Rec8 cohesin-mediated loop-axis chromatin architecture is required for meiotic recombination

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Summary

During meiotic prophase, cohesin-dependent axial structures are formed in the synaptonemal complex (SC). However, the functional correlation between these structures and cohesion remains elusive. Here, we examined the formation of the cohesin-dependent axial structure in fission yeast, which forms atypical SCs composed of linear elements (LinEs) resembling the lateral elements of SC but lacking the central elements. The results demonstrated that Rec8 cohesin is crucial for the formation of the loop-axis structure within the atypical SC. Furthermore, the Rec8-mediated loop-axis structure is formed in the absence of LinEs and provides a structural platform for aligning homologous chromosomes. We also identified a \textit{rec8} mutant that lost the ability to assemble the loop-axis structure without losing cohesion. Remarkably, this mutant showed defects in the LinE assembly, resulting in a significant reduction in meiotic recombination. Collectively, our results demonstrate an essential role for the Rec8-dependent loop-axis structure in LinE assembly, facilitating meiotic recombination.

Keywords

meiosis, cohesin, Rec8, chromosome, axis-loop structure, recombination, fission yeast, linear element, miscarriage, developmental disorders
Introduction

Meiosis is a unique type of cell cycle for sexual reproduction, which halves the chromosome number by two successive nuclear divisions after a single round of DNA replication to produce haploid gametes. In the first meiotic division (meiosis I), homologous chromosomes (homologs) are segregated to opposite poles. This type of division is called reductional segregation and is essentially different from mitosis or meiosis II, in which sister chromatids are segregated to opposite poles (Petronczki et al., 2003). Crossovers formed by reciprocal recombination constitute a physical link between homologs, ensuring reductional segregation (Hunter, 2015; Zickler and Kleckner, 1999).

Failure to establish such interhomolog links leads to the mis-segregation of chromosomes during meiosis I, resulting in aneuploidy of gametes. This is a major cause of human miscarriage or developmental disorders caused by chromosome trisomy, such as Down syndrome (Nagaoka et al., 2012; Webster and Schuh, 2017).

During meiotic prophase, homologs become closely associated or aligned through an elaborate proteinaceous structure called the synaptonemal complex (SC), followed by crossover recombination. The SC consists of two lateral elements, each along the axis joining a pair of sister chromatids, with one central element connecting the lateral elements (Grey and de Massy, 2021; Kleckner, 2006). The process of chromatin loop formation is thought to be essentially mediated by meiotic cohesin (Klein et al., 1999; Petronczki et al., 2003). The cohesin complex is responsible for sister chromatid cohesion. The complex consists of two structural maintenance of chromosome (SMC) proteins, a kleisin subunit, and a stromal antigen (SA) subunit. Replacement of kleisin commonly occurs in most organisms, in which the somatic kleisin Rad21/Scc1 is replaced by Rec8.
during meiosis (Ishiguro, 2019; Mehta et al., 2013; Nasmyth, 2001). Rec8 cohesin is responsible for sister chromatid cohesion and provides a physical link between homologs as a result of crossover (Moore and Orr-Weaver, 1998; Petronczki et al., 2003). Additionally, Rec8 cohesin is required for the assembly of lateral elements in diverse eukaryotes (Cai et al., 2003; Fujiwara et al., 2020; Fukuda et al., 2014; Golubovskaya et al., 2006; Hopkins et al., 2014; Ishiguro et al., 2014; Klein et al., 1999; Llano et al., 2014; Pasierbek et al., 2001; Winters et al., 2014; Xu et al., 2005). A recent study using high-throughput chromosome conformation capture (Hi-C) (Lieberman-Aiden et al., 2009) in the budding yeast \textit{Saccharomyces cerevisiae} clearly revealed that Rec8 cohesin is essential to construct meiosis-specific chromatin loop structures (Schalbetter et al., 2019).

As studied in somatic cells, cohesins can form higher-order structures by pulling together distant regions of the chromosome, for which a loop extrusion model has been proposed (Davidson and Peters, 2021; Fudenberg et al., 2016; Kim et al., 2019; Mizuguchi et al., 2014; Rao et al., 2017; Sanborn et al., 2015). Pds5 and Wpi/Wapl are cohesin-associating factors conserved in yeasts and vertebrates. These are considered to dissociate cohesin from chromatin (Challa et al., 2019; Peters and Nishiyama, 2012), thus affecting higher-order chromosome structures (Challa et al., 2016; Crawley et al., 2016; Ding et al., 2016a; Ding et al., 2006; Haarhuis et al., 2017; Hong et al., 2019; Silva et al., 2020; Tedeschi et al., 2013; Viera et al., 2020).

The fission yeast \textit{Schizosaccharomyces pombe} forms an atypical SC composed of a structure called the linear element (LinE). The LinE resembles the lateral element of the SC, but lacks central element components, such as \textit{S. cerevisiae} Zip1 (Bähler et al., 1993; Davis et al., 2008; Estreicher et al., 2012; Loidl, 2006; Lorenz et al., 2004; Olson et al.,
1978). Full alignment of homologs in *S. pombe* is finally achieved by chiasmata formation following crossover recombination (Loidl, 2006). The recognition and alignment of homologs in *S. pombe* relies on oscillatory movement of the nucleus (horsetail movement) in a bouquet orientation with all telomeres clustered to the spindle pole body (SPB) (Chikashige et al., 1994; Ding et al., 1998; Ding et al., 2019; Hiraoka, 2020; Loidl, 2006; Yamamoto and Hiraoka, 2001). More recently, a mechanism was proposed in which noncoding RNA on chromatin contributes to the initial recognition of correct homolog pairs (Chikashige et al., 1994; Ding et al., 1998; Ding et al., 2019; Hiraoka, 2020; Loidl, 2006; Yamamoto and Hiraoka, 2001).

