The bakers's yeast Msh4-Msh5 associates with double-strand break hotspots and

chromosome axis during meiosis to promote crossovers

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ABSTRACT

Segregation of homologous chromosomes during the first meiotic division requires at

least one obligate crossover/exchange event between the homolog pairs. In the baker's yeast

Saccharomyces cerevisiae and mammals, the mismatch repair-related factors, Msh4-Msh5 and

Mlh1-Mlh3 generate the majority of the meiotic crossovers from programmed double-strand

breaks (DSBs). To understand the mechanistic role of Msh4-Msh5 in meiotic crossing over, we

performed genome-wide ChIP-sequencing and cytological analysis of the Msh5 protein in cells

synchronized for meiosis. We observe that Msh5 associates with DSB hotspots, chromosome

axis, and centromeres. We found that the initial recruitment of Msh4-Msh5 occurs following

DSB resection. A two-step Msh5 binding pattern was observed: an early weak binding at DSB

hotspots followed by enhanced late binding upon the formation of double Holliday junction

structures. Msh5 association with the chromosome axis is Red1 dependent, while Msh5

association with the DSB hotspots and axis is dependent on DSB formation by Spo11. Msh5

binding was enhanced at strong DSB hotspots consistent with a role for DSB frequency in

promoting Msh5 binding. These data on the *in vivo* localization of Msh5 during meiosis have

implications for how Msh4-Msh5 may work with other crossover and synapsis promoting factors

to ensure Holliday junction resolution at the chromosome axis.

AUTHOR SUMMARY

During meiosis, crossovers facilitate physical linkages between homologous chromosomes that ensure their accurate segregation. Meiotic crossovers are initiated from programmed DNA double-strand breaks (DSBs). In the baker's yeast and mammals, DSBs are repaired into crossovers primarily through a pathway involving the highly conserved mismatch repair related Msh4-Msh5 complex along with other crossover promoting factors. *In vitro* and physical studies suggest that the Msh4-Msh5 heterodimer facilitates meiotic crossover formation by stabilizing Holliday junctions. We investigated the genome-wide *in vivo* binding sites of Msh5 during meiotic progression. Msh5 was enriched at DSB hotspots, chromosome axis, and centromere sites. Our results suggest Msh5 associates with both DSB sites on the chromosomal loops and with the chromosome axis to promote crossover formation. These results on the *in vivo* dynamic localization of the Msh5 protein provide novel insights into how the Msh4-Msh5 complex may work with other crossover and synapsis promoting factors to facilitate crossover formation.

INTRODUCTION

During meiosis in baker's yeast, crossover formation is initiated from programmed DNA double-strand breaks (DSBs) made by Spo11 and accessory proteins [1-3]. Crossovers, together with sister chromatid cohesion, facilitate homologous chromosome segregation by physically linking the homologs and properly orienting them towards opposite poles of the bipolar spindle [4]. A fraction of the DSBs are repaired using the homologous chromosome as a template to generate crossovers, and the rest are repaired either as noncrossovers using the homolog or using the sister chromatid as template [5, 6]. In humans, failure to obtain at least one crossover per homolog pair can lead to aneuploid gametes that result in birth defects in offspring [7].

Meiotically induced DSBs are non-randomly positioned in the genome. Multiple factors, including open chromatin structure, the presence of certain histone modifications, and the binding of sequence-specific transcription factors are known to influence the spatial positioning of DSBs [8, 9]. In *S. cerevisiae*, during meiotic prophase, chromosomes are organized into a series of loops that are tethered at the base by proteinaceous axial elements mainly consisting of proteins Red1 and Hop1 and the meiotic cohesion complex containing Rec8 [10-15]. The chromatin loops originating from the cohesin enriched axis regions have conserved density (~20 loops per micron of axis length) across organisms [12, 16]. In *S. cerevisiae* loops are between 0.2 to 0.6 um long (from axis to the distal-most sequence in the loop) containing up to 20 kb of DNA [12, 17]. Within the Red1-rich axis domains, DSBs are made at a distance from the axis on the loops [16]. But DSB-promoting proteins are associated with axis regions as well, indicating that DSB formation takes place in the context of the loop-axis structure of the chromosomes [18, 19]. Based on these findings, it has been hypothesized that chromosomal

loops are recruited to the proximity of the axis for DSB formation and repair [20-22]. This

allows events at both loop and axis levels to be functionally linked and coordinated [16, 17].

The chromosome axis proteins influence the formation of DSBs, promote interhomolog bias, and

facilitate the biased resolution of double Holliday Junction (dHJ) intermediates [23].

In *S. cerevisiae*, the major pathway for the repair of DSBs as crossovers is mediated by

the ZMM group of proteins (Zip1, Zip2, Zip3, Zip4, Mer3, Msh4, Msh5, and Spo16) along with

MutLγ (Mlh1 and Mlh3), Exo1, and the STR complex (Sgs1, Rmi1, Top3) to facilitate the

formation of ~80% of crossovers [24-28]. The ZMM-mediated crossovers show interference and

are called class I crossovers. A minor set of crossovers that are interference-independent (class

II) are produced by a pathway involving the Mms4-Mus81 protein complex, which can directly

act on D-loops or Holliday junction intermediates to form crossovers [29, 30].

