tested the impact of absence of Nse6 on meiotic recombination in three genomic intervals. In the large ade7–leu1 interval, frequency of crossover among viable spores from a wild-type cross was 41.5%, not significantly different from the 40.8% in nse6Δ mutants (Figure 3A). In two shorter intervals, lys4–his4 and ura1–prh1 (which contains the mbs1 DSB hotspot used in DNA analyses below (61)), frequencies of crossover were reduced by a factor of about 2 in nse6Δ mutants, relative to wild-type (Figure 3A), a statistically significant reduction (P < 0.0001). However, in contrast to the reductions by factors of 20–100 in mus81Δ cells (8,26), the meiotic crossover defects of nse6Δ cells are mild.

Through genetic methods, we can measure crossovers only in the ~0.5% of spores that are viable after nse6Δ meiosis; thus, our observed frequencies may overrepresent the frequencies in most of the cells. To circumvent this limitation, we physically measured the crossovers between two markers closely flanking the DSB hotspot.
mbs1 in the entire meiotic population, as previously analyzed in mus81A mutants (8). The crossover-specific product R2 (Figure 3B) was quantified in wild-type, mus81A, nse6A and nse6Δ mus81A strains at 7 h after induction of synchronous meiosis. Consistent with previous data (8), there was a severe defect, a reduction by a factor of 7, in crossover formation in mus81A and nse6Δ mus81A cells (Figure 3B). Importantly, in nse6Δ cells, crossover formation was reduced by a factor of about 2, as in the genetic assay for ura1–mbs1–prh1 recombintants (Figure 3A), suggesting that Nse5–Nse6 promotes but is not absolutely essential for meiotic crossover formation (Figure 3B).

Recombination intermediates (DNA joint molecules) accumulate in nse6A mutants

The similarities of the vegetative and meiotic phenotypes of mus81A and nse6A led us to investigate whether DNA JMs accumulate during meiosis in nse6A, as they do in mus81A (8). Synchronous meiosis was induced in pat1-114 nse6A and nse6Δ mus81A diploids, and the formation of branched DNA structures, indicative of both replication and recombination intermediates, was analyzed by 2D gel electrophoresis as previously performed for mus81A (8). Branched DNA structures produced during replication were visible at 2.5 h and decreased toward 3 h (Figure 4A and B), the time at which DNA content shifts from 2n to 4n as measured by flow cytometry (8); our unpublished data). After replication was complete and DSB formation had begun (Figure 4C), the amount of X-shaped DNA increased again at 4 h, persisted at 4.5 h and disappeared after 5 h in the wild-type strain (Figure 4A and B), as expected for the formation and resolution of HJs. In the nse6Δ strain, the recombination intermediates were formed with the same timing as in wild-type, but they did not disappear; the same was true for mus81A and nse6Δ mus81A mutants, although the double mutant showed a slight delay in JM formation (Figure 4A and B; (8)). As expected, early but not late appearing JMs were detected in an nse6Δ rec12Δ haploid double mutant (haploid and diploid analyses are indistinguishable (74) and our unpublished data). This result indicates that, as in mus81A mutants (8), the early arising JMs are replication derived, and the late arising JMs are recombination intermediates and are consistent with the meiotic phenotype of nse6Δ depending on DSB formation by Rec12 (Figure 2B). Thus, both nse6Δ and mus81A mutants accumulate recombination intermediates with a similar timing during meiosis.