Importantly, it has been shown that the phosphorylation of Rec11, a homolog of the meiosis-specific SA3 subunit of Rec8 cohesin in *S. pombe*, by casein kinase 1δ/ε (CK1), is required for the assembly of LinEs (Phadnis et al., 2015; Sakuno and Watanabe, 2015). The Rec8 cohesin complex containing phosphorylated Rec11 specifically interacts with Rec10, a homolog of the lateral element factor Red1/SYCP2. Previous studies in mouse spermatocytes have shown that depletion of the SYCP2 or SYCP3 structural components of axial elements causes defects in axial element formation despite normal cohesin-axis (Fujiwara et al., 2020; Fukuda et al., 2010; Pelttari et al., 2001). Accordingly, the structural property of chromatin to trail the horsetail movement is maintained in LinE-defective mutants of *S. pombe* (Ding et al., 2006; Sakuno and Watanabe, 2015). Based on these observations, it should be emphasized here that the Rec8-dependent chromosomal structure is strictly independent of lateral elements (or LinEs in *S. pombe*). However, the significance of the Rec8-dependent axis-loop structure in meiotic recombination remains unclear.
In this study, we performed Hi-C analysis to describe the meiotic chromosome structures of *S. pombe* in detail. We then sought to clarify the functional significance of the Rec8-dependent loop-axis structure by identifying *rec8* mutants that specifically show defects in axis formation, but not in cohesion. The collective data demonstrate that the Rec8-dependent loop-axis structure itself provides the scaffold to assemble the LinE axis, facilitating the execution of reciprocal recombination.

**Results**

**Induction of highly synchronized meiosis under physiological conditions**

To achieve Hi-C analysis of chromosomal configurations during meiotic prophase, we first developed an experimental system to prepare *S. pombe* cell populations undergoing highly synchronized meiotic progression under physiological conditions. For this purpose, we employed an *h-/h- pat1-as2/pat1-as2* diploid strain harboring one copy of the *mat-Pc* gene cassette (Cipak et al., 2012; Funaya et al., 2012; Yamamoto and Hiraoka, 2003). Meiosis can be induced using this ATP analog-sensitive allele of the Pat1 kinase (*pat1-as2*) upon inactivation of the Pat1 kinase by addition of the ATP analog, instead of the temperature-sensitive allele (*pat1-114*), in which raising temperature has detrimental effects on meiosis (Bähler et al., 1991; Cipak et al., 2012). In addition, MDR-sup alleles (Aoi et al., 2014; Kawashima et al., 2012), which allow efficient cellular uptake of ATP analogs, were also exploited. As shown in Figure S1A, the meiotic progression of wild type diploid cells harboring Rec8-green fluorescent protein (GFP) was highly synchronous (~70%), as indicated by the timing of nuclear division in meiosis I. Premeiotic DNA replication was almost completed approximately 1.5 hours after the
induction of meiosis by the addition of an ATP analog after G1-arrest via nitrogen starvation (Figure S1A, left panel).

**Hi-C analysis recapitulates meiotic bouquet chromosomes in S. pombe**

For Hi-C analysis, cells were collected at 0 h (G1) and 2.5 h (meiotic prophase) after meiosis induction, along with cells in vegetative growth (VG; mainly in mitotic G2 phase). Notably, even at 0 h, a significant amount of Rec8-GFP protein was expressed and loaded onto chromatin. The amount of Rec8 peaked at approximately 2.5–3 h after meiotic induction (Figures S1B and S1C). Hi-C contacts of all three chromosomes in VG cells detected obvious centromere clustering (indicated by green arrowheads in Figures 1B and 1C), suggesting the organization of the Rabl orientation (Figure 1A, left). In contrast, as cells progressed from G1 to meiotic prophase, centromere clustering became vague, while telomere clustering became evident (Figures 1B and 1C; blue arrowhead). Remarkably, as cells entered the meiotic prophase, X-shaped inter- and intra-chromosomal contacts appeared (Figures 1B and 1C). These X-shaped contact patterns represent chromosomal arms bundled at the telomere (Figure 1A; right), as previously demonstrated in S. pombe meiotic prophase (Chikashige et al., 1997; Ding et al., 1998). The degree of alignment along the intra-chromosomal arms from the telomere was quantified by the “alignment index” (Figure S1D). The alignment index of chromosome 1 increased from 1.6 (onset of meiosis at 0 h) to 2.5 (meiotic prophase at 2.5 h) compared with 0.55 in VG (Figure 1D).

A similar structure was observed during meiotic prophase in mouse spermatocytes by Hi-C analysis, although it was not as evident as in S. pombe (Patel et al., 2019).

Rec8 cohesin produces a chromosome shape suitable for alignment
Next, we examined three factors that play a central role in meiotic recombination. Rec10 is a major component of LinE. Rec12 is a Spo11 homolog that induces DNA double-strand breaks (Keeney, 2001; Sharif et al., 2002). Rec8 is a meiotic cohesin. To investigate their contributions to meiotic chromosome structures, we confirmed synchronous progression of meiosis in these mutants and performed Hi-C analyses (Figure S2). rec10Δ and rec12Δ cells showed slight defects in formation of the X-shaped contacts (Figures 2A and 2B), with an alignment index of 2.8 (rec10Δ), 2.5 (rec12Δ), and 2.5 (wild type, wt). In contrast, rec8Δ cells showed prominent defects with almost no X-shaped contacts (Figures 2A and 2B; alignment index 1.5). These findings suggest that Rec8 regulates the alignment of homologs in a manner distinct from LinE formation to modulate recombination. The bulk of the chromosomes do not follow the horsetail movement at meiotic prophase in rec8Δ, while only telomere regions follow the leading edge of the moving nucleus (Ding et al., 2016b; Ding et al., 2006). These observations imply that the traction force generated by the horsetail movement is not transmitted to the chromosomes in rec8Δ, leading to the loss of alignment along the chromosomes. Therefore, Rec8 cohesin likely provides the chromosome with appropriate structural properties to counteract the pulling force, independent of LinEs (Figure 3F).