The Msh4 and Msh5 proteins are homologs of the bacterial MutS family of mismatch

repair proteins with no known function in mismatch repair [31]. The Msh4 and Msh5 proteins

function together as a heterodimer [32]. In *S. cerevisiae*, both *msh4Δ* and *msh5Δ* mutants show a

substantial reduction in meiotic viability along with crossover defects and Meiosis I non-

disjunction [30, 33]. *In vitro* biochemical studies using purified human Msh4 and Msh5 show

that these proteins bind to Holliday junction substrates and stabilize them [34]. Similarly, *in*

vitro biochemical and physical studies in *S. cerevisiae* show that Msh4-Msh5 stabilizes 3'-

overhangs, single-end invasion (SEI) intermediates, and Holiday junctions [24, 35]. Further,

Msh4 has a degron, which is phosphorylated by cdc7 kinase to stabilize the Msh4-Msh5 complex

and promote a crossover outcome [36]. Recent genetic, biochemical, and physical studies

suggest that the Msh4-Msh5 complex acts in the same pathway as the Mlh1-Mlh3 complex,

Exo1, and PCNA to process inter homolog joint molecule structures into crossover products [27,

37-41]. The Msh4-Msh5 complex has a role in directing the activity of the Mlh1-Mlh3

endonuclease to generate crossovers from double Holliday junctions [27, 40, 41]. In mammals,

mutation of *Msh4* or *Msh5* results in sterility [42, 43]. Cell biological observations in the mouse

show a large number of Msh4/Msh5 foci (~ 140 per nucleus) during an early stage (Zygotene) of

meiosis [44]. As meiosis progresses, the number of Msh4/Msh5 foci decreases, and at mid

pachytene stage, they are present at almost twice the number of actual crossing over sites. At

this stage, almost half of the Msh4/Msh5 foci co-localize with Mlh1/Mlh3 foci, which are

considered to be the sites for crossover [42, 45, 46]. Although few aspects of Msh4-Msh5

function are known, an understanding of the *in vivo* DNA binding sites, recombination substrates

and localization with reference to the chromosomal organization during meiosis is lacking.

We analyzed the chromosome-binding properties of the Msh4-Msh5 complex using

chromatin immunoprecipitation and sequencing (ChIP-Seq), ChIP-qPCR, and cytology in

synchronized meiotic time courses. We found that Msh5 is specifically enriched at DSB

hotspots [18]. Additionally, Msh5 binding is also observed at the chromosome axis and

centromeres. Msh5 binding at DSB hotspots and chromosome axis is disrupted in *spo11*

mutants, while axis association is disrupted in *red1Δ*. Msh5-binding sites on chromosomes are

similar to Zip3 and the ZZS complex (Zip2, Zip4, Spo16) [47-49]. Further Msh5 binding at

chromosome axis and centromeres also suggest crossover-independent functions related to

chromosome synapsis. Our observations suggest that Msh5 is enriched at strong DSB hotspots

that have high DSB frequency. These results shed novel insights into the role of the

chromosome loop-axis structure on the assembly of the Msh4-Msh5 complex during meiosis.

They are also useful for developing models for how Msh4-Msh5 may coordinate both crossover

and synapsis events.

RESULTS

Genome-wide analysis shows Msh5 binding at DSB hotspots, chromosome axis, and

centromeres

Msh5 protein expression starts at 2h after induction of meiosis and remains till 8h with

peak expression at 5h (**Fig 1A**). To characterize the dynamic binding of Msh5 during the

different stages of meiosis, we performed ChIP-Seq from meiotic extracts using an anti-Msh5

antibody. The Msh5 antibody was generated in rabbit and is polyclonal. 5ul Msh5 serum was

used for each ChIP-Seq experiment. No Msh5 protein band was observed in the *msh5Δ* lysate or

eluate fractions, showing the specificity of the antibody (**Fig 1B**). Msh5 ChIP-Seq was

performed in quadruplicate at six-time points, from 2h to 7h at one-hour intervals. The Msh5

binding data from the four replicates (a-d) at the 5h time point was highly reproducible (r = 0.72-

0.93) (**S1 A-F Fig**). Msh5 ChIP-Seq data from the four replicates were normalized using input,

and genome-wide plots were generated to detect Msh5-peak positions along the chromosomes

(Materials and Methods). A total of 3397 Msh5 peaks were observed from all four replicates

(with $p < 10^{-5}$, see Materials and Methods) at 5h post entry into meiosis (**S1 Table**). Previous

studies of the ZMM proteins have shown that Zip3, as well as the ZZS complex (Zip2, Zip4,

Spo16) mostly associate with DSB hotspots and show weak and transient association with the

chromosome axis [47]. Msh5 (and Msh4) functions as a distinct complex from Zip3 and ZZS

[47, 50]. We therefore analyzed Msh5 binding with reference to DSB hotspots [18] and

chromosome axis sites [15]. Representative Msh5 binding plot from all four replicates for

chromosome III at 3h, 4h, and 5h, shows Msh5 associates with DSB hotspots, chromosome axis

sites, and also centromere position while being depleted from DSB coldspots (**S2A Fig**). The

Msh5 binding sites were validated using qPCR. Binding of Msh5 at three representative DSB

hotspots (*BUD23*, *ECM3*, *CCT6*), three axis sites (*Axis I*, *Axis II* and *Axis III*), two centromere

locations (*CENI, CENIII*) and one coldspot (*YCRO93W*) were analyzed from the same time

course in one of the replicates (d). The qPCR results confirm that Msh5 is specifically enriched

at DSB hotspots, chromosome axis and centromeres relative to DSB cold spots (**Fig 1C** and **S2B**

Fig). The qPCR data was further used to scale the corresponding Msh5 ChIP-Seq replicate (d)

(Materials and Methods) (**S3A Fig**). From the scaled Msh5 ChIP-Seq data, maximum Msh5

binding is observed between 3-5h relative to other time points (6h, 7h) across all chromosomal

locations consistent with Msh5 expression (**Fig 2A**). The scaled Msh5 ChIP-Seq plot is shown

for chromosome III as a representative example (**Fig 2B**). We observed that Msh5 binds weakly

to DSB hotspots at an early stage (3h) after induction of meiosis (**Fig 2B**). At later stages of

meiotic progression (4h and 5h) maximum Msh5 binding was seen at DSB hotspots (**Fig 2B**).

Statistical analysis of the scaled Msh5 coverage data at DSB hotspots (non-overlapping with

Red1 sites) showed binding differences at 3h, 4h, and 5h are significantly different (t-test) (**Fig**

2C). Further, maximum Msh5 binding at DSB hotspots is seen at 4h -5h (**Fig 2C**). Msh5 peaks

from all four replicates associated with DSB hotspots at 5h showed a median width of 0.5kb

(mean 0.9kb) (**Fig 2D**). This result suggests that Msh5 binding at DSB hotspots is confined to

the width of the heteroduplex region during DSB repair (1-2 kb) [51].