Next, we determined whether the JMs that accumulate in the nse6Δ mutant specifically form between sister...
chromatids or between homologs, and whether they are single HJs, as they are in a mus81D mutant (8), and not double HJs, a plausible alternative. A strain with heterozygous restriction site mutations that flank the DSB hotspot mbs1 was used to assay the relative interhomolog (IH) and intersister (IS) HJs formed during DSB repair. As was the case in the mus81D strain (8,9), IS HJs outnumbered IH HJs by \( \frac{3.5}{24} \) (Figure 5A). To distinguish single versus double HJs, the DNA in the agarose gel after the first dimension of electrophoresis was heated to 65°C to promote branch migration. The unequal length arms of homologous DNA in single HJs, which have two recombinant DNA strands, prevent the junction from migrating past the ends of the arms; the absence of recombinant DNA strands in double HJs and the equal length homologous arms of IS HJs allow their junctions to readily migrate off the end (8). When the DNA was heated, the IS HJs readily branch migrated and disassociated into the linear forms, whereas the IH HJs were resistant and remained intact (Figure 5B). This stability indicates that the IH-branched DNA structures are single HJs. This is the same behavior observed previously for branched DNA that accumulates in the mus81D mutant (8). These physical analyses further support a role for Nse6 in the resolution of DNA JMs during meiosis.

The X-shaped DNA structures seen in the 2D gel assays could be either HJs or a related four-stranded DNA structure, a hemicatenane, with two single DNA strands, or two pairs of strands, interwound. We first tested the sensitivity of these structures to the E. coli HJ resolvase RuvC (75). The JMs of the nse6D and mus81D strains were similarly sensitive to RuvC (Figure 6A and B), indicating that at least some of the X-shaped structures formed in both mus81D and nse6D strains are the same and are HJs. However, the JMs of the nse6D mus81D strain were largely resistant, suggesting that these X-shaped structures are not HJs but might be related structures such as hemicatenanes.

Although structurally similar to HJs, hemicatenanes have exposed ss DNA that would be susceptible to S1, a nuclease more active on ss DNA than on double-stranded DNA (76). To test this possibility, meiotic DNA prepared at 4.5 or 5 h after induction of mus81D or nse6D strains was treated with S1 before 2D gel electrophoresis. The accumulated branched DNA structures from the mus81D strain were insensitive to S1 treatment, but those from the nse6D and nse6D mus81D strains were partially sensitive.

**Figure 3.** Effect of nse6D mutation on the frequency of meiotic crossovers. (A) Crossover (CO) frequencies in three intervals in the wild-type and nse6D backgrounds. The percent of recombinant spore colonies was converted into genetic distance in centimorgans with Haldane’s equation. The number of colonies analyzed is the total from at least three independent experiments. (B) Amount of crossover DNA generated at the mbs1 hotspot (8). Diploid pat1-114 wild-type (GP656), mus81D (GP657), nse6D (GP0234) and nse6D mus81D (GP7765) strains with heterozygous restriction sites PmlI (L) and XbaI (R) flanking mbs1 were assayed. Digestion with these enzymes and PvuII (black arrowheads), which cuts outside L and R, gives two parental DNA fragments [9.2 kb (P1) or 6.8 kb (P2)] and two recombinant DNA fragments [11.2 kb (R1) or 4.8 kb (R2)] detected by the Southern blot hybridized probe (black lines at mbs1). The crossover DNA was measured at 7 h, after JM resolution in wild-type strains (Figure 4A, top); a pre-miotic 0 h is shown as a control. Because R1 can also arise from incomplete digestion (R1^) at either L or R, the frequency of crossover DNA (% CO) was calculated as two times the amount of R2 DNA divided by the amount of total DNA. DNA from two independent inductions of strain GP7765 (nse6D mus81D) are shown. The means of two to three experiments (some from additional unpublished experiments) with the range or standard error of the mean are given. The asterisk indicates a non-specific cross-hybridization band.
We propose that both HJs and an S1-sensitive form of DNA, such as hemicatenanes, accumulate in \textit{nse6} \textit{D} mutants but only HJs accumulate in \textit{mus81} \textit{D} mutants during meiosis (Figure 6A and B).