Rec8 cohesin is crucial to form meiosis-specific chromatin loops

Magnification of Hi-C data (2 kbp binning) in chromosome arm regions revealed that punctate Hi-C interactions occurred at the onset of meiosis (0 h) and became more prominent as meiosis progressed (2.5 h) in the wild type (arrows in Figures 3A and S3A). The positions at the bases of these interactions markedly coincided with the previously identified Rec8 accumulation sites (Ito et al., 2014) (Figures 3B and S3B). Consistently,
as meiosis progressed in the wild type, $P(s)$ (the contact frequency versus distance) indicated a “shoulder” at approximately 50 kbp, representing an increase in interactions within a region shorter than that length (Figure 3C). Crucially, punctate Hi-C interactions observed in the wild type were mostly lost in rec8Δ, but were retained in rec10Δ or rec12Δ (Figures 3A and S3A). In addition, the shoulder in $P(s)$ was lost in the rec8Δ mutant, but not in rec10Δ or rec12Δ (Figure 3D). These data suggest the linear compaction of chromosomes and the emergence of a Rec8-dependent cis-loop structure upon entry into meiosis in the wild type. In the Hi-C data of 5 kbp binning, topologically associating domain (TAD)-like signals were observed in wild type VG cells (dotted lines in Figure 3E), representing relatively long-range interactions manifest in mitotic chromosomes (Kim et al., 2016; Mizuguchi et al., 2014; Patel et al., 2019; Tanizawa et al., 2017). Interestingly, as Rec8-dependent loop structures appeared during meiosis, these TAD-like signals disappeared. Thus, *S. pombe* chromosomes undergo a structural transition through Rec8-dependent formation of chromatin loop-axis structures upon entry into meiosis (Figure 3F).

**Wpl1 in *S. pombe* regulates the length of chromatin loops in meiotic prophase**

In Rad21/Scc1 cohesin-dependent chromatin organization with TADs in somatic cells, Wapl depletion lengthens the chromatin loops and thickens the chromatin axis (Haarhuis et al., 2017; Tedeschi et al., 2013). However, it is unknown whether Rec8-dependent chromatin loops are affected by Wapl during meiosis. As shown in Figure 4A, the meiotic chromosome axial structures observed by Rec8-GFP were more prominent in *wpl1Δ* than in the wild type, suggesting that the axial structure becomes thicker in *S. pombe* meiosis. To examine the chromosome structure in *wpl1Δ*, we performed Hi-C analysis as in the
wild type (Figure S4A). In the Hi-C contact maps in \textit{wppl}\textDelta\ (Figure 4B, upper), the background was lower than that in the wild type (compare with Figure 1B and the lower panel of Figure 4B). These findings suggest that the inter- and intra-chromosomal long-range interactions, including X-shaped contacts, were reduced in \textit{wppl}\textDelta, which has a thicker chromatin axis, compared to the wild type (see Figure S4E).

In the amplified view of Hi-C data (2 kbp binning) in \textit{wppl}\textDelta, the number of grid-like punctate signals increased and the size of the diagonals of each grid increased upon entry into meiosis (Figures 4C and S4B). These findings imply that the nearby chromatin loops are in contact with each other at their bases with longer loops and thicker axes, leading to the linear compaction of chromosomes (Figure S4C). Consistently, compared to that of the wild type, a rightward shift of the shoulder in the \textit{P(s)} (blue dotted line for \textit{wt} and green dotted line for \textit{wppl}\textDelta in Figure 4D) and an increase in longer loop formation (Figure 4E) were observed in \textit{wppl}\textDelta. Note that, because axial signals of Rec8-GFP were still thicker in the absence of LinEs (\textit{wppl}\textDelta \textit{rec10}\textDelta double mutant, Figure S4D), Rec8 loop-axis structures formed independently of LinEs, even under the condition of \textit{wppl}\textDelta. These results strongly indicate that Wpl1/Wapl can antagonize the construction of chromosomal axes restricting chromatin loop extension, even when mediated by meiotic Rec8 cohesin in \textit{S. pombe} (Figure S4C).

\textbf{Identification of rec8 mutants showing defects in axis formation}

The foregoing results demonstrate that Rec8 cohesin is crucial for the formation of the meiosis-specific axis-loop structure in \textit{S. pombe} meiosis. Importantly, ectopic expression of Rad21 cohesin in meiosis partly compensates for the cohesion defect in the absence of
Rec8 (Kitajima et al., 2003; Yokobayashi et al., 2003). However, Rad21 signals were obscure along the chromatin axis (Figure S5A). Thus, besides cohesion, Rec8 may have an additional function that is more robust than Rad21 in chromatin axis formation. Therefore, it is possible to identify the mutant of rec8 that specifically shows defects in axis formation without affecting its cohesion function. To explore this, we examined the S. pombe strain in which Rec8 and Rec11 were ectopically expressed as the sole source of cohesin during mitosis, replacing Rad21 and Psc3 (Figure 5A). Using this strain as a screening host, randomly mutagenized rec8 cDNAs fused with GFP-coding DNA were transformed into the host strain. Since Rec8 is the only available kleisin protein in this strain, transformants will show a lethal or slow-growth phenotype if there is a rec8 mutation that causes loss of cohesion. Moreover, using the h90 mating-type and mei4Δ background, each transformant arrests at the meiotic prophase on agar plates of sporulation medium (Horie et al., 1998). We also introduced wp11Δ in the host strain to produce the prominent axis of Rec8-GFP (Figure 4A) so that defects in axis formation can be easily evaluated by microscopy observation (Figure 5A). Deletion of mei4+ represses the expression of recombination-related factors such as Rec12, resulting in almost no recombination (Miyoshi et al., 2012; Murakami-Tonami et al., 2007; Young et al., 2004). However, in the present study this did not seem to affect the Rec8 axis formation, at least in the wp11Δ background (Figure S5B).

We searched approximately 3000 viable transformants of the rec8 mutant library for mutants that showed axis formation defects with normal cohesion function in fluorescence microscopy. We identified a mutant that displayed normal growth (Figure S5C) but lost the Rec8 axis structure (Figure 5B). In this mutant, phenylalanine at amino
acid residue 204 of Rec8 was substituted with serine (rec8-F204S). Strikingly, sister chromatid cohesion was preserved in the rec8-F204S mutant, as assessed at the cut3 gene locus (Figure 5C) and other chromosomal loci (Figure S5D). In the cohesion assays, we used rec8-S552P or rec8Δ, which showed the cohesion defect, as a control strain (Figures 5C and S5D). The rec8-S552P mutant showed normal growth during mitosis (Figure S5C), but displayed defects in axis formation and cohesion during meiosis, probably due to low protein expression or low stability of the Psm1 subunit in the cohesin complex (Figure S5E and S5F). Although cohesion defects are expected to occur during mitosis of the P_adih-derived rec8-S552P strain (Figure S5C), growth would be ensured by their overexpression. Since the backup of Rad21 is likely to function only in rec8Δ cells, the rec8-S552P mutant was more deficient in cohesion than rec8Δ (Figures 5C and S5D). The cohesin complex seemed to be formed normally in the rec8-F204S mutant with comparable expression of Rec11 (Figures S5G and S5H). The F204 of Rec8 seemed to be conserved among Rec8 species in other organisms (Figure 5D), implying the significance of this residue in regulating Rec8 functions. Furthermore, the analysis of mutants in which F204 was replaced by amino acids other than serine indicated that the hydrophobicity of this position of Rec8 may be important for its axis-forming function, independent of its cohesion function (Figure S5I).

