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A Protein Complex Containing Mei5 and Sae3 Promotes the Assembly of the Meiosis-Specific RecA Homolog Dmc1

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Summary

Meiotic recombination requires the meiosis-specific RecA homolog Dmc1 as well as the mitotic RecA homolog Rad51. Here, we show that the two meiosis-specific proteins Mei5 and Sae3 are necessary for the assembly of Dmc1, but not for Rad51, on chromosomes including the association of Dmc1 with a recombination hot spot. Mei5, Sae3, and Dmc1 form a ternary and evolutionary conserved complex that requires Rad51 for recruitment to chromosomes. Mei5, Sae3, and Dmc1 are mutually dependent for their chromosome association, and their absence prevents the disassembly of Rad51 filaments. Our results suggest that Mei5 and Sae3 are loading factors for the Dmc1 recombinase and that the Dmc1-Mei5-Sae3 complex is integrated onto Rad51 ensembles and, together with Rad51, plays both catalytic and structural roles in interhomolog recombination during meiosis.

Introduction

During meiosis, recombination gives rise to crossovers and noncrossovers. Only a crossover between homologous chromosomes ensures the segregation of the chromosomes during meiosis division I. Meiotic recombination is initiated by the formation of double-strand breaks (DSBs) at recombination hot spots (Keeney et al., 1997). The ends of the DSB are processed to produce 3′OH overhanging single-stranded (ss) DNAs. For the crossover-specific pathway, one of the DSB ends interacts with a homologous duplex in homologous chromosomes but not with that in sister chromosomes, resulting in the formation of a product referred to as a single-end invasion intermediate (SEI; Hunter and Kleckner, 2001). The capture of the second DSB end by the SEI, accompanied by further processing, generates an intermediate with double Holliday structures. The Holliday structures are programmed to resolve into crossovers (Allers and Lichten, 2001; Schwacha and Kleckner, 1995). The formation of SEIs is proposed as a critical commitment step for "interhomolog"-specific crossover pathways (Hunter and Kleckner, 2001; Börner et al., 2004). Interestingly, only one end participates in SEI formation, suggesting a functional difference between the DSB ends and/or the independence of the ends. In addition, all of the biochemical processes of meiotic recombination are tightly coupled with chromosome morphogenesis (for a review, see Zickler and Kleckner [1999]). The crossovers mature into a chromosome axis exchange, i.e., chiasmata.

Meiotic recombination requires the two RecA homologs Rad51 and Dmc1 (Bishop et al., 1992; Shinohara et al., 1992), which are conserved from yeast to humans. The coordinated actions of Rad51 and Dmc1 are considered to play a critical role in homology searches and strand exchange during recombination. Rad51 forms a right-handed helical filament on ssDNAs, known as the nucleoprotein filament, and promotes a robust strand exchange in vitro (Symington, 2002). However, Dmc1 shows poor in vitro strand exchange activity compared to Rad51 (Hong et al., 2001; Masson et al., 1999). Dmc1 forms an octameric ring structure, which binds to DNAs (Passy et al., 1999; Kinebuchi et al., 2004). Dmc1-ring might be an inactive form. A recent biochemical study shows that human Dmc1 promotes robust strand exchange in vitro by forming a filament on ssDNA under particular conditions (Sehorn et al., 2004). Irrespective of the biochemical similarities between Rad51 and Dmc1, they play distinct roles in meiotic recombination (Shinohara et al., 1997). Dmc1 seems to be specialized to promote recombination between homologs.

Both Rad51 and Dmc1 are detected as an immunostaining structure on chromosomes, known as the "focus." Genetic analyses of focus formation suggest that the foci are sites of recombination and DSB repair (Gasior et al., 1998). Indeed, we recently showed that a focus containing Rad51 marks a site of mitotic DSBs and is an intermediate during DSB repair (Miyazaki et al., 2004). Rad51 and Dmc1 foci are readily colocalized on chromosomes. Interestingly, under some conditions, Rad51 and Dmc1 show a side-by-side staining pattern, suggesting independent complex formation of the two RecA homologs (Shinohara et al., 2000). Immunoelectron microscopy reveals that Rad51 and Dmc1 are components of a densely stained structure on the chromosome axes in zygotene, referred to as a recombination nodule (RN; Anderson et al., 1997; Tarsounas et al., 1999). The presence of proteineous structures containing the RecA homologs on the chromosome axis suggests that recombination is carried out by a large protein complex, which might play both a catalytic role and a structural role in the interaction between homologous chromosomes.

The assembly pathway for Rad51 on ssDNAs is well documented based on cytological and biochemical analyses (Gasior et al., 2001; e.g., Figure 7). The assembly and activity of Rad51 is promoted by various factors, such as RPA, Rad52, Rad55-57, Rad54, and Tid1/Rdh54 (Pâques and Haber, 1999; Symington, 2002). Once an ssDNA is formed, RPA binds to the DNA to remove the secondary structure of the ssDNA, which indirectly

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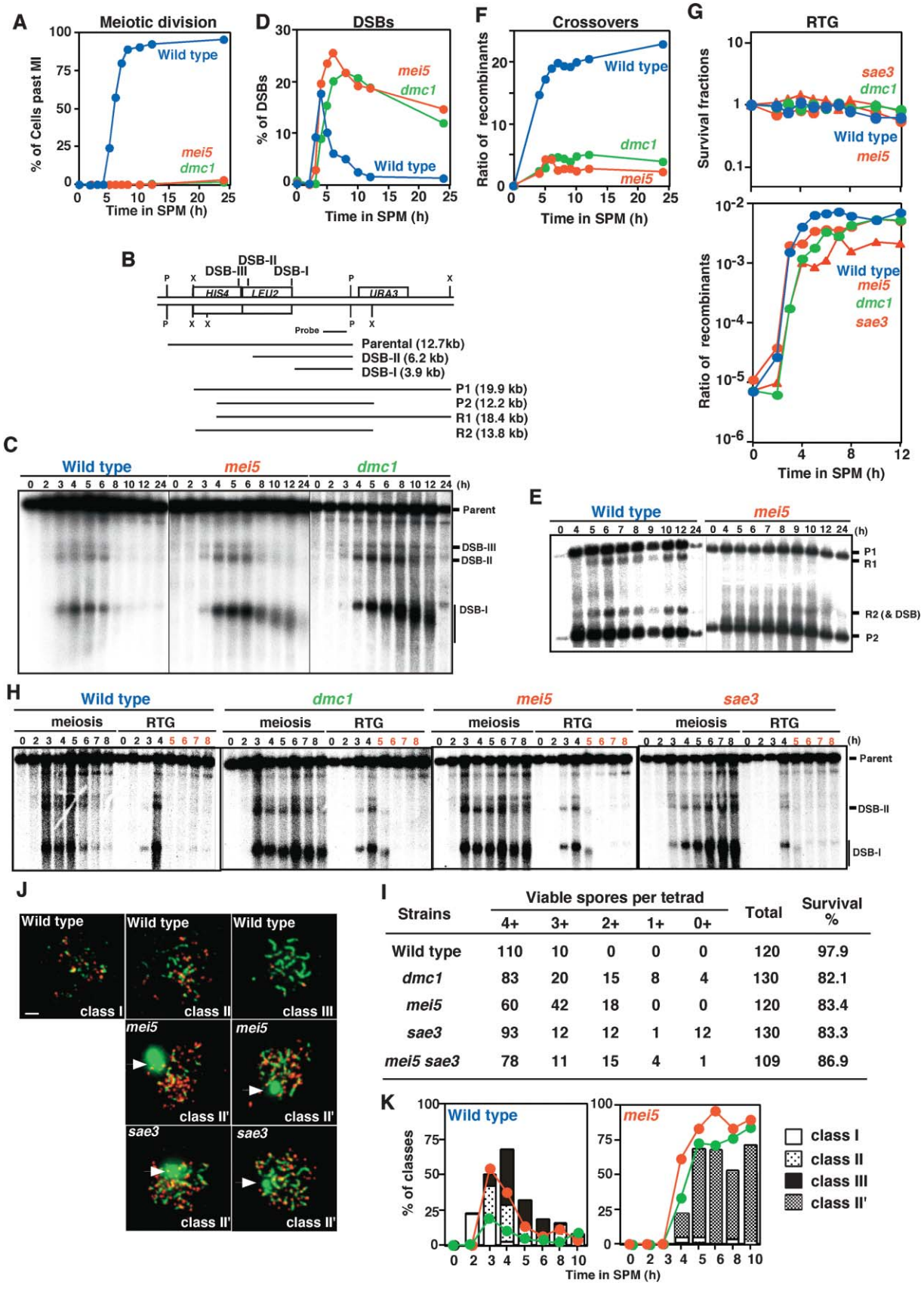


Strains	Viable spores per tetrad					Total	Survival %
	4+	3+	2+	1+	0+		
Wild type	110	10	0	0	0	120	97.9
dmc1	83	20	15	8	4	130	82.1
mei5	60	42	18	0	0	120	83.4
sae3	93	12	12	1	12	130	83.3
mei5 sae3	78	11	15	4	1	109	86.9

promotes the formation of a Rad51 nucleoprotein filament. However, Rad51 by itself cannot bind to RPA-coated ssDNAs. Rad52 helps the loading of Rad51 on RPA-coated ssDNA and the assembly of the Rad51 nucleoprotein filament. Rad55-57 might help the assembly of Rad51 and/or stabilize the Rad51 filament. Proteins that help the loading of Rad51 and the assembly of the nucleoprotein filaments on DNAs are referred to as mediators (Gasior et al., 2001). Rad51 nucleoprotein filament, working with Rad54, carries out homology search between the ssDNA and a homologous duplex DNA and exchanges the strands.