Suppression of \textit{nse5} \textit{A} and \textit{nse6} \textit{A} meiotic defects by bacterial resolvase RusA

Our biochemical analyses provide evidence that \textit{nse6} \textit{A} mutants accumulate meiotic HJs, as observed in \textit{mus81} \textit{D} mutants (8,15). For \textit{in vivo} validation of this conclusion, we used the RusA resolvase to probe the nature of the meiotic impediment in \textit{nse5} \textit{D} and \textit{nse6} \textit{D} cells. Our biochemical analyses provide evidence that \textit{nse6} \textit{D} mutants accumulate meiotic HJs, as observed in \textit{mus81} \textit{D} mutants (8,15). For \textit{in vivo} validation of this conclusion, we used the RusA resolvase to probe the nature of the meiotic impediment in \textit{nse5} \textit{D} and \textit{nse6} \textit{D} cells. Our laboratories and others have shown that heterologous expression of \textit{E. coli} RusA reduces the level of HJs that accumulate either on treatment with various DNA damaging agents or during meiosis in various DNA repair mutants (15,26,30–32). We expressed RusA and its catalytically inactive mutant, D70N, in \textit{nse6} \textit{D} cells and analyzed both spore formation and viability. Strikingly, RusA expression significantly rescued the meiotic defects of \textit{nse6} \textit{D} cells, yielding asci that often contained a wild-type complement of four spores (Figure 6C). This rescue required RusA endonuclease activity, as the RusA-D70N catalytically inactive mutant provided no benefits (Figure 6C). The spore viability was increased.
Failure of the HR pathways underlies many human diseases including cancer and can cause birth defects through aberrant meiotic chromosome segregation. Here, we have identified a novel function for Nse5–Nse6 of the Smc5–Smc6 complex in processing mitotic and meiotic HR intermediates. Genetic and physical analyses indicate that nse6Δ mutants accumulate JMs resembling the HJs that accumulate in mus81Δ during meiosis. Thus, to our knowledge, Nse5–Nse6 is only the second factor in fission yeast required for the endonucleolytic processing of HJs, besides Mus81–Eme1, which has the catalytically active site for HJ resolution.

The key data supporting a function for Nse5–Nse6 in the resolution of JMs are as follows: (i) like mus81Δ mutants (15), nse6Δ mutants form few viable spores (Figure 2), due to the failure of chromosome segregation; (ii) like mus81Δ (15), rec12Δ is epistatic to nse6Δ in meiosis, demonstrating that Nse5–Nse6 acts after DSB formation (Figure 2); (iii) the double nse6Δ mus81Δ mutant also forms few viable spores and is suppressed, at least partially, by rec12Δ, indicating that Mus81 and Nse6 act in the same or closely related steps of meiosis (Figure 2); (iv) the rad51Δ dmc1Δ combination is epistatic to nse6Δ, demonstrating that Nse5–Nse6 acts after the formation of JMs (Figure 2); (v) JMs accumulate in meiotic nse6Δ and nse6Δ mus81Δ cells, and these JMs are temporally, genetically and electrophoretically indistinguishable from the HJs that accumulate in mus81Δ cells (Figures 4 and 5, (8)); (vi) at least some of these JMs are sensitive to E. coli RuvC HJ resolvase (Figure 6) and to RusA HJ resolvase, because, as for mus81Δ cells (15), expression of RusA partially rescues the meiotic defects of nse6Δ cells (Figure 6C and D) and (vii) crossovers are modestly reduced in nse6Δ cells, indicating that Nse5–Nse6 is important but not absolutely essential for HJ resolution, which requires Mus81–Eme1 (Figure 3).