Thereafter, to delineate the details of axis formation defects caused by rec8-F204S, we observed chromosome morphology during horsetail movement in meiotic prophase. In the rec8-F204S mutant, only the nuclear leading edge followed the horsetail movement with the bulk of chromosomes remaining, similar to rec8Δ, in which axial assembly and chromosome compaction were lost (Figure 5E, Supplemental Movies 1, and 2). To
determine the degree of chromosome compaction more directly, we measured the distance between the telomere and the ade8 gene locus during meiotic prophase using a GFP-tagged telomere protein (Taz1) and a lacO/lacI-GFP tag at the ade8 gene locus (Figure 5F). As in rec8Δ, the telomere-ade8 distance was longer in the rec8-F204S mutant than in the wild type, suggesting that the chromatin of the rec8-F204S mutant is flexible and is abnormally stretched by the traction of horsetail movement.

**rec8-F204S mutant is defective in chromatin loop formation**

Next, we performed Hi-C analysis in the rec8-F204S mutant as in the rec8-wt (Figure S6A). In the Hi-C contacts of three chromosomes at meiotic prophase (2.5 h), formation of X-shaped contacts representing the bouquet orientation were apparently compromised in the rec8-F204S mutant, similar to rec8Δ (Figure 6A). The alignment index of chromosome 1 at meiotic prophase (2.5 h) decreased in rec8-F204S (1.6) compared with the wild type (rec8-wt, 2.6) (Figure 6B). The findings were consistent with the fact that chromatin is too flexible to resist the traction of horsetail movement in the rec8-F204S mutant (Figures 5E and 5F). Moreover, in the amplified 2 kbp binning view of Hi-C data, a clear decrease in the punctate Hi-C interactions was observed in rec8-F204S, although it was not as prominent as in rec8Δ (Figure 6C). In addition, the shoulder in P(s) became flattened in rec8-F204S compared with rec8-wt, both at 0 h and 2.5 h after meiosis induction (Figure 6D). Consistently, chromatin loops with a length of approximately 20 kbp that formed in a Rec8-dependent manner were diminished at meiotic prophase in rec8-F204S (Figure 6E). These findings suggest that in the rec8-F204S mutant, as in rec8Δ, the Rec8-dependent meiosis-specific short chromatin loop structures are lost, resulting in a concomitant loss of the structural property of the chromosome required for...
proper alignment. Next, we performed a chromatin immunoprecipitation (ChIP) assay to
determine the localization pattern of Rec8 on chromatin. The accumulation of Rec8-
F204S protein apparently decreased compared with wild type Rec8 at the loci of known
cohesin-enriched regions. In contrast, the localization in other chromosome arm regions
seemed to be normal (Figure S6B). Rec8-enriched regions mostly coincided with each
base of the chromatin loops (Figures 2B and S2B). In the case of rec8-F204S, the amount
of Rec8 of in the non-enriched region appeared normal. Thus, the reduced Rec8
probability at known enriched regions possibly reflects the noticeable loss of chromatin
loop formation (Figure S6C).

Rec8-dependent axis-loop structure is required for LinE formation and
recombination

To delineate the significance of the Rec8-dependent axis-loop structure in terms of
meiotic recombination, we examined the formation of LinEs during meiotic prophase. In
wild type cells, the LinE component Rec10, marked by mCherry, localized within the
entire nucleus, similar to Rec8 (Figures 7A and S7A; CK1+, rec8-, rec11-wt). In rec8-
F204S cells, however, Rec10-mCherry formed aberrant dotty or filamentous aggregates
within the nucleus (Figures 7A and S7A), as with rec8Δ or rec11-5A, in which LinE was
completely lost (Sakuno and Watanabe, 2015). Interestingly, Rec11 phosphorylation by
CK1, which is required for the assembly of LinEs (Sakuno and Watanabe, 2015), occurred
normally in the rec8-F204S mutant, similar to the wild type (Figure 7B). We also
expressed mimetic mutations of CK1-mediated phosphorylation of Rec11 (rec11-5D),
which can suppress the defects caused by the loss of Rec11 phosphorylation via CK1
(Sakuno and Watanabe, 2015). Strikingly, the expression of Rec11-5D protein did not
suppress defects in LinE formation or meiotic recombination in rec8-F204S (Figures S7A and S7B). These results strongly suggest that axis-loop formation along sister chromatids via Rec8 cohesin is still essential for LinE assembly, even in the presence of active CK1-dependent regulation. Consistent with the LinE formation defect, rec8-F204S cells showed a decreased frequency of crossover recombination at several loci (Figure 7C). Intragenic recombination between the genomic ade6-M26 allele and plasmid harboring the ade6-469 allele was also significantly reduced in rec8-F204S, similar to rec8Δ (Figure S7C). Collectively, these results demonstrate the essential role of the Rec8-dependent loop-axis structure, which may function as a scaffold for LinE assembly, thus facilitating meiotic recombination.

Discussion

We demonstrate the Rec8-dependent formation of axis-loop structures during meiosis in S. pombe, an asynaptic organism. The Rec8 cohesin functions as a scaffold for the formation of the LinE axis, thereby ensuring meiotic recombination during meiosis.

Pairing of homologous chromosomes in S. pombe meiosis

Our Hi-C analysis revealed the emergence of an intriguing X-shaped contact map representing tight contacts between chromosome arm regions near the telomere in S. pombe meiotic prophase. This is consistent with the previous microscopy observation of telomere clusters at the SPB (the bouquet orientation of chromosomes) during meiotic prophase (Chikashige et al., 1994; Ding et al., 1998). The Hi-C data indicate that contacts also occur between non-homologous arms, not only between homologous arms. It should be noted that chromosomes recognize their homologous partners to pair within the bundle.
of all chromosomes. Therefore, there must be mechanisms to eliminate non-homologous pairing, while simultaneously promoting homologous pairing. Such selective forces between chromosomes can be provided by the oscillatory movement of telomere-bundled chromosomes (Chacón et al., 2016; Takao et al., 2019). Additionally, noncoding RNAs play a robust role in recognizing homologous pairs (Ding et al., 2019; Ding et al., 2012). Importantly, Rec8 dominates noncoding RNA-mediated robust pairing (Ding et al., 2016a; Ding et al., 2016b; Hiraoka, 2020). Therefore, Rec8-mediated chromatin structures are an absolute requirement in the pairing homologous chromosomes during meiosis.