We analyzed binding data of Msh5 in meiotic time courses at axis sites (Red1 binding

regions) [15]. Representative plot for chromosome III shows the overlap of Msh5, and Red1

reads from 3h to 5h (**Fig 2B**). Msh5 binding at Red1 sites (non-overlapping with Spo11 sites)

from 3h to 5h is significantly different between the time points with maximum binding at 5h (**Fig**

3A). These observations suggest Msh5 binds to both DSB hotspots and axis from 3h to 5h.

Together, these observations support Msh5 association with both DSB sites at chromosomal

loops and axis regions.

184   In addition to DSB hotspots and axis, ChIP-qPCR and ChIP-Seq analysis also showed

185   binding of Msh5 at representative centromere regions **(Fig 1C, 2B, S2A, B).** Also, earlier

186   binding of Msh5 at centromeres (e.g. at 3h) is observed compared to the DSB hotspots and axis

187   sites **(Fig 3B**). Individual chromosome plots show Msh5 associates with centromeres on all

188   chromosomes **(S4 Fig).** Association with centromeres is also observed for other ZMM proteins

189   like Zip3 [48].

190   qPCR analysis showed no Msh5 enrichment in *msh5Δ* mutant at DSB hotspots relative

to the coldspot (**S5A Fig**). Axis and centromeres (which show reduced Msh5 enrichment

compared to DSB hotspots) showed background levels of Msh5 binding in *msh5Δ*. qPCR

analysis also showed no enrichment of Msh5 in *msh4Δ* mutant at DSB hotspots (**S5B Fig**).

However, weak Msh5 binding is observed at axis and centromere in *msh4Δ* (**S5B Fig**) consistent

with earlier cytological observations showing residual Msh5 signals in *msh4Δ* [25]. Therefore,

Msh4-Msh5 complex formation is required for normal Msh5 enrichment at DSB hotspots, axis,

and centromeres.

198   Since other ZMM proteins (Zip2, Zip3, Zip4, Spo16) have been observed to associate

with the centromere, chromosome axis and DSB hotspots in ChIP-array and ChIP-Seq studies

[47, 48], we re-analyzed Zip3 binding to facilitate comparison with the Msh5 binding data in the

same strain background and experimental conditions. We used a Zip3 construct C-terminally

tagged with three copies of the FLAG epitope [48]. The diploid strain expressing *ZIP3-His6-*

FLAG3 allele showed a spore viability of 97% (40 tetrads dissected), as reported previously

[48]. Zip3 expression was detected using an anti-FLAG antibody (mouse) from 2h after

induction of meiosis till 8h with peak expression at 5h (**S6A Fig**). Zip3 ChIP from meiotic

extracts was performed at 3h, 4h, and 5h using an anti-FLAG antibody. No Zip3 protein band

was observed in the untagged wild-type lysate or eluate fractions showing the specificity of the

antibody (**S6B Fig**). Zip3 ChIP-Seq was performed once for 3h time point and in duplicates for

4h and 5h time points. Representative binding of Zip3 on chromosome III is shown in **S6C Fig**.

Like Msh5, Zip3 shows binding to DSB hotspots, chromosome axis sites, centromere and is

absent from DSB cold spots (**S6C Fig**) as observed previously [47, 48].

Msh5 binding at DSB hotspots is stimulated by DSB repair intermediates

We performed cytology in meiotic time courses with the Msh5 antibody in several

mutant backgrounds that affect DSB formation and processing to determine genetic requirements

for Msh4-Msh5 localization at DSB hotspots. In wild-type cells, we observed a peak in the

average number of Msh5 foci/cell (48 ± 6.5) at 5h (**Fig 4A, C**). Previous studies have also

observed a similar number of Msh5 foci in wild type [36, 49]. In a *spo11Δ* mutant where DSBs

are not formed, cytological observations showed no Msh5 foci (**Fig 4B, C**) at any of the time

points in meiosis. Consistent with previous studies, these observations suggest that Msh5

binding to the DNA is dependent on DSB formation [49]. We asked if DSB formation and

resection are sufficient for the binding of Msh5 protein. In *dmc1Δ* mutant, cells arrest after DSB

formation and resection [52]. The joint molecule formation is blocked completely in *dmc1Δ*

mutant [53]. These cells cannot repair DSBs because of the loss of strand exchange activity [52,

54]. Compared to wild type (48 ± 6.5 Msh5 foci), on average 22 ± 3.8 Msh5 foci were observed

at 5h in a *dmc1Δ* mutant **(Fig 4A-C).** These results indicate that an event prior to the Dmc1

function, such as DSB resection, promotes Msh5 binding. Such an observation is also consistent

with *in vitro* data showing Msh4-Msh5 can bind to 3' overhangs [35]. It has been shown that in

the *dmc1Δ* mutant, the DSBs appear at the normal time but persist longer, and the cells get

arrested at this stage [52]. The reduced number of Msh5 foci (22 ± 3.8) in *dmc1Δ* mutant

compared to wild type (48 ± 6.5, p < 0.001) is therefore not due to reduced DSBs. Instead, this

observation suggests that even though resected DSBs can recruit Msh5, the binding efficiency to

resected DSB structures is reduced. Dmc1-dependent strand invasion steps, therefore, promote

stable binding of Msh5.

We tested Msh5 binding in a *rad52Δ* mutant where cells efficiently proceed to strand

invasion and make wild-type level of SEI intermediates, but dHJ formation is significantly

reduced [55, 56]. The timing and frequency of DSBs are similar in *rad52Δ* and wild type [55].

We observed an average of 31 ± 6 Msh5 foci at 5h in the *rad52Δ* mutant (**Fig 4B, C**), which is

less compared to wild type (p < 0.001). Since SEIs are similar in *rad52Δ* and wild type, but SEI

+ dHJs are about two times more in wild type, the number of Msh5 foci in *rad52Δ* (31 ± 6) and

wild type (48 ± 6.5) suggests that SEI intermediates are efficient for recruiting Msh4-Msh5.

Taken together with previous observations of lack of Msh5 foci in *spo11Δ* and *rad50S* mutants

[49], our results in *dmc1Δ* and *rad52Δ* suggest in *S. cerevisiae* Msh4-Msh5 recruitment *in vivo*

happens after DSB resection, which is consistent with *in vitro* biochemical data showing binding

of *S. cerevisiae* and human Msh4-Msh5 at SEI structures [34, 35].