In addition to HJ resolution, Nse5–Nse6 appears to have a second role because (i) the double nse6Δ mus81Δ mutant grows more slowly and is more CPT sensitive than either single mutant (Figure 1D); (ii) the double mutant is more than 20-fold when RusA was expressed in nse6Δ cells compared to an empty vector control (Figure 6D). Spore viability was also increased in nse5Δ cells (Figure 6D). Likewise, although more dramatic due to their initial lower viability, mus81Δ spore viability was increased more than 800-fold by RusA expression. Interestingly, spore viability was around 1.5% in nse5Δ and 3% in nse6Δ mutants expressing RusA, which was significantly lower (P < 0.005) than in mus81Δ-expressing RusA (7%; Figure 6D). This result is consistent with the accumulation of both HJs and an S1-sensitive form of DNA, such as hemicatenanes, in nse5Δ and nse6Δ meioses, but only HJs in mus81Δ meiosis (Figure 6A and B). RusA expression did not so strongly improve the ascus morphology or spore viability in the nse6Δ mus81Δ double mutant (Figure 6C and D), which is consistent with the RuvC insensitivity of DNA isolated from these cells (Figure 6A and B). Overall, the remarkably similar suppression of both the ascus morphology and spore viability defects in nse5Δ or nse6Δ and mus81Δ by RusA endonuclease suggests that Nse5–Nse6 plays a role in facilitating the HJ resolvase activity of Mus81–Eme1.
not quite as well suppressed to CPT resistance by expression of RusA as the single mutants (Figure 1D); (iii) unlike mus81Δ, nse6Δ is defective for mitotic DSB repair, as is rad51Δ (Figure 1); (iv) crossovers are formed, although at reduced level compared to wild-type, in nse6Δ but not in mus81Δ or nse6Δ mus81Δ mutants (Figure 3) and (v) the JMs that accumulate in nse6Δ and nse6Δ rad51Δ meiotic cells are at least partially sensitive to S1 nuclease, whereas those that accumulate in mus81Δ meiotic cells are not (Figure 6A and B), suggesting that Mus81–Eme1 and Nse6 have distinct, and overlapping, roles.

To reconcile these observations, we propose that in meiotic cells Nse5–Nse6 stimulates the resolution of HJs by Mus81–Eme1 and the resolution of other structures, such as hemicatenanes, which may arise during mitotic growth or during meiosis in the absence of Mus81–Eme1. These structures may arise during mitotic replication, as suggested by others (45,77), thereby partially accounting for the mitotic phenotypes of nse6Δ mutants. The structure of the JMs that accumulate in nse6Δ and nse6Δ mus81Δ mutants is not entirely clear, although those that accumulate in mus81Δ mutants are clearly single HJs (8). The JMs that accumulate in nse6Δ mutants are sensitive to both S1 nuclease and RuvC HJ resolvase, whereas those that accumulate in mus81Δ mutants are sensitive to RuvC and those in nse6Δ mus81Δ mutants are sensitive to S1 nuclease (Figure 6A and B). The stimulation of Mus81 HJ resolvase activity by the Nse5–Nse6 complex may be direct or indirect.

We propose that in nse6Δ mutants, Mus81–Eme1 slowly resolves HJs while some are converted into another structure and that in nse6Δ mus81Δ mutants, most or all of the HJs are converted into this structure. This proposal is consistent with the slight reduction in meiotic crossover frequency, among the few viable spores that arise, in nse6Δ mutants but strong reduction in mus81Δ mutants (Figure 3A (8,23,26)); with the slight reduction in total crossover DNA in nse6Δ mutants but strong reduction in mus81Δ and nse6Δ mus81Δ mutants (Figure 3B (8)); and with the suppression of nse6Δ, but not nse6Δ mus81Δ, by expression of the RusA HJ resolvase (Figure 6C and D).

The resistance to branch migration of the IH JMs in nse6Δ mutants suggests that these JMs, between heterozygous restriction sites, are single HJs with recombinant length strands (Figure 5B). This is because either double HJs, which have parental length strands, or hemicatenanes

![Figure 6.](https://academic.oup.com/nar/article-abstract/40/19/9633/2414720)
with either recombinant or parental length strands should dissociate into separate duplexes on heating (e.g. (8,76,78)). IS JMs would dissociate in any case, as observed. IH and IS JMs may differ in structure, but their low level has precluded our determining their sensitivity to S1 nuclease and RuvC. Although the S1 nuclease sensitivity is consistent with some JMs being hemicatenanes, to our knowledge, this structure has not been clearly demonstrated to arise in cells, for example by electron microscopy or comparison with synthetic DNA molecules. Further investigation is required to establish the structure of the population of non-HJ containing JMs in the absence of Nse6.