Loop-axis structure of chromatin in *S. pombe* meiosis

Upon entry into meiosis in *S. pombe*, chromosomes form a loop between the Rec8 binding sites (Figures 3B and S3B), which is also evident in *S. cerevisiae* meiosis (Muller et al., 2018; Schalbetter et al., 2019). Although this Rec8 geometry is undetectable via Hi-C in mammalian meiosis (Luo et al., 2020; Patel et al., 2019; Wang et al., 2019), it is argued that the chromosomal position of Rec8 or loop location varies from one cell to another in mammals (Patel et al., 2019). Importantly, the Rec8 axis-loop structure formed normally without LinEs in *S. pombe* (rec10Δ in Figure 3A). Therefore, we emphasize that the Rec8-dependent axis-loop structure is distinct from LinEs and not a part of them. This relationship seems to be valid between cohesin-dependent axis-loop structures and lateral elements in other SC-forming organisms (Fujiwara et al., 2020; Fukuda et al., 2010; Pelttari et al., 2001). With the emergence of Rec8-dependent loop structures, TAD-like signals observed in mitotic interphase cells mostly disappeared during meiotic prophase (Figures 3A and 3E), similar to mammalian spermatocytes (Luo et al., 2020; Patel et al., 2019).
The emergence of a loop structure with the disappearance of TADs is probably due to the switch of kleisin subunits from Rad21 to Rec8 that occurs upon the commitment to meiosis.

Notably, in the meiotic prophase of wp1lΔ cells, the Rec8 axis became thicker, while each chromatin loop became longer (Figures 4A, 4C, 4D, and 4E), as has been described in mammalian somatic interphase cells (Haarhuis et al., 2017). In the case of S. cerevisiae, WPL1 deletion was also reported to cause chromosomal compaction and recombination defects with an increase in Rec8 amounts on chromatin (Challa et al., 2016). If the axial structures become thicker and the chromosomes become more compact, one would intuitively expect that the alignment of homologs would be easier. However, the opposite was true: X-shaped interactions tended to be reduced in the Hi-C analysis of wp1lΔ cells, and alignment of chromosome arms also became defective in wp1lΔ (Figure S4F), similar to pds5Δ (Ding et al., 2016b). Pds5 associates with the cohesin complex and is required for the chromosomal localization of Wpl1 (Ding et al., 2006; Goto et al., 2017; Lin and O’Connell, 2017), explaining the similar phenotypes of pds5Δ and wp1lΔ, such as thicker Rec8 axis formation and the alignment of chromosome arms during meiosis (Figures 4A, S4E, and S4F). These alignment defects are presumably caused by the overall stiffness of the chromosomes with thicker axes in wp1lΔ. In fact, examination of the chromosomes during horsetail movements in wp1lΔ rarely revealed torsional turning (Figure S4G, Supplemental Movies 1, and 3). Thus, our findings imply an interesting mechanism by which S. pombe maintains proper chromosome stiffness through the function of the Rec8 cohesin.
Possible mechanisms for formation of the Rec8 chromatin axis

The Rec8-dependent emergence of punctate Hi-C contacts only during meiosis suggests mechanisms by which the chromatin loop structure is formed differently from mitotic Rad21 cohesin. Fluorescence microscopy revealed that the Rec8 axis in the wpl1Δ background is reproduced in the mutant of Rec10, Hop1 (a homolog of HORMAD), or Mek1 (Figure S5J). All are required for LinE formation (Lorenz et al., 2006). The same was true in the Rec12 mutant and regulators of Rec12, such as Rec7, Rec15, and Mei4 (Figures S5B and S5J). Our Hi-C analysis demonstrated that the chromatin loop structure was also maintained in rec10Δ and rec12Δ (Figures 3A and S3A). Therefore, critical regulators of Rec8 axis formation remain to be identified among the factors expressed in a meiosis-specific manner and are required for recombination.

Mechanisms of chromatin loop formation have been proposed in mammalian somatic cells. cis-Looping of distal chromatin or TAD formation is thought to be established by the action of the Rad21 cohesin complex anchored on chromatin via the CCCTC-binding factor (CTCF) (Li et al., 2020; Nora et al., 2017; Rao et al., 2014; Wutz et al., 2017). More recently, a loop extrusion model was proposed for the formation of chromatin loops by SMC protein complexes, including cohesin (Davidson and Peters, 2021; Fudenberg et al., 2016; Goloborodko et al., 2016; Sanborn et al., 2015). In this model, the SMC protein complex interacts with DNA, reels, and extrudes it through its own ring structure to form a DNA loop (Davidson et al., 2019; Ganji et al., 2018; Kim et al., 2020; Kschonsak et al., 2017; Terakawa et al., 2017). At least in vitro, human Rad21 cohesin is able to extrude DNA, but it requires the cohesin loading factors Scc2 and Scc4 (Davidson et al., 2019). Interestingly, CTCF is a conserved factor only in vertebrates (Hore et al., 2008). Thus,
there are no homologs of CTCF in yeasts. In contrast, Scc2 and Scc4 are conserved from yeasts to humans (Mis4 and Ssl3, respectively, in S. pombe). Interestingly, chromatin loop structures were already observed in S. pombe at meiosis entry (0 h) when the Rec8 cohesin is loaded by Mis4 and Ssl3 before pre-meiotic DNA replication (Figures 3A and S1A). Furthermore, the rec8-F204S mutant isolated in this study showed a significant defect in the formation of loop structures, despite maintaining the cohesion function. Strikingly, phenylalanine at position 204 in Rec8 is located in the domain where kleisin interacts with Scc2/Mis4, as previously shown by an in vitro binding assay (Kikuchi et al., 2016).