Next, we asked if dHJs stimulate maximum Msh5 binding using the *ndt80Δ* mutant that

accumulates joint molecules, but the dHJs are not resolved [5]. An average of 47 ± 6.4 Msh5

foci is observed at 5h in *ndt80Δ* mutant which is similar to the maximum number of Msh5 foci

seen in the wild type at 5h (p = 0.44, **Fig 4A-C**) indicating that the SEI and dHJ structures

together facilitate maximum binding of the Msh4-Msh5 complex to the DNA. These results

suggest that even though resected DSBs can initiate binding of the Msh4-Msh5 complex,

maximum binding is achieved with joint molecule structures comprising SEIs and dHJs (see

discussion). In addition, the average Msh5 foci count (51) at 6h in *ndt80Δ* is higher than wild

type (40.7) due to the accumulated levels of SEIs and dHJ structures. Msh5 aggregates were

observed in all the above mutants (*spo11Δ, dmc1Δ, rad52Δ, ndt80Δ*) and is quantified in **S7 Fig.**

Msh5 binding depends on DSB strength

We tested the correlation between DSB frequency and Msh5 association with

chromosomes. ChIP-qPCR analysis corroborated cytological observations of the absence of

Msh5 binding in *spo11Δ*. ChIP-qPCR analysis showed no enrichment of Msh5 at DSB hotspots

and axis relative to DSB coldspot (**S5C Fig**). But early binding (3h) at centromeres was

observed, suggesting Msh5 enrichment at DSB hotspots and chromosome axis is dependent on

DSB formation (**S5C Fig**). In budding yeast 3600 DSB hotspots have been identified in high

resolution [18]. These DSB hotspot positions (Spo11 cutting sites) were obtained from Pan et

al., 2011[18]. We defined DSB hotspots that show Msh5 peak at 5h time point ($p < 10^{-5}$) in at

least one of the four replicates as Msh5 enriched DSB hotspots. DSB hotspots that did not show

Msh5 peak (or $p > 10^{-5}$) in any of the four replicates were defined as Msh5 depleted DSB

hotspots. Our analysis suggests that about half of the 3600 identified DSB hotspots (1734/3600)

are enriched for Msh5 protein (**S2 Table**). We observed that Msh5 enriched DSB hotspots

showed higher Spo11 oligo counts (**Fig 5A, S2 Table**). Among the top 10% of DSB hotspots

(based on Spo11 oligo counts,[18]), 96% were Msh5 enriched DSB hotspots and 4% were Msh5

depleted DSB hotspots ($p < 0.01$) (**S2 Table**). Among the bottom 10% of DSB hotspots, 27%

were Msh5 enriched DSB hotspots, and 73% were Msh5 depleted DSB hotspots ($p < 0.01$). This

observation suggests Msh5 is easily detected at active DSB hotspots as more cells in the

population would have the break.

Small chromosomes have a higher density of crossovers than long chromosomes, which

is correlated with a higher Spo11 oligo density on the smaller chromosomes [18, **Fig 5B**]. Msh5

read density (reads / Kb) analyzed from all four replicates was negatively correlated with

chromosome size (r = -0.72; p = 0.0016) consistent with more crossovers / kb and higher DSB

activity on smaller chromosomes (**Fig 5C**). These observations also support Msh5 enrichment is

dependent on DSB activity. Zip3 read density showed weaker correlation with chromosome size

(r = -0.56, p = 0.02) (**Fig 5C**). A recent study shows that the three smallest chromosomes (I, III,

and VI) have unusually high DSB density due to distinct regulation of DSB formation that

ensures their accurate segregation [57]. Although Spo11 oligo density (and crossover density) is

negatively correlated with chromosome size even after excluding the three smallest

chromosomes [57], Msh5 read density showed weak negative correlation (r = -0.6, p = 0.03),

while Zip3 read density (r = -0.18, p = 0.55) was uncorrelated with chromosome size (**Fig 5D**).

Msh5 binding to the chromosome axis is disrupted in *red1Δ* mutant

Since Msh5 binding is positively correlated with the Red1 axis associated sites, we

tested whether loading of Msh5 is dependent on the Red1 protein. Red1, an axial element

protein, is essential for normal DSB formation by recruiting DSB factors to the axis, synapsis,

and for promoting interhomolog DSB repair through its role in meiotic checkpoint signaling [19,

58-61]. Cytological observations showed that the number of Msh5 foci in *red1Δ* was

significantly less than the number of Msh5 foci in wild type at all time points (3h, 4h, 5h, 6h) in

meiotic time courses with no overlap in the standard deviation (**Fig 6A, S1 Data**). At 5h an

average of 16 ± 5.3 Msh5 foci is observed in *red1Δ* compared to 48 ± 6.5 foci (p < 0.001) in wild

type (**Fig 6A**). Since the number of Msh5 foci is less compared to wild type, we also examined

the DSB frequency using Rad51 foci counts. We observed an average of 20 ± 4.3 Rad51 foci at

a three-hour time point when the numbers of DSBs are at peak level (**Fig 6B**). Previous studies

have also shown that DSBs are decreased in *red1Δ* mutant [60]. The average number of Rad51

foci in *red1Δ* is significantly less compared to the number of Rad51 foci in wild type at 3h (49 ±

15, p <0.001) [58]. The reduced number of Msh5 foci in *red1Δ* is most likely due to overall

reduction in DSB levels, and since DSB repair is mostly off the sister chromatid [61-63].

305   ChIP-qPCR analysis showed no enrichment of Msh5 at representative axis sites relative

to the DSB coldspot in *red1Δ* at an early time point (3h) (**S5D Fig**). Msh5 association was

maintained at representative DSB hotspots and centromeres (**S5D Fig**). ChIP-Seq analysis of

Msh5 protein in the *red1Δ* mutant scaled with qPCR data (at 5h, **S3B Fig**) confirmed Msh5 is

depleted at axis sites and maintained at DSB hotspots and centromere sites (**Fig 6C**). Overall

these observations suggest that Red1 is essential for Msh5 binding at axis regions but not at DSB

sites and centromere regions.