As Nse5–Nse6 acts as part of the Smc5–Smc6 complex, which has multiple roles in chromosome metabolism (44), it is no surprise that Nse5–Nse6 has multiple roles. One tempting hypothesis is that during meiosis, the primary role of Nse5–Nse6 is to stimulate the Mus81–Eme1 HJ resolvasome and that during mitotic growth it plays both this and a second role. During both stages of the life cycle, expression of RusA suppresses at least partially the nse6Δ phenotype (Figures 1D and 6C and D), indicating that HJ resolution is stimulated by Nse5–Nse6 in both stages. The second role of Nse5–Nse6 might be to regulate any of the multiple functions of the Smc5–Smc6 complex (44). Further investigation is required to elucidate this function, but the requirement for Rad51 and Nse6 but not Mus81–Eme1 in mitotic DSB repair (Figure 1) suggests that Nse5–Nse6 is also important for the synopsis phase of mitotic DSB repair.

Although the Nse1 and Nse2 E3 ligase activities, like Nse5–Nse6, facilitate mitotic DNA repair (79–81), neither of these E3 ligases is required for meiotic nuclear division and recombination [(82) our unpublished data]. Thus, although the mitotic functions of Nse1, Nse2 and Nse5–Nse6 have not been dissected, in meiosis, Nse5–Nse6 acts independently of posttranslational modifications catalyzed by Nse1 and Nse2. Nevertheless, on the basis of our previous analyses showing that hypomorphic mutants of the essential Smc5–Smc6 subunits exhibit catastrophic meioses (83), we propose that Nse5–Nse6 acts in conjunction with the Smc5–Smc6 holocomplex to execute its meiotic HR role.

Budding yeast smc5–smc6 mutations also disrupt meiotic nuclear division (84), but the underlying defects appear strikingly different from those of nse6Δ fission yeast. Key differences are that a spo11 (rec12 homolog) mutation is not epistatic to an smc5–smc6 mutation, and crossovers are not affected in smc6 temperature-sensitive (Ts) mutant cells (84). Thus, the authors concluded that the crucial role of Smc5–Smc6 is executed during premeiotic S phase in budding yeast and not after the initiation of meiotic recombination. It is unclear whether this reflects real differences in the functions of Smc5–Smc6 between species or if the unavoidable disruption of both the essential and repair roles in smc6 (Ts) budding yeast masks functions analogous to those of fission yeast Nse5–Nse6.

HJ resolution must be carefully controlled, both during mitotic growth and in meiosis. When a DNA strand lesion blocks mitotic replication, the fork can regress and form a structure whose center is identical to that of an HJ. Were it resolved by Mus81–Eme1, a DSB would be formed and require further processing to allow completion of replication. Alternatively, the regressed fork can migrate back to the original position and allow immediate continuation of replication. Thus, Mus81–Eme1 may be kept inactive during this time. If strand exchange between sisters or homologs forms an HJ that is present at the time of mitosis, HJ resolution would appear to be the most expedient means to allow chromosome segregation, and Mus81–Eme1 may be activated at this time [e.g. (19)]. Similarly, during meiosis, many dozens of HJs must arise to account for the ~45 crossovers in a fission yeast cell (85), and Mus81–Eme1 is clearly highly active in meiotic cells. We surmise that Nse5–Nse6, likely as part of the Smc5–Smc6 complex, regulates directly or indirectly the activity of Mus81–Eme1 allowing it to function at the proper time and place to maintain chromosome integrity and cell viability.

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