The assembly process for Dmc1 is poorly understood. Previous studies showed that Rad51 is necessary for efficient Dmc1 focus formation, while Dmc1 is not necessary for Rad51 focus formation (Bishop, 1994; Shinohara et al., 1997). Tid1/Rdh54, which interacts with both Rad51 and Dmc1 (Dresser et al., 1997), is required for the coordinated actions of Rad51 and Dmc1 (Shinohara et al., 2000). In addition, the meiosis-specific complex Mnd1-Hop2 works in a *DMC1*-dependent recombination pathway (Chen et al., 2004; Tsubouchi and Roeder, 2002; Zierhut et al., 2004). However, Tid1/Rdh54 and Mnd1-Hop2 are necessary for the postassembly stage of Rad51 and Dmc1 on chromosomes.

In this report, we identified two meiosis-specific proteins, Mei5 and Sae3, which facilitate Dmc1 loading to a Rad51 complex on DNAs. Mei5 and Sae3 form an evolutionary conserved heterocomplex, which works together with Dmc1 during meiotic recombination.

Results

The *mei5* and *sae3* Mutants Show Identical Phenotypes to the *dmc1*

To look for the genes that may function in meiotic recombination together with Dmc1, we searched for mutations conferring a *dmc1*-like phenotype, e.g., meiotic prophase arrest. Previous whole-scale genome analyses indicated that the *mei5* mutant exhibits a prophase arrest (Enyenihi and Saunders, 2003; Marston et al., 2004; C. Giroux, personal communication). We constructed a null mutant for the *MEI5* gene, whose open reading frame (ORF) is deleted in the SK-1 background. The *mei5* null mutant shows an arrest at the prophase of meiosis I (Figure 1A), as reported, and accumulates meiotic DSBs at a recombination hot spot (the *HIS4-LEU2* locus; Figure 1B) with more resected ends than the wild-type (Figures 1C and 1D), as seen in the mutant of the *DMC1* gene (Bishop et al., 1992). In addition, the mutant forms very few crossover recombinants at the *HIS4-LEU2*, as measured by physical analysis (Figures 1E and 1F). These observations suggest that *MEI5* facilitates the conversion of the DSBs into recombinants.

A previous study showed that the *sae3-1* mutant accumulates meiotic DSBs that trigger meiotic prophase arrest, similar to *dmc1* and *mei5* (McKee and Kleckner, 1997). We confirmed these previous observations for a *sae3* null mutant. The *sae3* null mutant exhibits prophase arrest (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/7/927/DC1/). DSBs in the *sae3* null mutant appear as in the wild-type, but, during further incubation, they accumulate more processed ends than those in the wild-type (Supplemental Figure S1).

We further compared the meiotic phenotypes of the *mei5* and *sae3* mutants with those of *dmc1*. Previous studies showed that a high copy of either the *RAD51* or *RAD54* gene suppresses the spore inviability of the *dmc1* mutant (Bishop et al., 1999; Tsubouchi and Roeder, 2003). We found that a high copy of *RAD51* largely suppresses the meiotic cell arrest conferred by either the *mei5* or the *sae3* and increases the viability of the mutants as high as 82%–83% (Figure 1I). In addition, return-to-growth experiments indicate that, as for *dmc1*, both the *mei5* and *sae3* mutants maintain the viability of cells during incubation with sporulation medium (SPM) and can repair the meiotic DSBs when they resume mitotic growth, which was shown by an increase in prototroph formation and the disappearance of DSBs in Southern blotting (Figures 1G and 1H).

The *dmc1* mutant is defective in synaptonemal complex (SC) formation in the SK-1 strain background (Bishop, 1994). We examined the SC formation in both *mei5* and *sae3* mutants by staining with an antibody against Zip1, a central component of SC (Sym et al., 1993), together with Rad51. The wild-type shows Zip1 foci early in the process (class I; leptotene) and then partially elongated Zip1 staining with a number of Rad51 foci (class II; zygotene). Later, fully elongated Zip1 linear elements, which correspond with full-length SCs, appear with a few Rad51 foci (class III; pachytene) and

Figure 1. Mei5 Is Required for Meiotic DSB Repair and Shares the Same Properties with Dmc1 and Sae3

(A) DAPI analysis of meiosis progression in various strains. Wild-type, blue circles; *mei5*, red circles; *dmc1*, green circles.

(B) Schematic drawing of a recombination hot spot, the *HIS4-LEU2*. P, PstI; X, XhoI.

(C and D) Meiotic DSBs in wild-type, *mei5*, and *dmc1* cells were analyzed by Southern blotting (C) and quantified (D).

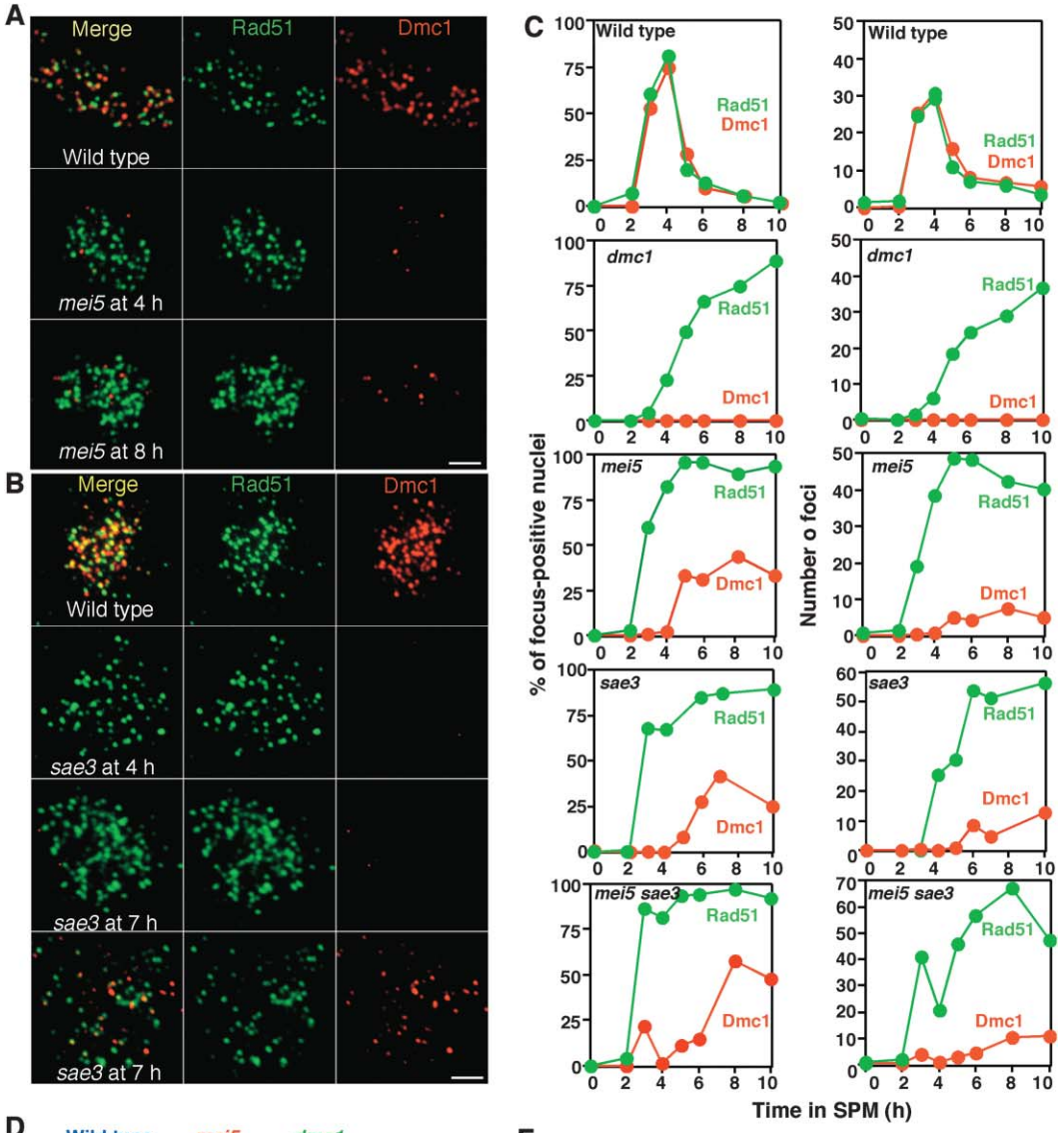
(E and F) Crossover formation was examined in the wild-type and the *mei5* mutant (E), and ratios of the product, R1, to a parental fragment, P1, were quantified (F).