DISCUSSION

314   Previous studies using cytological, biochemical, and physical approaches in various

organisms have given insights into the timing of Msh4/Msh5 action, and it's *in vitro* DNA

binding properties. Purified human Msh4-Msh5 has a high affinity towards SEI and dHJs [34],

and the *S. cerevisiae* Msh4-Msh5 has also been shown to bind 3' overhangs, pro-Holliday

junctions and Holliday junction structures [34, 35]. But the genome-wide dynamic localization

of the Msh4-Msh5 complex with reference to its *in vivo* binding sites, meiotic chromosomal

organization, and mutations in other meiotic recombination genes is not well understood. The

current model of meiotic recombination based on *in vitro* biochemical analysis suggests Msh5

associates with 3' overhangs, SEI, and dHJs. We demonstrate that *in vivo,* Msh5 associates with

DSB hotspots, chromosome axis, and centromere sites. Recruitment of Msh5 to DSB hotspots

and chromosome axis requires DSB formation and is observed as early as the stage of DSB

resection (**Fig 4**).

Msh5 foci in *dmc1Δ* mutants, where DSBs are formed and resected but cannot undergo

strand invasion is consistent with *in vitro* studies (**Fig 4**) [35]. It is possible that Msh5 binding in

dmc1Δ may reflect aberrant binding due to the hyperresected DSBs. Further studies are required

to explain whether this binding has any functional relevance or is just related to its recruitment.

Although resected DSBs are sufficient for the initial recruitment of Msh5, the optimal binding of

Msh5 requires the formation of dHJs (**Fig 4**). ChIP-Seq results also suggest weak binding of

Msh5 at DSB hotspots at the early stages of meiosis (3h) and maximal binding at DSB hotspots

around 4-5h that corresponds to joint molecule formation. These results, along with previous

studies showing Msh2 associates with DSBs *in vivo* [64] suggest a general property of MSH

proteins to bind DNA that facilitates specific binding to their major substrates.

Msh5 binding is observed at chromosome axis sites from 3 to 5h with maximum

binding at 5h. This observation supports previous cytological studies that show localization of

Msh5 and Msh4 at the chromosome axis [50, 65]. The observation that Msh5 associates with

both DSB and chromosome axis sites suggest that Msh5 may participate in both the processing

of recombination intermediates and the assembly of the synaptonemal complex like some of the

other ZMM proteins [66]. Thus it may have a role in crossover formation and synapsis. It is

also possible that Msh5 binding to the axis is indirect via interactions with axis proteins. A

recent study has implicated interactions between Msh5 and Red1 [47].

The Msh5 protein also shows binding to centromere sites on all chromosomes. Other

ZMM proteins like Zip2, Zip3, Zip4, and Spo16 also show centromere localization. It is

suggested that Zip1, Zip2, Zip3, and Spo16 binding to centromeres promotes synapsis initiation

from centromeres, besides crossover sites [67]. It is also suggested that Zip1 localization to

centromere facilitates non-exchange chromosome segregation. Just as Msh5 association with

chromosome axis, may facilitate synapsis, it is possible that Msh5 localization to centromeres

may also facilitate synapsis initiation, especially as it is observed even in *spo11Δ* mutant at early

time points (**S5C Fig**). Alternatively, it is possible that Msh5 binding at centromere may reflect

a docking site for the protein or is indirect through interactions with other ZMM or centromeric

proteins.

Cytological and ChIP-array studies also show that some of the ZMM proteins Zip1 and

Zip3 associate with the chromosome axis [19, 48]. A recent study by De Muyt et al. 2018 [47]

suggests that ZZS (Zip2, Zip4, Spo16) and Zip3 show strong association with DSB hotspots and

weak and transient association with chromosome axis. Similar to Zip3 and the ZZS complex,

which associate with the centromere, chromosome axis, and DSB hotspots in a dynamic manner

[48], Msh5 associates with centromeres, chromosome axis and DSB hotspots (**Fig 1C**, **Fig 2B**).

Further, Msh5 loading is dependent on Zip3 protein [25], and Zip3 and Msh4 foci co-localize

cytologicaly. This is supported by our observation that Zip3 and Msh5 occupy similar

chromosomal features (**S6C Fig**, **Fig 1C**, **Fig 2B**). Although there are similarities between Zip3

and Msh5 binding sites, Msh5 binding is also stimulated by DSB frequency (**Fig 5**, **S2 Table**).

These observations support a direct role for Msh5 in the mechanism of crossover formation.

Model for Msh4-Msh5 function in crossing over

Chromosome domains with high DSB activity are enriched for Red1 at the axis. But within the

Red1 enriched domain, there is a negative correlation between Spo11/Dmc1 and Red1 sites since

DSBs occur on the loop while Red1 sites are on the axis [16, 19]. We observed that about half of

all DSB hotspots are detectably enriched for Msh5. The Msh5 localization supports the model

that recombination occurs in sequences that are at a distance from the base of the chromosome

loops [16]. The Msh5 binding data also suggests that within axis protein-enriched regions, the

Msh4-Msh5 complex binds to the DSB hotspots that are known to occur at a distance from the

axis. We also observe Msh5 association with the chromosome axis from 3h to 5h time point.

The axial association is absent in a *spo11Δ* mutant (**S5C Fig**), which suggests, DSBs may

promote Msh5 assembly on the chromosome axis. At 4-5h, Msh5 binds both DSB and axis

optimally, which may facilitate communication between DSB sites and axis through Msh5 for

crossover formation. Msh5 may be part of the recombinosome for loops tethering to the axis.

Based on our observations that the Msh4-Msh5 complex binds to DSB hotspots on chromosomal

loops and also on the axis, a model for how Msh4-Msh5 may work in crossover formation is

shown in **Fig 7**.