Thus, as an alternative scenario for an appealing molecular mechanism in the establishment of meiosis-specific chromatin loop structures, Rec8 and Mis4 with Ssl3 may also play an important role. Mis4 with Ssl3 may replace the function of CTCF in anchoring cohesin to chromatin. It is also plausible that some changes in chromatin may have occurred upon entry into meiosis, such as the modification of histones suitable for the formation of axes with chromatin loops by the Rec8 cohesin.

**Rec8 axis as a scaffold for LinE assembly**

In the rec8-F204S mutant, which was unable to form a loop structure, LinE formation failed to occur and the recombination frequency was markedly reduced (Figures 7C, S7B, and S7C). However, phosphorylation of Rec11, which is required for LinE formation, was intact and the distribution of Rec8-F204S along the chromosomes was largely normal (Figures 7B and S6B). Based on the observations of the localization of Rec10 (LinE) by ChIP and fluorescence microscopy, Rec10 and Rec8 overlap in some regions of the chromosome, but not in many other chromosome regions (Ding et al., 2021; Miyoshi et al., 2012; Sakuno and Watanabe, 2015). It is likely that the vast majority of Rec10 and
other LinE factors initially localize to chromosomes through interaction with Rec8 cohesin, and then are redistributed along the chromosome appropriately. We speculate that the oligomerization of LinE factors, such as Rec10, is necessary for the formation of LinE, which requires a certain amount of Rec8 to be present in a continuous axial structure. The Rec8 axis acts as a scaffold to trigger the axial formation of LinEs. Otherwise, LinE factors associated with the chromosome are not stabilized (Figure 7D).

As a result, LinE factors are released from the chromosome, and finally form aggregates in the nucleus (Figures 7A, S7A, and 7D), due to their propensity to adhere to each other, as in the polycomplex formed by mutants with defects in SC formation during meiosis of *S. cerevisiae* (Zickler and Kleckner, 1999). Crucially, in addition to Rec11-5D mutant protein, expression of the Rec11-Rec10 fusion protein, which can suppress the defects caused by the loss of Rec11 phosphorylation (Sakuno and Watanabe, 2015), did not suppress the LinE formation defect of *rec8*-F204S (Figures S7A, S7B and S7D). This result strongly supports the view that the interaction between Rec8 and Rec10 is not stabilized in the absence of the Rec8 axis along the chromatin. LinEs in *S. pombe* are crucial in the assembly of complexes on chromatin that induce double-strand breaks on DNA. This explains why a Rec8-dependent axis-loop structure is required for meiotic recombination. Importantly, F204 in *S. pombe* Rec8 appears to be conserved among some eukaryotes, including mammals. Although the relevant residue in mouse is leucine, we demonstrated that the phenylalanine to leucine substitution in *S. pombe* Rec8 showed almost no defects in axis formation (Figures 5D and S5I). Rec8 and Rec11/SA3 are required for lateral element formation in mammals, as well as for axial element formation in *S. pombe* (Fukuda *et al.*, 2014; Hopkins *et al.*, 2014; Llano *et al.*, 2014; Sakuno and Watanabe, 2015; Winters *et al.*, 2014). Thus, there may be a mechanism by which
meiosis-specific cohesin-dependent axial structures can function as a scaffold in the formation of lateral elements in mammals.

In conclusion, the current results obtained in S. pombe identify crucial functions of Rec8-dependent assembly of chromatin structures that facilitate the execution of meiotic recombination essential for faithful propagation of genetic materials into the progenies during meiosis. The diverse and essential functions of the Rec8 cohesin in supporting meiotic recombination may provide a basis for understanding the molecular mechanisms of meiotic chromosome axis maturation and regulation of recombination in mammals, potentially related to miscarriages and congenital abnormalities in humans (Nagaoka et al., 2012; Webster and Schuh, 2017).

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Author contributions

T.S., S.T., T.H., N.K., and Y.H. conceived and designed the experiments; T.S. conducted almost all of the experiments except for the Hi-C, under supervision of T.H. and Y.H; S.T.
and O.I. performed Hi-C related analyses, under supervision of N.K; Bioinformatics-related works were implemented by H.T; D.-Q.D. provided the data about the pairing state in fission yeast meiosis; T.S., T.H., K.N., and Y.H. wrote the manuscript with the input from all the authors.

Declaration of interest

The authors declare no competing interests.
Figure legends

Figure 1. Hi-C analysis firmly recapitulates the structure of meiotic bouquet chromosomes

(A) Chromosome arrangement in mitosis and meiosis in *S. pombe*. In mitosis (left), centromeres are clustered at the spindle pole body (SPB) and display the Rabl orientation. The grey lines indicate sister chromatids. In meiotic prophase (right), grey and black lines indicate a homolog pair of chromosomes. Chromosomes are bundled by the telomeres clustered at the SPB via telomeres, representing the bouquet orientation. (B) Hi-C contact maps at 20 kbp resolution for three chromosomes prepared at vegetative growth (VG), 0 h, and 2.5 h after meiosis entry. Schematic views of each chromosome are shown on the top and the left. Green arrowheads represent the centromere clustering. Blue arrowheads in meiosis 0 h and 2.5 h represent the telomere clustering as depicted by the left lower diagonal lines. The color scale on the right represents the percent ranking of contact scores. (C) Heat maps showing the difference of contacts detected by Hi-C. Left, subtraction of VG data from 0 h data. Right, subtraction of 0 h data from 2.5 h data. The color scale on the right represents the percent ranking of difference scores. (D) Degree of alignment of the two chromosome arms of telomere-bundled chromosome 1 is quantified as the alignment index (Figure S1D) and is shown as the value along with a black vertical line in the Hi-C contact map.

Figure 2. Rec8 cohesin shapes chromosomes into a structure suitable for its alignment

(A) Hi-C contact maps of three chromosomes for the indicated strains, prepared from the cells 0 h and 2.5 h after meiosis entry. The color scale on the right represents the percent
ranking of contact scores. (B) The alignment index for the indicated strains at meiotic prophase (2.5 h after meiosis induction) is shown as the value along with a black vertical line in the Hi-C contact map of chromosome 1.