(G) Return-to-growth (RTG) assay. Cell survival (upper panel) and His^+ prototroph formation (lower panel) were analyzed at each time point. Wild-type, blue circles; *mei5*, red circles; *dmc1*, green circles; *sae3*, red triangles.

(H) DSB repair in RTG. DSBs at the *HIS4-LEU2* were analyzed by Southern blotting. Media were changed from SPM to YPD after a 4 hr incubation. Times after YPD shift are shown in red.

(I) Spore viability of *dmc1*, *mei5*, and *sae3* single and *mei5* and *sae3* double mutants with a high copy number of Rad51 (YEp-based vector) was analyzed as described (Shinohara et al., 2003b).

(J and K) SC formation in the *mei5* and *sae3* mutants. Nuclear spreads were stained with anti-Zip1 (green) and anti-Rad51 (red) and categorized as described (Börner et al., 2004; Shinohara et al., 2003b). Class I (open bars), Zip1 dots with a few Rad51 foci; class II (dotted bars), partial Zip1 linear with lots of Rad51 foci; class III (closed bars), linear Zip1 staining with a few Rad51; class II′ (hatched bars), partial Zip1 linear with lots of Rad51 foci with Zip1 aggregates. Rad51 foci, red circles; Zip1 polycomplex, green circles. A polycomplex is shown by an arrow. Scale bars, 2 μm.


A
Merge
Rad51
Dmc1
Wild type
mei5 at 4 h
mei5 at 8 h
B
Merge
Rad51
Dmc1
Wild type
sae3 at 4 h
sae3 at 7 h
sae3 at 7 h
C
Wild type
dmc1
mei5
sae3
mei5 sae3
Rad51
Dmc1
% of focus-positive nuclei
Number o foci
Time in SPM (h)
D
Wild type
mei5
dmc1
0 4 0 4 0 4 (h)
Dmc1
tubulin
E
DSB-II
DSB-I
HIS4
LEU2
0.9kb
2.1kb
F
Wild type
dmc1
mei5
sae3
WCE
IP
0 4 5 8 0 4 5 8
(h)
Rad51
Dmc1
G
IP:Rad51
IP:Dmc1
% of IP per total
Wild type
dmc1
sae3
mei5
(h)

then disappear (Figures 1J and 1K). Both the *mei5* and *sae3* mutants are defective in the formation of full-length SCs. The chromosome spreads of the mutants rarely contain Class III, which is often found after 4 hr of incubation in the wild-type (Figure 1K). On the other hand, the *mei5* and *sae3* mutants accumulate partially elongated Zip1 staining with a lot of Rad51 foci (Figures 1J and 1K, and see Supplemental Figure S1 on the *Cell* web site). However, this is not the typical class II observed for the wild-type (referred as to class II′), since they accumulate an aggregated Zip1 protein known as a polycomplex. These data indicate that the mutants are defective in Zip1 elongation. Thus, like the *dmc1* mutant, both *mei5* and *sae3* mutants are defective in chromosome synapsis.

The *mei5* and *sae3* mutants are different from those of the genes that are specifically required for the crossover-specific pathway, called *zmm* mutants (Börner et al., 2004). The *mei5* and *sae3* mutants are deficient in the formation of noncrossovers as well as crossovers (Supplemental Figure S2), while the *zmm* mutant is only defective in crossover formation. In addition, *mei5* and *sae3* mutants do not show a temperature-sensitive defect in meiosis, which is a characteristic of the *zmm* mutants (Börner et al., 2004). The *mei5* mutant is defective in DSB repair and shows a prophase arrest at both 23°C and 33°C (A.H. and A.S., unpublished data).

Both *MEI5* and *SAE3* Are Required for the Assembly of Dmc1

Immunostaining analysis of the chromosome spreads reveals that the *mei5* mutant accumulates Rad51 foci (Figure 2A), which mark ongoing recombination sites (Gasior et al., 1998; Miyazaki et al., 2004), consistent with the accumulation of meiotic DSBs in the mutant. In the *mei5* mutant, Rad51 foci appear that are the same as the wild-type, but these never disappear and in fact become brighter during further incubation (Figures 2A and 2C), indicating that Mei5 is necessary for the timely disassembly of Rad51. On the other hand, the *mei5* mutant is defective in the assembly of Dmc1 on meiotic chromosomes (Figure 2A). The mutant shows no Dmc1 foci on the chromosomes after 4 hr in SPM, while a wild-type strain exhibits a peak of Dmc1 focus formation at this time. There is a gradual increase in Dmc1 focus-positive cells as time progresses, but the percentage of Dmc1-positive nuclei and the average focus number per nucleus are greatly reduced compared to those of Rad51 (Figure 2C). These results indicate that *MEI5* is required for the efficient loading of Dmc1 but not for that of Rad51. Western blotting analysis indicates that *mei5* cells do express Dmc1 protein during meiosis (Figure 2D). Thus, the effect of the *mei5* on Dmc1 assembly is not due to the inability of the mutant cells to express Dmc1. These data suggest that Mei5 is a novel assembly factor for the meiosis-specific Dmc1 protein.

Cytological analysis also reveals that the *sae3* cells form Rad51 foci on meiotic chromosomes but never dismantle the foci (Figures 2B and 2C). More importantly, the *sae3* null mutant is deficient in Dmc1 focus formation, as is the *mei5* mutant (Figures 2B and 2C). There is no Dmc1 focus formation after 4 hr in the *sae3*, where wild-type cells show a peak in the focus formation. The *sae3* mutant also exhibits residual Dmc1 focus in late stages. These data suggest that Sae3 as well as Mei5 is necessary for efficient assembly of Dmc1 on meiotic chromosomes.

We found that a *mei5 sae3* double mutant showed identical phenotypes to either single mutant. The *mei5 sae3* double mutant is proficient in Rad51 focus formation but deficient in Dmc1 focus formation, as in the case of the *mei5* and *sae3* single mutants (Figure 2C). Furthermore, spore inviabiity of the *mei5 sae3* double mutant is suppressed by a high copy number of *RAD51* (Figure 1I). These results indicate that *MEI5* and *SAE3* belong to the same epistasis group.

The Association of Dmc1 to a Recombination Hot Spot Depends on Mei5 and Sae3

To extend the results of the above cytological analysis, we examined binding of the Dmc1 protein to recombination hot spots by chromatin immunoprecipitation (ChIP) analysis. Previous ChIP results indicated that Dmc1 associates with meiotic recombination hot spots (Blat et al., 2002; D. Bishop, personal communication). We examined the binding of Dmc1 to a recombination hot spot called *HIS4-LEU2* (Figure 2E). In the wild-type, both Dmc1 and Rad51 transiently bind to a site 0.9 kb from a major DSB locus but not to a site 3 kb from the DSB locus (Figures 2F and 2G). The kinetics of the bindings are very similar to those of focus formation for Rad51 and Dmc1. Furthermore, both *mei5* and *sae3* mutations abolish the binding of Dmc1 to a site near the DSB. On the other hand, in the case of the mutants, Rad51 binds to the site but does not dissociate from the DSB even after an extended period. These observations suggest that Mei5 and Sae3 promote the loading of Dmc1 to DSBs.

Mei5 Is Colocalized with Dmc1 and Rad51 on Meiotic Chromosomes

To analyze the behavior of Mei5 protein during meiosis, a strain with a Flag epitope tag at the C terminus of the *MEI5* ORF was constructed that shows wild-type spore

Figure 2. Mei5 and Sae3 Are Required for Assembly of Dmc1 and Binding of Dmc1 to Hot Spots

(A and B) Chromosome spreads from various strains were immunostained with anti-Rad51 (green) and anti-Dmc1 (red). (A) Wild-type and the *mei5*; (B) wild-type and the *sae3*. Scale bars, 2 μm.

(C) Percentages of focus-positive cells (with more than five foci; left panels) and average numbers of foci per nucleus (right panels) were plotted. More than 100 randomly selected nuclei were counted at each time point. Rad51 foci, green; Dmc1 foci, red.