Similar to the loop – axis interaction required for meiotic DSB formation, we speculate a

loop-axis interaction model for crossover formation. Specifically, Msh4-Msh5 association on

chromosome loops and axis following DSB formation may facilitate the recruitment of the

Mlh1-Mlh3 complex at these locations. Recent *in vitro* studies suggest that the Mlh1-Mlh3

complex forms unidirectional polymers on DNA and that polymerization is essential for its

nicking activity [27, 68]. Since Msh4-Msh5 encircles both interacting duplexes during joint

molecule formation, it could direct polymerization on both duplex arms. The Mlh1-Mlh3

complex could polymerize directionally from the loop associated Msh4-Msh5 complex towards

the axis. Since the Msh4-Msh5 complex is also associated with the axis, it could facilitate loop-

axis interaction for crossover formation near the axis by Mlh1-Mlh3. Also, human Msh4-Msh5

is known to stimulate human Mlh1-Mlh3 endonuclease activity [40, 41]. Crossover formation at

the axis can facilitate long-range interference through the dissipation of mechanical stress along

the axis [69, 70]. This axis-loop interaction during crossover formation may also be required to

convert crossovers into chiasma. In conclusion, these data shed novel insights into the *in vivo*

assembly and functions of the Msh4-Msh5 complex in relation to meiotic chromosome

organization and DSB activity.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains used in this study are derivatives of *S. cerevisiae* SK1 strain and are listed in

the **S3 Table.** Yeast strains were grown on either yeast extract–peptone–dextrose (YPD) or

synthetic complete media at 30^0 [71-73]. Sporulation medium was prepared as described in

Argueso et al. (2004) [30]. Mutants were generated by direct transformation using standard

techniques [74]. When required, the drugs geneticin (Invitrogen), nourseothricin (Werner

BioAgents,Germany), and hygromycin (Sigma) were added to the media at prescribed

concentrations [75].

Meiotic synchronization

Meiotic synchronization was performed using SPS method as described [76]. In short,

diploid colonies were inoculated in 5 ml YPD medium and grown for 24h at 30^0 C to reach

saturation. From the saturated YPD culture $\sim 5 \times 10^6$ cells/ml were inoculated to liquid SPS

(0.5% yeast extract, 1% peptone, and 0.67% yeast nitrogen base without amino acids, 1%

potassium acetate, 0.05M potassium biphthalate, and pH 5.5) medium and grown for 7h at 30^0 C.

This culture was used to inoculate 500 ml of liquid SPS at cell density $\sim 3 \times 10^5$ cells/ml and

grown for 12-16h at 30^0 C until the cell density reached $\sim 4 \times 10^7$ cells/ml. Cells were collected

by centrifugation, washed twice with 1% potassium acetate and re-suspended in 400 ml of

sporulation (2% Potassium Acetate) medium at cell density $\sim 4 \times 10^7$ cells / ml and incubated at

30^0 C with shaking. Meiotic progression was monitored by examining the nuclear divisions

using DAPI staining.

Chromatin Immunoprecipitation

422   Msh5 ChIP was performed as described in [19, 76, 77] with minor modifications, using

native Msh5 antibody and Protein A Sepharose beads (GE Healthcare). Briefly, from a

synchronous meiotic culture, 50 ml of samples were collected at various time points (as

described in the results) and crosslinked using formaldehyde. Cells were lysed using

Beadbeater. The cell extract was sonicated using Bioruptor (Diagenode) and the debris were

removed by centrifugation. 50 ul of Protein A sepharose beads were used to preclear the cell

lysate from each sample. To the pre-cleared lysate, Msh5 antibody and Protein A sepharose

beads blocked with BSA was added. After incubation, lysate was removed by centrifugation and

beads were washed to remove non-specific binding. The immunoprecipitated material was

eluted from the beads and decrosslinked along with RNAse A treatment. The DNA was purified

following Proteinase K treatment using QIAGEN PCR purification kit. Lysate samples were

collected before and after the immunoprecipitation and after elution to check for IP efficiency by

Western blotting. Zip3 ChIP was performed using anti-FLAG antibody and Protein G

Dynabeads (Life technologies). For Msh5 western blot, anti-Msh5 antibody was used at 1:4000

dilution, and anti-rabbit HRP secondary antibody was used at 1:10000 dilution. For Zip3

western blot, anti-FLAG antibody was used at 1:4000 dilution, and anti-mouse HRP secondary

antibody was used at 1:10000 dilution. Anti-PGK1 antibody was used at 1:30000 dilution, and

anti-mouse HRP secondary antibody was used at 1:40000 dilution. The immunoprecipitated

DNA was used for sequencing as well as qPCR. For wild type, four ChIP samples were

sequenced for each time point (2-7h). For the *red1Δ* mutant, three ChIP samples were sequenced

at the indicated time points. The DNA fragments were sequenced on Illumina HiSeq 2500/4000

platform at Fasteris, Switzerland. qPCR was performed on a fraction of the DNA sample used

for ChIP-Seq for wild type and mutants. The DNA enrichment of the ChIP samples were

calculated with respect to the input for wild type and mutants at each time point from three

technical replicates.

Cytology

Yeast chromosome spreads were prepared as described [25, 56] and stained with anti-

Msh5 and anti-Rad51 antibody (Rabbit polyclonal antibody from Akira Shinohara) at 1: 500

dilution followed by anti-Rabbit TRITC conjugated secondary antibody (Jackson

ImmunoResearch) at 1: 1500 dilution. Immunofluorescence images were captured using a 63x

oil immersion objective on a Leica SP5 confocal microscope. For each time point,

approximately 50 images were analyzed from two independent experiments. For *spo11Δ*, only

30-50 images were analyzed at each time point from a single experiment as previous studies

show the absence of Msh5 foci in *spo11Δ*. Image analysis and 3D deconvolution were done

using Leica Application Suite (LAS) Advanced Fluorescence (AF) Lite 2.8.0 software. All foci

counts are shown as mean ± standard deviation.

Bioinformatic analysis of Illumina sequence data

Quality and statistics of raw reads were analyzed using FastQC (version 0.11.5).

Preprocessing was decided based on the FastQC report summary. Removal of Illumina adapters

and trimming of raw sequence reads was done using Trimmomatic (version 0.36). Processed

reads (~2-8 Million reads per samples) were aligned to the S288c reference genome (version

R64-1-1) using bowtie2 (version 2.3.0) since the SK1 reference genome assembly is incomplete.