**Figure 3. Rec8 cohesin is crucial to construct the meiosis-specific chromatin loops**

(A) Amplification of the Hi-C contact map in the 200 kbp region of chromosome 1 plotted at 2 kbp resolution for the indicated strains at meiosis (0 h) and 2.5 h (at VG only for wild type). The black arrow depicts the loop structure. (B) A merged view of the Hi-C contact map and ChIP-chip data of Rec8 at the 200 kbp region of chromosome 1 shown in (A). ChIP-chip data were adopted from the previously described source (Ito et al., 2014). (C) Contact probability versus genomic distance, $P(s)$, for VG (red), meiosis 0 h (blue) and 2.5 h (orange) of the wild type. The “shoulder” marked by the dotted line at approximately 50 kbp represents an increase in interactions within a region shorter than that length. (D) Contact probability versus genomic distance, $P(s)$, for wild type (red), rec8Δ (blue), rec10Δ (orange), and rec12Δ (green) 2.5 h after meiotic induction. (E) Amplification of Hi-C contact maps at the 500 kbp region of chromosome 1 and 2 plotted at 5 kbp resolution for wild type strain at VG, meiosis 0 h and 2.5 h. The dotted line depicts the TAD-like structure. (F) Schematic diagram of chromosome conformation at meiotic prophase in *S. pombe*. All chromosomes are tightly aligned from the telomeres to the centromeres. Rec8 establishes the meiosis-specific chromatin loop structure, irrespective of LinEs or recombination.

**Figure 4. Wpl1 regulates chromatin loop length during meiotic prophase in *S. pombe***

(A) Serial Z sections (0.3 μm each) of Rec8-GFP signals at meiotic prophase in wild type
and \( wpl1 \Delta \). Axis-like signals of Rec8 are clearly observed in \( wpl1 \Delta \). (B) Upper panel displays Hi-C contact maps of three chromosomes at 20 kbp resolution in \( wpl1 \Delta \) at vegetative growth (VG) and 0 h, and 2.5 h after meiosis entry, with schematic views of each chromosome. The color scale on the right represents the percent ranking of contact scores. The lower panel displays heat maps showing the difference of contacts detected in each genome region by Hi-C in wild type and \( wpl1 \Delta \) at VG, and meiosis 0 h and 2.5 h. The color scale on the right represents the percent ranking of difference scores. (C) Amplification of Hi-C contact maps showing the 200 kbp region of chromosome 2 plotted at 2 kbp resolution for the indicated strains at VG and meiosis 0 h and 2.5 h. (D) \( P(s) \)'s for meiosis 0 h and 2.5 h of wild type (red and blue) and \( wpl1 \Delta \) (orange and green). The blue or green dotted line represent a shoulder of wild type or \( wpl1 \Delta \) at 2.5 h after meiosis entry, respectively. (E) The number of loops per chromatin distance in \( wpl1 \Delta \) and wild type was calculated. The value obtained by dividing \( wpl1 \Delta \) data by wild type data is shown as the log\(_2\) ratio.

**Figure 5. Isolation of rec8 mutant showing defect in axis formation with intact cohesion function**

(A) The left panel shows a schematic view of cohesin complexes in fission yeast in mitosis and meiosis. In mitosis, Rad21-Psc3 cohesin complex ubiquitously locates along the whole chromosomes. In meiosis, Rec8-Psc3 cohesin complex exists only at the centromeres and Rec8-Rec11 cohesin complex at the chromosomal arm regions. The right panel shows the scheme for isolation of rec8 mutants that specifically show defects in axis formation, but normal cohesion in meiotic prophase. The promoter of the rec8 gene is replaced by the \( adh1 \) promoter, and the promoter of the rec11 gene is replaced by
the adh41 (a weaker version of adh1) promoter, to allow their expression both in mitosis and meiosis. After transformation of a library of GFP-fused rec8 mutants with a selective marker bsdR (not shown here), the obtained blasticidin-resistant colonies were subsequently induced to meiosis to observe the axial Rec8-GFP signals. Growth rate of the blasticidin-resistant clone acts as an indicator of the functionality of Rec8-Rec11 cohesin. (B) Representative pictures of Rec8-GFP with a cell shape (white dotted line) during meiotic prophase in wild type and the isolated rec8-F204S mutant in this screening. (C) Cohesion defects in the chromosome arm region (cut3+ locus, left arm of chromosome 2) were examined in the indicated cells arrested at prophase I by the mei4Δ mutation. After fixation by methanol, the number of heterozygous cut3-GFP signals was counted (n > 150). A score of 2 or 1.5 cut3-GFP signals represents the complete or partial loss of sister chromatid cohesion, respectively. The rec8-S552P identified in this screening displays a remarkable defect in cohesion that is stronger than that of rec8Δ (see the text). Error bars show standard deviations (SD) from three independent experiment. P values were obtained from Chi-square test for statistical significance. (D) The upper panel is a schematic diagram of S. pombe Rec8. Domains for the known interaction with cohesin-related factors are shown. The position of F204 is also indicated by the red arrowhead. The lower panel depicts the alignment of Rec8 homologs in Shizosaccharomyces pombe (Sp), S. octosporus (So), human (h), mouse (m) and frog (Xenopus leaves) (x). (E) Time-lapse observation (5 min intervals) of horsetail nuclear movement in the indicated cells stained with histone H2B-mCherry. The right cartoon represents a model of the chromatin state for each strain. In rec8Δ, Rad21 partially compensates the cohesion defect. (F) The upper left panel shows a schematic drawing of the distance of ade8-lacO inserts from the telomere of chromosome II. The upper right panels represent images of GFP signals for
the indicated strains. Brighter GFP signals at the edge of nucleus represent telomeres (Taz1-GFP) and weaker GFP signals represent the ade8 locus. The lower graph displays a plot of the distance between the telomere and the ade8 locus in each cell during meiotic prophase for the indicated strains with average distance (n=55).

Figure 6. rec8-F204S mutant displays defective chromatin loop formation

(A) Hi-C contact maps of three chromosomes at 20 kbp resolution in wild type rec8-3×Pk and rec8-F204S-3×Pk cells at 0 h and 2.5 h after meiosis entry, with schematic views of each chromosome. The color scale on the right represents the percent ranking of contact scores. (B) The alignment index for indicated strains at meiotic 0 h and 2.5 h after meiosis induction is shown as the value along with a black vertical line in the Hi-C contact map of chromosome 1. (C) Amplification of Hi-C contact maps of the 200 kbp region of chromosome 1 (upper) and chromosome 2 (lower) at 2 kbp resolution, for the indicated strain at meiosis 0 h and 2.5 h. (D) Contact probability versus genomic distance, P(s), for meiosis 0 h and 2.5 h of rec8-wt (red and blue) and rec8-F204S (orange and green). A shoulder observed in meiosis 2.5 h of rec8-wt was shown with dotted line. (E) Number of each loop per length quantified using genome-wide loop caller HiCCUPS for the indicated strains at meiosis 0 h (left) and 2.5 h (right).