(D) Expression of Dmc1 (upper panel) and tubulin (lower panel) in various strains was analyzed by Western blotting.

(E–G) ChIP analysis of binding of Rad51 and Dmc1 to meiotic DSBs. Positions of primer sets in a schematic diagram of the *HIS4-LEU2* locus are indicated by red and green (E). DNAs from whole-cell extracts (WCE) and immunoprecipitates (IP) with either anti-Rad51 (upper panel) or anti-Dmc1 anti-sera (lower panel) were analyzed at each time point (F) and quantified (G) as described in Experimental Procedures.

viability and similar repair kinetics for the meiotic DSBs to those observed in the wild-type (data not shown). The expression of Mei5-Flag protein is induced during meiosis (Figure 3A). There is little expression of the Mei5-Flag in vegetative cells. These data are consistent with the fact that the *MEI5* mRNA is induced in meiotic cells (*Saccharomyces* genome database, http://www.yeastgenome.org). Next, using the Mei5-Flag construct, we analyzed the localization of the Mei5 protein on the nuclear spreads by staining with an anti-Flag antibody. Mei5-Flag exhibits punctate staining on chromosomes after a 4 hr incubation with SPM (Figure 3B). The presence of the Mei5 foci is dependent upon the addition of the tag to the *MEI5* gene (data not shown). This result indicates that the Mei5 protein is associated with chromosomes during meiosis. The staining pattern of Mei5 is confirmed by immunostaining analyses using two independent anti-Mei5 antibodies (Figure 3C). The staining detected with anti-Mei5 is abolished in the *mei5* null mutant (data not shown), confirming the specificity of these antibodies. Mei5 foci begin to appear after 2 hr incubation with SPM, peak at 4 hr, and then disappear (Figure 3D). The kinetics of assembly and disassembly of the Mei5 foci are very similar to those of Rad51 or Dmc1 and thus to the kinetics of meiotic DSBs (Figure 3D). The average number of Mei5 foci at 3 hr is 43 $\pm$ 11 (n [*n*umber of focus-positive nucleoids analyzed] = 72), while that of Rad51 is 44 $\pm$ 9.2 (n = 72). Mei5 focus assembly requires DSB formation. The *spo11-Y135F* mutation at a catalytic residue of Spo11 for DSB formation (Keeney et al., 1997) abolished Mei5 focus formation (Figure 3D). We also studied Mei5 focus formation in the *ndt80* cells, which shows an arrest in pachytene (Xu et al., 1995). With the *ndt80*, as with the Rad51 foci, Mei5 foci appear and disappear as seen in wild-type (Figure 3D). No Mei5 foci are observed late in the incubation for the *ndt80* mutant, consistent with the formation of Mei5 foci in leptotene and zygotene.

Next, we examined the colocalization of Mei5 with Rad51. Mei5 cohabited with Rad51 with a frequency of 62% (n = 51) after a 4 hr incubation (Figures 3B and 3C). This frequency is higher than that of the random colocalization frequency (12%, calculated with a Dotstat program; Gasior et al., [1998]). Independent staining reveals 63% (n = 45) colocalization of Rad51 and Dmc1 under these conditions. We could not analyze the colocalization of Mei5 with Dmc1 directly, because both of the antisera against both of the Dmc1 and Mei5 were raised in rabbits. The colocalization of Mei5 with Dmc1 could be studied using the *MEI5-Flag* diploid. The Mei5-Flag shows 57% (n = 36) colocalization with Dmc1 after 4 hr. These results suggest that Mei5 functions together with both Rad51 and Dmc1 on meiotic chromosomes. The fact that the colocalization frequencies are not 100% suggests that each focus contains different compositions of proteins and/or that each protein shows different kinetics of assembly and disassembly from focus to focus. Alternatively, the reactivity of the antibodies might vary from focus to focus. The result that more than 90% of Rad51 and Mei5 are finally colocalized in the *mnd1* mutant (see below), which stalls recombination at the postassembly phase of the RecA homologs, supports the former possibility.

ChIP indicates that Mei5 is transiently associated with a meiotic recombination hot spot, the *HIS4-LEU4* locus. The kinetics of association of Mei5 to the hot spot are similar to those of Rad51 and thus to those of DSBs (Figure 4A). Indeed, the *spo11-Y135F* mutation not only eliminates the binding of Rad51 but also of Mei5 to the DSB site. Taken together, this observation suggests that Mei5 is a component of meiosis-specific recombination machinery working together with the RecA homologs.

DMC1 and *MEI5* Are Interdependent for Assembly on Chromosomes

In order to understand the relationship between Mei5 and Dmc1, we next examined the effect of a *dmc1* null mutation on Mei5 focus formation. The *dmc1* mutant severely impairs the formation of Mei5 foci (Figures 3C–3E). There is no Mei5 focus formation after 4 hr, when wild-type cells show a peak in the focus formation. As a control, we confirmed the accumulation of Rad51 foci in the mutant. Similar results were obtained for the staining of *dmc1 MEI5-Flag* mutant cells with anti-Flag (data not shown). These indicate that the *DMC1* function is required for efficient loading of Mei5 on chromosomes. Thus, Dmc1 and Mei5 are mutually dependent for chromosome association.

The *mnd1* and *hop2* mutants show similar phenotypes to the *dmc1* as well as to the *mei5* mutant, i.e., accumulation of meiotic DSBs (Tsubouchi and Roeder, 2002; Zierhut et al., 2004). To determine the relationship of Mei5 with Mnd1-Hop2, we examined the effect of an *mnd1* mutation on Mei5 focus formation. The *mnd1* mutant forms Mei5 foci as in the wild-type and eventually accumulates Mei5 foci together with Rad51 (Figures 3C and 3D), in agreement with the fact the *mnd1* mutant accumulates Dmc1 foci (Zierhut et al., 2004). The number and intensity of Mei5 foci in the *mnd1* are increased during further incubation. After an 8 hr incubation, more than 90% of the Mei5 is colocalized with Rad51. Therefore, the Mnd1 function is not necessary for assembly but is required for the timely disassembly of Mei5 (and RecA homologs). This result suggests that the Mnd1 (and possibly Hop2) is required for the step after the Mei5 and Dmc1.

Previous studies indicated that efficient loading of Dmc1 onto meiotic chromosomes is largely dependent upon Rad51 (Bishop, 1994; Shinohara et al., 1997). We studied the effect of a *rad51* null mutation on the formation of Mei5 foci. Mei5 focus formation is greatly reduced in the *rad51* mutant (Figure 3F). Therefore, *RAD51* is not only necessary for loading of Dmc1 but also for Mei5. It is known that the *rad51* mutant exhibits a residual assembly of Dmc1 (Shinohara et al., 1997). Even under conditions in which Dmc1 foci could be detected, the *rad51* mutant shows very little Mei5 focus formation (Figure 3G).

The above cytological experiments suggest that Mei5 forms a functional unit with Dmc1 in vivo. In order to examine possible complex formation of Dmc1 with Mei5, we carried out the immunoprecipitation (IP) of lysates from Mei5-Flag cells with anti-Dmc1 antibodies. An endogenous Flag-specific protein was found in precipitated fractions with anti-Dmc1 in meiotic cells but not in mitotic cells (Figure 4B, upper panel). The protein was not recovered when cells without the Flag on Mei5 and