Previous studies have used both S288c and SK1 as reference genomes and showed that the

results are similar [15, 18]. Samples having more than 75% alignment rate and with more than 2

million aligned reads were used for the downstream analysis. Statistics of the mapped reads

generated from the alignment program were analyzed for uniquely mapped reads as well as for

multiple mapped reads. Samples having an alignment rate of less than 30% and less than 0.5

million reads were discarded from the analysis. Unmapped reads were analyzed using blast to

detect any type of contamination in the sample. After analyzing the alignment statistics,

conversion of aligned file format (SAM) to its compressed file format (BAM), indexing and

sorting was done using samtools (version 1.3.1). All the downstream analyses were done using

R (version 3.3). All raw sequence data for this study are available from the National Centre for

Biotechnology Information Sequence Read Archive under accession number SRP129066.

Genome-wide profile normalization using NCIS

The S288c reference genome was partitioned into equal-sized bins (200 bp), and reads

per bin were calculated from the samples. Reads that are mapped to N locations were assigned a

score of 1/N for each read in case of multi-mapped reads. Normalization was performed in order

to account for factors affecting the data distribution, such as an error during library preparation,

sequencing, and sample preparation. For this study, we performed normalizations with the

control sample (Input for Msh5 ChIP and wild type untagged for Zip3 ChIP) using NCIS

(Normalization of ChIP-seq). NCIS estimates the fraction of background from the control

sample using a data-adaptive length of the window and a data-adaptive threshold assuming that

background tends to have lower counts than enriched regions [78]. After normalizing the counts

using NCIS, backgrounds were subtracted from their respective control samples and averaged,

followed by genome-wide smoothening by ksmooth with a bandwidth of 1 kb.

Peak calling using MACS

To identify the Msh5 peaks, only reads that are uniquely aligned were taken into account.

MACS (Model-based Analysis for ChIP-Seq) (http://liulab.dfci.harvard.edu/MACS/) [79] was

used to identify peaks from the sample. MACS uses a dynamic Poisson distribution to identify

local biases in peak detection. Msh5 peaks were called from the pooled set of four replicates.

Msh5 peaks with a p-value greater than 10^{-5} were filtered out, and the final set of Msh5 peaks is

shown in **S1 Table**. Spo11 DSB hotspots (non-overlapping with Red1 sites) and axis sites

(Red1 bound regions non-overlapping with Spo11 sites) were considered as Msh5 enriched if the

Msh5 peak showed minimum one base pair overlap with the Spo11 hotspot or the Red1 peak

respectively (**S2 Table**). The raw data for calling Red1 peaks were extracted from Sun et al.,

2015[15]. Red1 peaks were called following the same criteria described for calling Msh5 peaks,

except for p-value, which was set at less than 10^{-15} as per Sun et al., 2015[15]. Zip3 reads were

analyzed similarly to Msh5 reads.

ChIP-qPCR scaling of Msh5 ChIP-Seq reads

For wild type, one of the four Msh5 Chip-Seq replicates was scaled with the corresponding

qPCR data. We performed normalization of the Msh5 Chip-Seq data with respect to its input

using NCIS [78]. Linear regression model was generated between the normalized Msh5 Chip-

Seq data and the qPCR data [**S3A Fig**] using R scripts provided by Hajime Murakami and Scott

Keeney [80]. The linear regression model was used to scale the Msh5 ChIP-Seq data. A similar

process was used to scale Msh5 ChIP-Seq reads in the *red1*Δ mutant.

Statistical tests

For all count data (foci, box plots) student's t-test was used to test for statistical

significance. For all correlation analysis, Pearson's correlation coefficient was used. Numerical

data underlying all graphs are shown in **S1 Data.**

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FIGURE LEGENDS

Fig 1. Msh5 expression and DNA binding in wild-type meiosis. **(A)** Western blot analysis of

Msh5 expression pattern in synchronized wild-type meiosis. **(B)** ChIP using Msh5 antibody in

wild type and *msh5Δ* strains. Lanes 1 to 3 indicate lysate, lysate after overnight incubation with

Msh5 antibody and beads, and the eluate fractions in wild type. Lanes 4 to 6 indicate lysate,

lysate after overnight incubation with Msh5 antibody and beads and the eluate fractions in

msh5Δ. **C)** Msh5 enrichment at DSB hotspots (*BUD23*, *ECM3,* and *CCT6*), centromere regions

(*CEN I, CEN III*) and axis regions (*AXIS I, AXIS II, AXIS III*) relative to DSB coldspot

(*YCR093W*), measured using ChIP-qPCR. The samples are normalized using input. The error

bars represent the standard deviation.

Fig 2. Msh5 binds at DSB hotspots, chromosome axis, and centromere positions. A)

Density of scaled Msh5 ChIP-Seq reads at Msh5 peak locations (5h) from 3h to 7h.

B) Representative image to show chromosomal localization of Msh5 in wild type on

chromosome III (t=3, 4, and 5h after induction of meiosis) using qPCR scaled ChIP-Seq data.

Zoomed in image of the centromeric region, axis region, one DSB hotspot (*BUD23*), and one

DSB coldspot (*YCR093W*) is also shown. Red1 and Spo11 data are taken from, Sun et al

2015[15] and Pan et al., 2011 [18]. The black circle indicates centromere. **(C)** Coverage

analysis of qPCR scaled Msh5 ChIP-Seq reads at Spo11 DSB hotspots (*** indicates p < 0.001,

outliers are removed). **(D)** Width of the Msh5 binding peaks (5h time point) at DSB hotspots

(not overlapping with Red1 sites) from all four Msh5 ChIP-Seq replicates.

Fig 3. Msh5 association with chromosome axis and centromeres. A) Coverage analysis of

qPCR scaled Msh5 ChIP-Seq reads at axis sites (Red1 peaks) (*** indicates p < 0.001, outliers

are removed). **B)** Coverage analysis of qPCR scaled Msh5 ChIP-Seq reads at centromere sites

(* indicates p < 0.01, outliers are removed).