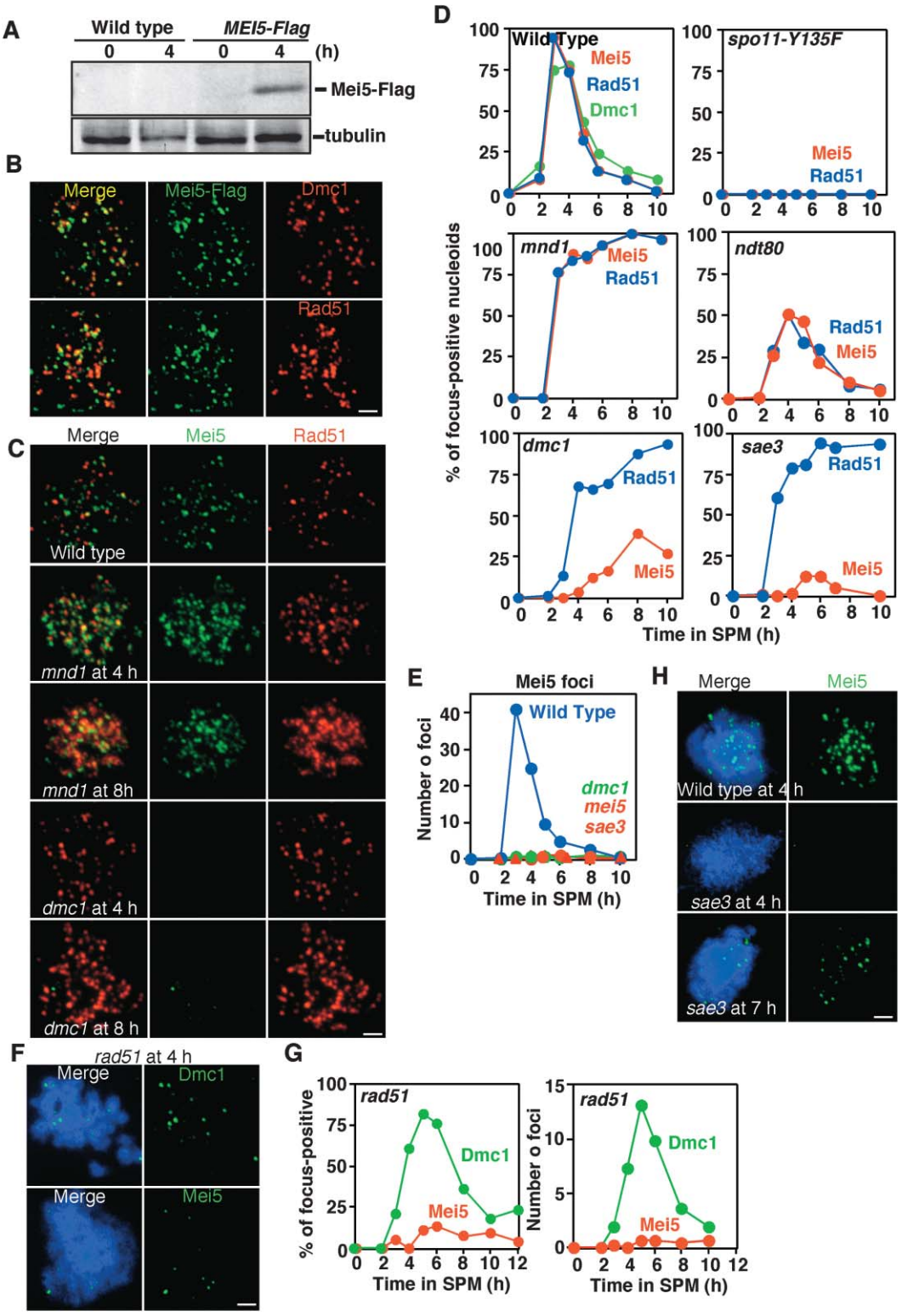


Figure 3. Mei5 Is Recruited to Immunostaining Structures

(A) Expression of Mei5-Flag (upper panel) and tubulin (lower panel) as controls in both mitosis and meiosis were analyzed by Western blotting.

(B) Nuclear spreads from *MEI5-Flag* diploid cells at 4 hr in SPM were stained with anti-Flag antibody (green) together with either anti-Rad51 or anti-Dmc1 (red). Scale bars, 2 μm.

(C–E) Focus formation of Mei5 in various strains was analyzed by immunostaining using anti-Mei5 (green) with control staining of Rad51 (red) (C). Scale bars, 2 μm. More than 100 nuclei were analyzed at each time. In wild-type, Dmc1 is stained independently to analyze the kinetics of focus formation. Percentages of focus-positive cells (with more than five foci; [D]) and average numbers of foci per nuclei (E) were plotted.

(F and G) Focus formation of Mei5 as well as Dmc1 in the *rad51* mutant was examined (F) and quantified (G) as described above. Dmc1 or Mei5, green; DAPI, blue.

(H) Mei5 focus formation in the *sae3* mutant. Mei5, green; DAPI, blue.

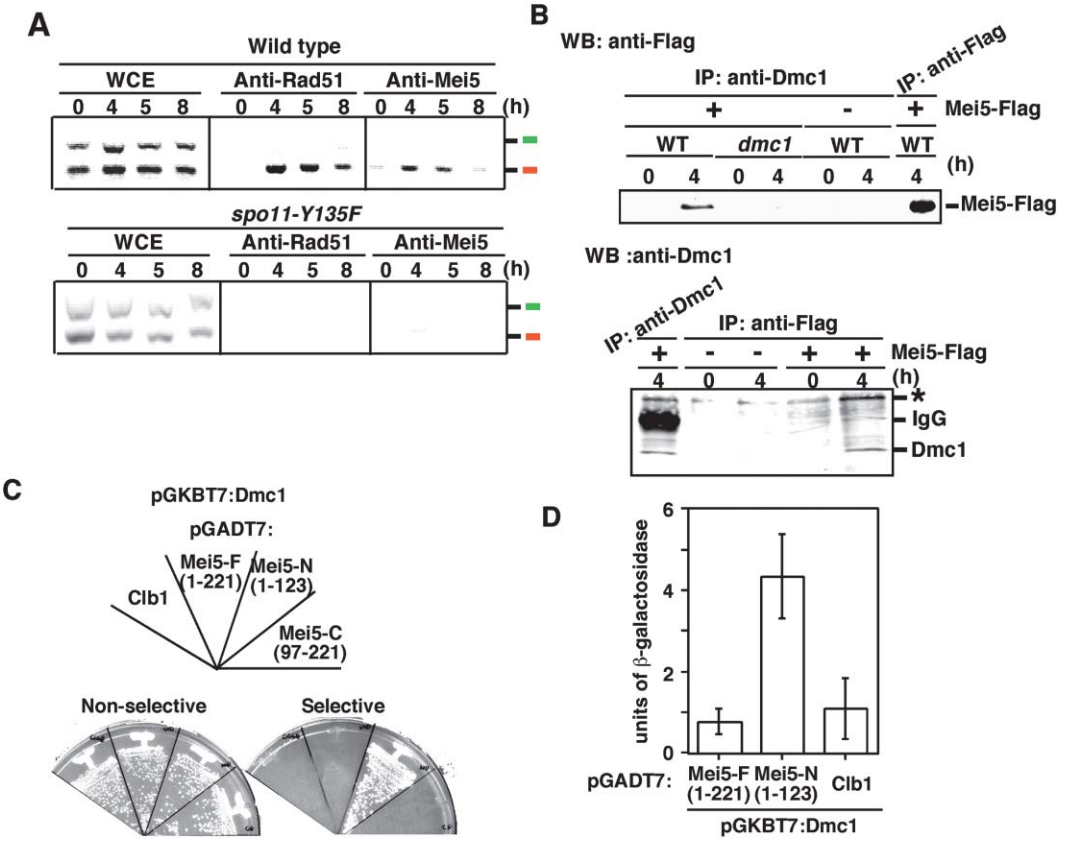


Figure 4. Association of Mei5 with DSB and Interaction with Dmc1

(A) ChIP analysis of Mei5 and Rad51 was carried out using wild-type (upper panel) and the *spo11-Y135F* (lower panel) cells as described in Figure 2E.

(B) Immunoprecipitation (IP) was carried out using *MEI5-Flag*, *dmc1 MEI5-Flag*, and wild-type (without the Flag tag) diploid cells at either 0 or 4 hr. Mei5-Flag (upper panel) or Dmc1 (lower panel) was detected by Western blotting (WB). Asterisks indicate nonspecific bands.

(C and D) Two-hybrid analysis of Dmc1 and Mei5. A full-length, an N-terminal half (1–123), and a C-terminal half (97–221) of Mei5 were cloned into pGADT7 and examined for the interaction with a full-length Dmc1 on pGKBT7 in AH109 strain. A nonselective plate and selective plates lacking both histidine and adenine were used (C). Quantification of the two-hybrid analysis is presented in (D) (an average of three independent experiments).

the *dmc1* mutant with the *MEI5-Flag* were used for IP. A reciprocal IP experiment indicates the presence of endogenous Dmc1 in the fractions precipitated with the anti-Flag antibody (Figure 4B, lower panel). Control experiments show that the anti-Flag and the anti-Dmc1 antibodies could precipitate Mei5-Flag and Dmc1, respectively. These data indicate the presence of a complex containing both Mei5 and Dmc1 in vivo.

Since the efficiency of IP is not high (only about 5% of Mei5-Flag in lysates was recovered in the IP fractions), we carried out two-hybrid analysis to elucidate the interaction between Dmc1 and Mei5. Although a full-length Mei5 protein (1–221) cannot interact with a full-length Dmc1 in the assay, we found that the N-terminal half (1–123) of Mei5 binds with a full-length Dmc1 but not with Clb1 as a control. On the other hand, the C-terminal (97–221) half of Mei5 did not interact with the Dmc1 (Figures 4C and 4D). This result supports and extends the above observation, suggesting that Dmc1 forms a complex with Mei5 through the N-terminal domain of Mei5.

Sae3, Mei5, and Dmc1 Are Mutually Dependent for Chromosome Loading

Similar phenotypes conferred by *mei5* and *sae3* mutations prompted us to examine the relationship between the two proteins. We first analyzed the effect of a *sae3* mutation on Mei5 focus formation. As expected, the *sae3* mutant greatly reduces focus formation of Mei5 (Figures 3D and 3H). Thus, *SAE3* is required for efficient assembly of Mei5, as well as Dmc1, on chromosomes.

Although the *SAE3* gene was reported to encode a putative 50 amino acid protein (Figure 5A; McKee and Kleckner, 1997), comparative analyses of different yeast genomes suggest conservation of a noncoding 3′ sequence of the hypothetical *SAE3* ORF (*Saccharomyces* genome database, http://www.yeastgenome.org). RT-PCR analysis reveals the presence of an intron in this gene (Figure 5B). The analysis of meiotic cDNAs confirms the presence of an intron with 86 nucleotides. The branched and 3′ acceptor sequences of the *SAE3* intron are different from the consensus sequences for yeast splicing (Figure 5A), suggesting that splicing of the *SAE3*

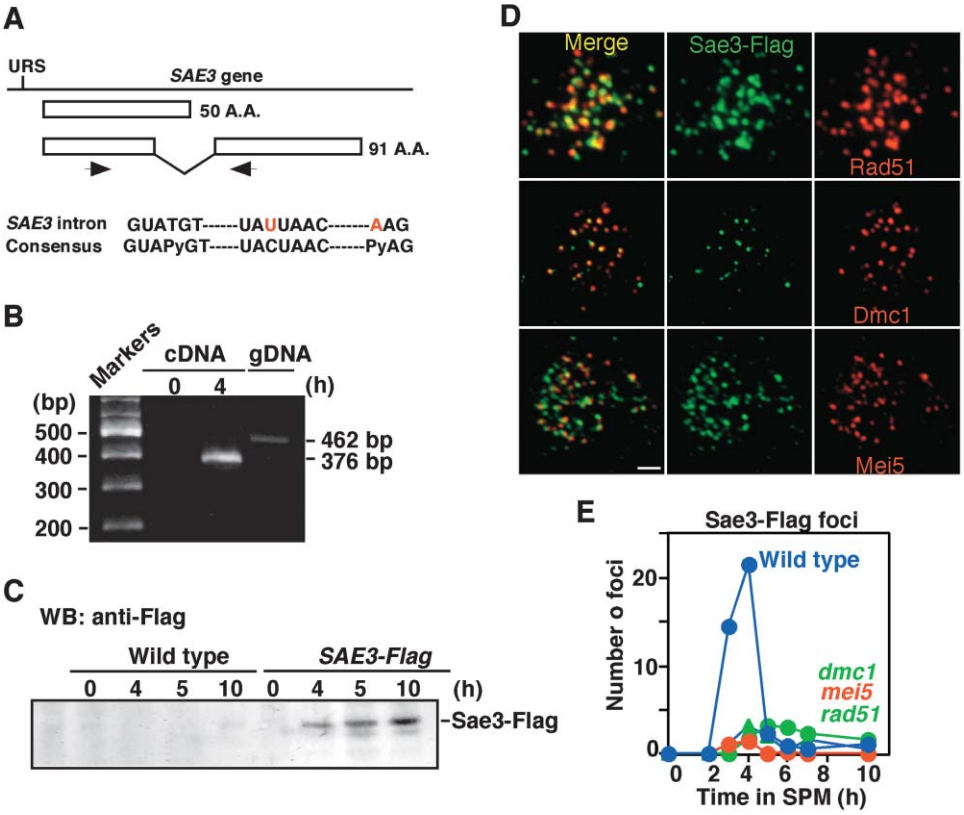


Figure 5. Expression of *SAE3* Gene and Sae3 Focus Formation

(A) Schematic drawing of the *SAE3* locus. The positions of ORF and putative intron sequences necessary for splicing of the *SAE3* gene are shown. Differences in nucleotides in the *SAE3* from consensus sequences for splicing are presented in red. Positions of primers used for RT-PCR analysis are shown by arrows.

(B) RT-PCR analysis of RNAs at 0 and 4 hr incubation with SPM. cDNAs at 0 and 4 hr and genomic DNAs (gDNA) were amplified by PCR using primers described above. DNA size markers are shown at left.

(C) Expression of Sae3-Flag during meiosis was analyzed by Western blotting using anti-Flag antibody.

(D) Sae3-Flag focus formation was examined in a wild-type background after a 4 hr incubation with SPM. Staining of Flag epitope (green) was carried out simultaneously together with that of Rad51, Dmc1, and Mei5 (red).

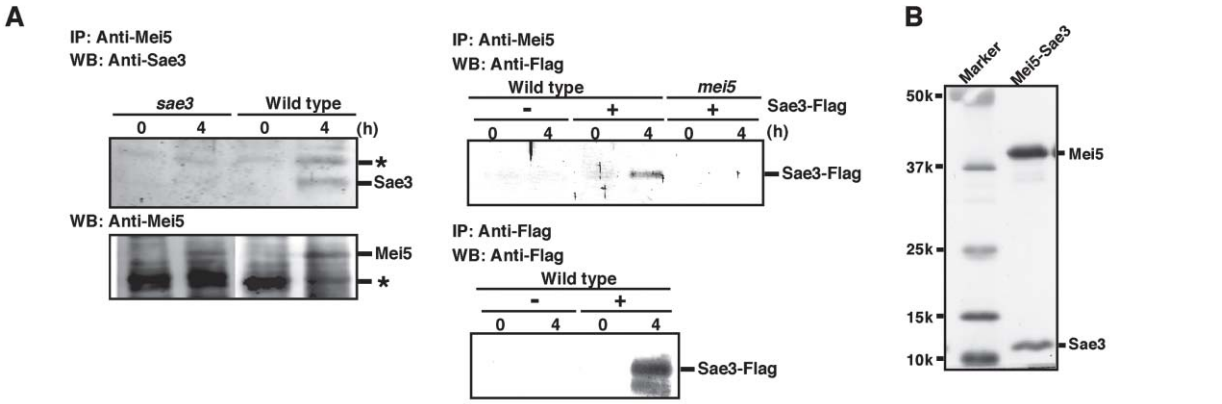
(E) Sae3-Flag focus formation in various strains. Kinetics of the number of Sae3-Flag foci were analyzed in wild-type, *dmc1*, *mei5*, and *sae3* mutants.

intron might be regulated in a meiosis-specific manner, as seen in other meiosis-specific recombination genes such as *MER2*, *MER3*, and *HOP2* (e.g., Nakagawa and Ogawa [1999]). As a result of splicing, the *SAE3* cDNA encodes a 91 amino acid protein. DNA sequencing shows that the *sae3-1* mutation is a C-to-T transition at position 48, which converts a glutamine codon at 16 to the ochre codon. We inserted a Flag tag into the C terminus of the ORF and confirmed the expression of this tagged protein during meiosis by Western blotting (Figure 5C), consistent with meiosis-specific expression of the *SAE3* mRNA (McKee and Kleckner, 1997). The protein detected on the blot has the expected relative molecular mass of 18,700 (with the tag). The presence of the Flag tag does not seem to interfere with Sae3 function, since *SAE3-Flag* diploid cells show a wild-type level of spore viability.

Immunostaining of the *SAE3-Flag* nuclear spreads reveals that Sae3-Flag also transiently forms foci on meiotic chromosomes (Figure 5D). The average number of Sae3-Flag foci at 4 hr is 30 $\pm$ 12 (n = 54), slightly lower than that of Rad51, Dmc1, and Mei5 foci. This might be due to epitope masking. Sae3 foci are often colocalized with Rad51 or Dmc1 foci with a frequency of 48% (n = 56; Figure 5D). We also examined the effect of the *dmc1*, *mei5*, and *rad51* mutations on the formation of Sae3-Flag foci. All three mutants greatly reduced the numbers of Sae3-Flag foci on the chromosome compared to those in wild-type (Figure 5E). Thus, Sae3, Mei5, and Dmc1 are mutually interdependent for chromosome association, consistent with the idea that Dmc1, Mei5, and Sae3 form a complex whose assembly is promoted by the action of Rad51.