Fig 4. **Msh5 binding is stimulated by meiotic DSB repair intermediates. A)** Representative

image of a spread meiotic nucleus from wild-type strain (t=5h) immunostained for Msh5 (red

foci). **B)** Representative image of a spread meiotic nucleus from *spo11Δ, dmc1Δ, rad52Δ,* and

ndt80Δ mutants (t=5h) immunostained for Msh5 (red foci). *spo11Δ* indicates the specificity of

the Msh5 antibody. **(C)** Kinetics of Msh5 foci and the percentage of meiotic nuclei positive for

Msh5 foci in synchronized meiosis in wild type and *dmc1Δ, rad52Δ, ndt80Δ* mutants.

Approximately 50 nuclei, each from two independent experiments, were assessed for each time

point (except *spo11Δ*, see Materials and Methods). The error bars represent the standard

deviation.

Fig 5. **Msh5 binding at DSB hotspots is dependent on DSB frequency. (A)** Log2 of Spo11

reads [18] at Msh5 enriched and depleted DSB hotspots. **B)** Spo11 peak density plotted as a

function of chromosome size [18]. **C)** Msh5 and Zip3 read density plotted as a function of

chromosome size. **D)** Msh5 and Zip3 read density as a function of chromosome size, excluding

the shortest chromosomes (I, III and VI).

Fig 6. Msh5 association with chromosomes in *red1Δ* mutant. Representative image of **A)**

Msh5 (t = 5h) and **B)** Rad51 (t = 3h) foci and kinetics of its accumulation in *red1Δ* mutant.

Approximately 50 nuclei, each from two independent experiments, were assessed for each time

point. The error bars represent the standard deviation. **(C)** Coverage analysis of qPCR scaled

Msh5 ChIP-Seq reads at Spo11 DSB hotspots, Red1 peaks, and centromere positions (***

indicates $p < 0.001$ and * indicates $p < 0.01$, outliers are removed).

Fig 7. Model for the chromosomal localization of the Msh4-Msh5 complex during meiosis.

During meiosis binding of the axis proteins (Red1/Hop1) define the chromosomal axis region

with intervening loop regions. The loops are recruited to the axis region for DSB formation.

Msh4-Msh5 associates with the DSB hotspots and axis after DSB formation at early stages (3h)

of meiosis. At later stages (4-5h), enhanced binding of Msh5 is observed at DSB hotspots

consistent with the generation of joint molecule structures that promote Msh5 binding. Also,

Msh5 is enriched at strong DSB hotspots. Msh5 binding to both loop and axis regions may

promote loop-axis interaction for crossover formation.

S1 Fig. Genome-wide pairwise comparison of the log2 (fold change) of Msh5 reads for

wild-type replicates (a-d) (panels A-F).

S2 Fig. Msh5 binding sites from ChIP-Seq and ChIP-qPCR data. A) NCIS normalized

Msh5 ChIP-Seq reads from all four replicates (not scaled with ChIP-qPCR) in wild type on

chromosome III (t=3, 4, and 5h after induction of meiosis). **B)** ChIP-qPCR analysis of Msh5

enrichment at representative DSB hotspots, axis regions, centromeres, and cold spot (*YCR093W*).

S3 Fig. Linear regression model of Msh5 ChIP-Seq and ChIP-qPCR data. A) Correlation

between Msh5 ChIP-qPCR and ChIP-Seq in wild-type strains at different time points (2 -7h) for

DSB hotspots (*BUD23*, *ECM3,* and *CCT6*), DSB coldspot (*YCR093W*), centromere regions

(*CEN I*, *CEN III*) and axis regions (*Axis I*, *Axis II*, *Axis III*). **B)** Correlation between Msh5 ChIP-

qPCR and ChIP-Seq in *red1*Δ (5h).

S4 Fig. ChIP-qPCR scaled Msh5 ChIP-Seq coverage at centromere location on all

chromosomes for 3-6h time points.

S5 Fig. ChIP-qPCR analysis of Msh5 in meiotic mutants. ChIP-qPCR analysis of Msh5

enrichment at DSB hotspots (*BUD23*, *ECM3,* and *CCT6*), DSB coldspot (*YCR093W*),

centromere regions (*CEN I, CEN III*) and axis regions (*Axis I*, *Axis II*, *Axis III*) in **A)** *msh5*Δ, **B)**

*msh4*Δ, **C)** *spo11*Δ and **D)** *red1*Δ mutants. The samples are normalized using input. The error

bars represent the standard deviation.

S6 Fig. Zip3 ChIP-Seq analysis. A) Western blot analysis of Zip3 expression in synchronized

wild-type meiosis. **B)** ChIP using anti-FLAG antibody in Zip3-FLAG and wild-type strains.

Lanes 1 to 6 indicate lysate fraction at 3, 4, and 5h time points. Lanes 7 to 12 indicate eluate

fractions at 3, 4, and 5h time points. **C)** Representative image of Zip3 binding on chromosome

III (t=3, 4, and 5h after induction of meiosis). Zoomed in image of the centromeric region, one

DSB hotspot (*YCR047C*), axis, and one DSB cold spot (*YCR093W*) is also shown.

S7 Fig. Percentage of meiotic nuclei showing Msh5 aggregate like staining. Approximately

50 nuclei, each from two independent experiments, were assessed for each time point (except

spo11Δ, see Materials and Methods).

S1 Table. Msh5 peaks pooled from the four wild-type replicates (WT-a,b,c,d). Peak start

and end positions are indicated.

S2 Table. DSB hotspots that are enriched or depleted for Msh5. DSB hotspot data (3600

hotspots) from Pan et al. 2011[18] were sorted based on overlap with Msh5 binding regions and

Spo11 oligo hits.

S3 Table. List of strains used in this study.

S1 Data. Numerical data underlying all graphs in the manuscript.

A

B

C

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Chromosomal loop

Chromosomal axis

Msh5 enriched DSBs
Msh5 depleted DSBs
Red1/Hop1
Synaptonemal complex
Msh4-Msh5
Centromeres

Figure 7

S1 Fig

A

B

S2 Fig

A **Wild type**

B *red1Δ*

S3 Fig

S4 Fig

S5 Fig

S6 Fig

S7 Fig