Mei5 and Sae3 Form a Complex Both In Vivo and In Vitro

In order to understand the relationship between Mei5 and Sae3, we analyzed colocalization of a Mei5 focus with a Sae3-Flag. Immunostaining reveals that the Sae3-Flag shows extensive colocalization with Mei5 after a 4 hr incubation (Figure 5D). Sixty-two percent (n = 34) of the Mei5 foci are colocalized with the Sae3 foci. Further-



C

Sae3/Swi5 homologs

```
Sae3   1-91  MNYLETQLNKKQKQIQEYESMNGNLIKMFEQLSKEKKNDETPKKISSTYEKELKEYNELRDAGLRLAQIIADEKQCKIKDVFEEIGYSMKD
Swi5   1-85        MEKSQLESRVHLLEQQKEQLESSLQDALAKLKNRLAKQTVQKHIDLLHTYNEIRDIALGMIGKVAEHEKCTSVELFDRFGVNGSE
mouse  1-89  MIDENNDVSE-EALSSDIKKLKEKHDMLDKEISQLIAEGYRVIELEKHISLLHEYNDIKDVSQMLLGKLAVTRGVTTKELYPDFDLNLND
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D

Mei5/Sfr1 homologs

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Mei5     1- 63        MHNQEEWLDKDKTLVNE-EENTCIN------------------HSYTKKDTNNYRVGKSGIKDLK-KPTNQKEIAIKNRELTK
Sfr1   105-191  KEFKSPLRQTASPQVADTNLKPSLAV-DNLNSDETNTSSEPVTSPLRTTPNSIKRQKRLFKSPISNCLNPKSDP-EITQLLSRRLKLEK
Human   73-148  KEASSTEENCLEFQESFKHIDSEFEENTNLKNTLKNLNVCESQS-LDSGSTRLTVSSPERCSALQNEFVSEKLP-KQR-LNAEKAKLVK

                                          W97
Mei5     64-190 QLTLLRQENNHLQQACKIL-SENKIIENRKSIEKWRTICEMELSFILNSTLIKINRMGGY-KDFLEKEMEAKKRRLEYQIDNGMEDQIC
Sfr1   192-279  EVRNLQEQLITAETARKVE-AKNEDKDLQTLIQKWKNAAQQAAEVLFKPMAERIRLAGGVTQSFRIEEGDNKGQIQEVRTEFTMSMFLN
Human  149-230  QVQEK-EDLLRRLKLVKMYRSKNDLSQLQLLIKKWRSCSQLLLYELQSAVSEE-NKKLSLTQLIDHYGLDDKLLHYNRSEEEFIDV*

Mei5   191-222  EIKESDDFRQLSEVEKQEWESQMNEQLKELEKKKIAELEKLNKVLHDSEGKDFGMAELC*
Sfr1   280-299  QFGVPVHLMSFDE-ENGDWKS*
```

Figure 6. Mei5 and Sae3 Form a Complex Both In Vivo and In Vitro

(A) In vivo complex formation of Mei5-Sae3 was analyzed by immunoprecipitation using wild-type, *sae3*, *SAE3-Flag*, and *mei5 SAE3-Flag* cells. Anti-Mei5 or Anti-Flag was used for the precipitation. Sae3 and Sae3-Flag were detected using anti-Sae3 and anti-Flag antibodies, respectively. Asterisks indicate nonspecific bands.

(B) Both Mei5 and Sae3 were expressed in *E. coli* and purified. Coomassie blue staining of purified Mei5-Sae3 (2 μg) with marker proteins. The sizes of the markers are indicated at left.

(C) Sequence alignment of Sae3, *S. pombe* Swi5, and a putative mouse homolog of Sae3/Swi5 (AAH21748.1). Identical and homologous amino acids between Sae3 and the possible homologs are boxed in red and blue, respectively. Identical amino acids between the homologs are boxed in black.

(D) Sequence comparison of Mei5, *S. pombe* Sfr1, and a putative human homolog of Mei5/Sfr1 (AAH20892.1). Identical and homologous amino acids between Mei5 and the possible homologs are boxed in red and blue, respectively.

more, endogenous Sae3 or Sae3-Flag proteins were immunoprecipitated with anti-Mei5 antibodies, indicating the presence of a complex containing both Mei5 and Sae3 in vivo (Figure 6A). The control experiments show that the anti-Mei5 does not precipitate the Sae3-Flag in the *mei5* mutant.

The expression of Mei5 protein alone in *E. coli* produces an insoluble Mei5 protein. When Mei5 was coexpressed with Sae3, which is soluble in *E. coli*, then the Mei5 also became soluble, suggesting a functional interaction between Mei5 and Sae3 (A.S., unpublished data). Indeed, both Mei5 and Sae3 were copurified together in at least three column chromatography experiments (Figure 6B). These results indicate that Mei5 and Sae3 can form a tight complex in vitro.

Discussion

Mei5 and Sae3 Form a Loading Complex Specific to Dmc1

Our studies described here have identified a protein complex involved in meiotic recombination, which contains two meiosis-specific proteins, Mei5 and Sae3. The *mei5* and *sae3* mutants share very similar phenotypes with each other and belong to the same epistasis group. Immunostaining shows that Mei5 and Sae3 are colocalized very well on meiotic chromosomes. The localization of Mei5 and Sae3 is mutually dependent. Indeed, Sae3 is coimmunoprecipitated with Mei5. Furthermore, when coexpressed in *E. coli*, Mei5 and Sae3 are purified as a complex. These observations indicate that the Mei5 and Sae3 form a functional complex.

The Mei5-Sae3 complex works specifically with a meiosis-specific RecA homolog Dmc1. The *mei5*, *sae3*, and *dmc1* mutants confer identical meiotic defects. Importantly, all three mutants are defective in the conversion of DSBs to recombination products. In addition, the three genes are transcribed specifically during the meiotic prophase, whose expression is under the control of a meiosis-specific enhancer sequence. Importantly, both Mei5 and Sae3 are necessary for the efficient loading of Dmc1 on recombination hot spots but are not required for Rad51. Furthermore, the association of Mei5 and Sae3 on chromosomes depends upon the *DMC1*.

The IP and two-hybrid experiments indicate a physical interaction between Dmc1 and Mei5, suggesting that Dmc1 and Mei5-Sae3 function together as a complex. This is supported by the mutual interdependence of Dmc1, Mei5, and Sae3 for focus formation.

The Mei5-Sae3 complex not only promotes the Dmc1 assembly but also works with the RecA homolog. The mediators are a class of proteins involved in the promotion of assembly on DNAs (Gasior et al., 2001). The defects of the *mei5* and *sae3* mutants in Dmc1 binding to the hot spots suggest that Mei5-Sae3 is a mediator specific for Dmc1. The colocalization of either Mei5 or Sae3 with Dmc1 in the wild-type implies the presence of a Dmc1 complex containing Mei5-Sae3 on the chromosomes. A complex containing all three proteins seems to work in homology search and strand exchange, since extensive colocalization of Mei5 and Rad51 (thus, Dmc1) is observed even in the mutants that are deficient in strand invasion, e.g., the *mnd1* mutant. Given that a Dmc1-dependent pathway is required for crossover interference in budding yeast (Shinohara et al., 2003b), Mei5-Sae3 might play a role in crossover control. Although Dmc1, Mei5, and Sae3 seem to work together, immunostaining analysis of the wild-type reveals that not all three proteins are colocalized very well on chromosomes. In addition, the brightness of each staining is different from focus to focus. This result suggests that each complex on the chromosomes is heterogeneous in the composition of its proteins. It is possible to consider that this reflects the different kinetics of the assembly and disassembly of the proteins in each focus, even in a single nucleus. Alternatively, this could be explained by the different accessibility of antibodies to the proteins in the focus.

Two RecA homologs are a component of the RN on zygotene chromosome axes (Anderson et al., 1997; Tarsounas et al., 1999). Therefore, Mei5-Sae3 is also likely to be a component of RNs. The RN bridges the homologous chromosomes during zygotene (Zickler and Kleckner, 1999). The protein machinery might play a critical role in promoting the formation of the interhomolog connection.

Dmc1 forms an octameric ring structure (Kinebuchi et al., 2004). Given the intimate relationship between Dmc1 and Mei5-Sae3, it is possible that Dmc1, associated with Mei5-Sae3, might form a different structure and might exhibit more enhanced activity. Recently, it was shown that human Dmc1 forms a filament on ssDNAs under high salt conditions (Sehorn et al., 2004). Mei5-Sae3 might assist Dmc1 filament formation to promote the recombination activity (Figure 7). Further biochemical and structural analyses of this complex might shed light on the mechanism of meiotic recombination, which depends on a unique RecA homolog, Dmc1.

Integration of Dmc1 into Rad51 Assembly Is a Critical Biochemical Transition in Meiotic Recombination

The phenotypes of the *mei5* and *sae3* mutants suggest the presence of a biochemical step that couples the Rad51 assembly with the Dmc1 assembly (Figure 7). This might be a conversion step of a mitotic recombinase with Rad51 into a meiotic version with both Rad51 and Dmc1. Two-hybrid analysis fails to detect any interaction between yeast Rad51 and Dmc1 (Dresser et al., 1997). Immunostaining reveals that the two RecA homologs show side-by-side localization on chromosomes (Shinohara et al., 2000), suggesting that Rad51 and Dmc1 bind to meiotic DSBs by forming independent complexes. It is proposed that the two RecA homologs form an asymmetrical complex at ends of the DSB (Shinohara et al., 2000). We do not currently know whether or not this is true. If true, how is Dmc1 recruited to the DSBs and distributed in an asymmetric way with respect to Rad51? Rad51 promotes the recruitment of Mei5-Sae3 as well as Dmc1. However, there is a significant difference in the effects of Rad51 on the assembly of Mei5-Sae3 and Dmc1 on chromosomes. The *rad51* mutant shows residual Dmc1 focus formation but greatly reduced focus formation of Mei5 and Sae3 (Figures 3G and 5E), suggesting the presence of a Dmc1 assembly

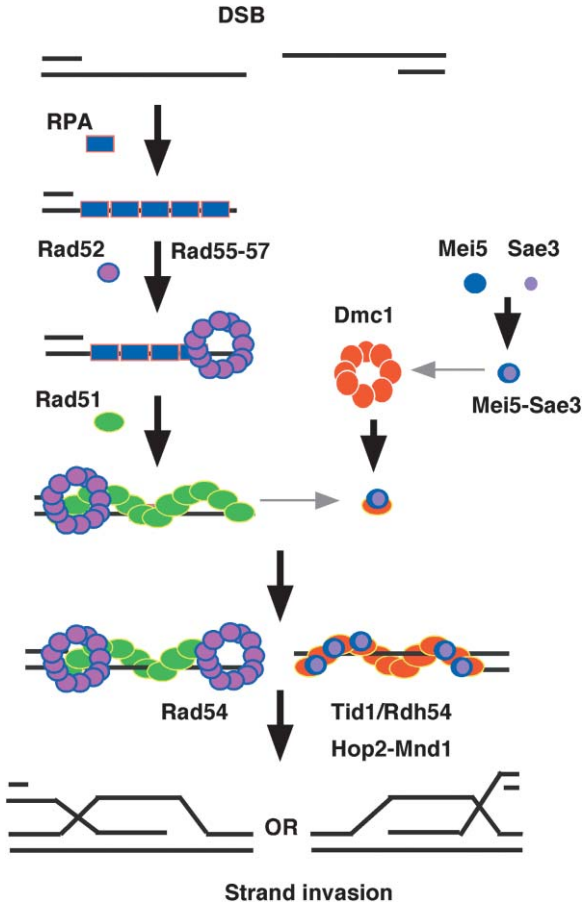

Figure 7. Assembly Pathway of Rad51 and Dmc1

Rad51 is recruited to RPA-coated ssDNAs in the aid of two mediators, Rad52 and Rad55-57 (Gasior et al., 1998; Miyazaki et al., 2004). Mei5 and Sae3 form a heterocomplex and may convert an inactive Dmc1 ring into an active complex by forming a ternary complex containing Dmc1 and Mei5-Sae3. Rad51 nucleoprotein filament then promotes the assembly of Dmc1-Mei5-Sae3 complex on a DSB end, resulting in the asymmetric distribution of Rad51 and Dmc1 complexes. Together with other proteins such as Rad54, Tid1/Rdh54, and Hop2-Mnd1, this large protein machinery with the two RecA homologs facilitates strand invasion of ssDNA into a homologous duplex.

pathway in the absence of Mei5 and Sae3 under these conditions. Although we cannot exclude differences in the sensitivity of the antibodies used in the immunostaining, the result indicates more strict dependency of Rad51 for Mei5-Sae3 assembly than for Dmc1 assembly. One attractive possibility is that Mei5-Sae3 might bridge the two RecA homologs structurally and/or functionally. Furthermore, since the *rad51* mutant does form a joint molecule between sister chromosomes rather than between homologous chromosomes (Schwacha and Kleckner, 1997), the Dmc1(-Rad51) complex formed in the absence of Mei5-Sae3 might lack the activity to carry out interhomolog recombination.

Sae3 and Mei5 Homologs Are Conserved among Organisms

A previous study suggests that the putative Sae3 protein is homologous to the *S. pombe* Swi5 protein, which is involved in DNA damage repair (Akamatsu et al., 2003). Correct assignment of the *SAE3* intron reveals more homology between Sae3 and Swi5 than that reported previously. Homology between the two is 65% over entirety of both proteins (Figure 6C). A putative homolog of Sae3/Swi5 is found in other organisms, including humans (Figure 6C). Swi5 is known to interact with Swi2 and Sfr1 proteins, both of which can bind to *S. pombe* Rad51. Swi2 and Sfr1 are partially redundant in DNA repair but have a distinct function in mating type switching (Akamatsu et al., 2003). Our initial database search detected Mei5 homologs, which only occur in *Saccharomyces* relatives and not in other organisms. The homology between Sae3 and Swi5 prompted us to undertake a comparison of Mei5 with Swi2 and Sfr1. Indeed, Mei5 shares a significant homology over 200 amino acids with Swi2 and Sfr1, particularly with Sfr1 (Figure 6D). Using the homologous sequence between Mei5 and Sfr1, we could find a possible protein homologous to Mei5 and/or Sfr1 in fish, frogs, rats, mice, and humans (Figure 6D; data not shown). The conserved region of these proteins contains a coiled-coiled motif (our unpublished data) and binding domains of Swi2 with both Swi5 and Rad51 (Akamatsu et al., 2003) as well as the Dmc1-interacting region of Mei5. Indeed, substitution of the conserved tryptophan residue at 97 in *S. cerevisaie* Mei5 abolishes the Mei5 function (T.M. and A.S., unpublished data).

Together with Sfr1-Swi5 and Swi2-Swi5, Mei5-Sae3 could form a new family of protein complexes involved in recombination and DNA repair, which would function as a loading factor for RecA homologs. Based on the analogy with Mei5-Sae3, we suggest that both Sfr1-Swi5 and Swi2-Swi5 promote the assembly and strand exchange activity of Rad51, possibly by forming a complex with Rad51. We stress that, despite the structural similarity between Sfr1-Swi5 and Mei5-Sae3, the functions of the complexes seem to be specialized for either Rad51 or Dmc1. Sfr1-Swi5 and Swi2-Swi5 seem to be specific mediators for Rad51, possibly even in meiosis. The *swi5* mutant reduces meiotic recombination and displays more similar phenotypes to *rad51* than *dmc1* single or *rad51 dmc1* double mutants (Young et al., 2004). Although fission yeast requires Dmc1 for meiotic recombination, the Dmc1 loading in this yeast might be mechanistically different from that in budding yeast.

Experimental Procedure

Strains and Plasmids

All strains described here are derivatives of SK-1 diploids and NKY1551 (*MATα/MATa, lys2/", ura3/", leu2::hisG/", his4X-LEU2-URA3/his4B-LEU2, arg4-nsp/arg4-bgl*) and are described in Supplemental Table S1. *mei5* and *sae3* null alleles were constructed by PCR-mediated gene disruption using the *KlURA3* gene (Reid et al., 2002) and the *KanMX4* (Wach et al., 1994), respectively. *MEI5-Flag* and *SAE3-Flag* were constructed by a PCR-based tagging methodology (De Antoni and Gallwitz, 2000).

Cytology

Immunostaining was performed as described previously (Shinohara et al., 2000). See Supplemental Data for details.

Analyses of Meiotic Recombination

Time course analyses of DNA events in meiosis and cell cycle progression were performed as described previously (Shinohara et al., 2003a). The return-to-growth experiment was carried out as previously (Shinohara et al., 1997). A high-copy suppression by Rad51 was assayed as reported (Shinohara et al., 2003b).

ChIP and Western Blotting

ChIP was carried out as described previously (Zeirhut et al., 2004). At each time point, 15 ml of cultures was withdrawn and fixed with 1% (V/V) of formaldehyde at 25°C for 15 min and then quenched by the addition of glycine (final 125 mM). Cell extracts were prepared by glass bead disruption and processed for ChIP. See Supplemental Data for details.

Two-Hybrid Analysis

A full length of Dmc1, Mei5, Clb1, and a N-terminal (1–123) and C-terminal half (97–221) of Mei5 are cloned into two-hybrid vector, pGBKT7, and pGADT7 (Clonetech) by inserting a PCR-amplified fragment into the vectors. See Supplemental Data for details.

cDNA Analysis of the *SAE3*

Meiotic cells (wild-type; NKY1551) after a 4 hr incubation with SPM were used for mRNA preparation using the RNeasy kit (Qiagen). A *SAE3* cDNA was reverse transcribed by the Superscript II reverse transcriptase (Invitrogen) using Oligo-$(dT)_{20}$ and PCR amplified with a *SAE3*-specific reverse primer.

Protein Purification

Mei5 and Sae3 were produced in *E. coli* and purified using three column chromatography. See Supplemental Data.

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