Figure 7. Rec8-dependent chromatin loop formation and meiotic recombination

(A) Representative pictures of Rec10-mCherry with cell shape (white dotted line) during meiotic prophase for the indicated strains. The yellow arrows depict the aggregates of LinE. (B) Western blot analysis. Whole-cell extracts were prepared from meiotic prophase (2.5 h after meiosis induction) expressing Rec8-3×Flag in wild type and rec8-
F204S, with Psm1-3×HA. Rec11 phosphorylation by CK1 was detected by anti-phosphorylated Rec11 (at T7). The monoclonal anti-tubulin (DM1A, Abcam) antibody was used as a loading control. (C) Intergenic meiotic recombination for ura1-lys3 (n > 700), cox7-vps38 (n > 500), and vps38-leu1 (n > 500) in the indicated zygotes. Error bars show standard deviations (SD) from three independent experiment. (D) Model for the Rec8 cohesin-dependent regulation of LinE assembly and meiotic recombination. After the commitment in meiosis, Rec8 cohesin shown in blue is loaded onto the chromosomes via cohesin loader complex (Mis4-Ssl3) shown in orange. Once loaded, Rec8 cohesin promotes the loop formation that is antagonized by Wpl1 shown by purple. By meiotic prophase, in parallel with the continuous loading of Rec8 and phosphorylation of Rec11 by CK1 (indicated by red triangles), chromatin looping with axis formation by Rec8 cohesin occurs to form chromatin to recruit LinE factors. See the detailed description in the Discussion.
STAR Methods

S. pombe strains

All media and growth conditions were as described previously (Moreno et al., 1991). Complete medium supplemented with uracil, lysine, adenine, histidine, and leucine (YE5S), minimal medium (EMM2), and sporulation-inducing medium (SPA and SSA) were used unless otherwise stated. The strains used in this study are listed in Table S1. Deletion of the endogenous genes (rec12+, rec11+, rec7+, rec15+, mek1+, rec8+, rec10+, hop1+, wpl1+, pds5+, and mei4+) and tagging (rec10+, rec8+, psm1+, rec25+, rad21+, and rec11+) using GFP, mCherry, 3×hemagglutinin (HA) tag, 3×Pk (V5 tag), or 3×Flag was performed according to the PCR-based gene targeting method for S. pombe using the ura4+, kanMX6 (kanR), hphMX6 (hygR), natMX6 (natR), and bsdR genes as selection markers (Bahler et al., 1998; Rabitsch et al., 2001; Sato et al., 2005). Histone H2B tagging by mCherry for time-lapse observation has been described previously (Ruan et al., 2015). The CK1 shut off mutant, rec11-5A mutant, rec11-5D mutant, and the strain expressing Rec10-GFP-Rec11 fusion protein have been described previously (Sakuno and Watanabe, 2015). For the assay of crossing over recombination frequency, the hygR cassette was inserted 1215 bp upstream from the start codon of the cox7+ gene and the natR cassette was inserted 1000 bp upstream from the start codon of vps38+ gene using the PCR-based gene targeting method. To generate strains of rec8 point mutants with tagging of GFP or 3×Pk tag, the endogenous rec8+ locus was cloned with its promoter and terminator into pUC119. Using the NEBuilder system (New England Biolabs), a DNA fragment encoding GFP or 3×Pk tag with kanR or bsdR was introduced immediately before the stop codon of the rec8 gene cloned in pUC119. Next, target sequences of rec8+ were subsequently mutated using the KOD One Master Mix Blue (TOYOBO) to generate an amino acid
substitution. In case of rec8-S552P, a C-terminal portion of rec8\(^+\) open reading frame including the mutation site and marker gene with the rec8\(^+\) terminator was amplified by PCR and transformed into the wild type rec8\(^+\) locus. Alternatively, in the case of N-terminal mutations such as rec8-F204S, the promoter to terminator of the rec8\(^+\) gene, including the mutation and marker gene, was amplified by PCR and transformed into the \(\Delta\)rec8::ura4\(^+\) strain. Correct replacement was confirmed using PCR and direct sequencing. The strain in which the mitotic cohesin, Rad21 and Psc3, were exchanged for meiotic cohesin (Rec8 and Rec11) has been described previously (Kitajima et al., 2003). To construct the pat1-as2 diploid with MDR-sup, haploid strains (\(h^+\) pat1-as2 MDR-sup and \(h^-\) pat1-as2 MDR-sup mat-Pc) were used (they were generously provided by Masamitsu Sato, Waseda University). After introducing tagging or disruption of a gene of interest in both haploids, protoplast fusion was performed as described previously (Flor-Parra et al., 2014), except for the use of Lysing Enzyme (Sigma-Aldrich) instead of Lallzyme or Zymolyase.

**Preparation of meiotic cells**

For microscopic observation of Rec8-GFP, Rec11-GFP, Rec10-mCherry, and Rec25-mCherry, \(h^{oo}\) cells cultured on YE plates at 26°C overnight were streaked on SSA plates, or suspended in 20 mg/ml of leucine and spotted on SPA or SSA plates. The cells were incubated at 26°C for 6–10 h to observe the cells at the horsetail stage. In case of \(lacO/lacI\)-GFP observation for cohesion assays (Figure 5C and S5D), \(h^+\) and \(h^-\) cells were mixed with 20 mg/ml of leucine and spotted on SSA or SPA plates. For the immunoprecipitation of Rec8-GFP with Psm1-3HA (Figures S5F), \(h^+/-\) diploid cells with mei4\(\Delta\) were used to arrest the cells at the meiotic prophase. In the case of diploid
cells, the cells were grown in EMM2 liquid medium containing 5 mg/ml NH₄Cl at a
density of 5×10⁶ cells/ml at 25°C and then resuspended in EMM2 (1% glucose) medium
lacking NH₄Cl at a density of 1×10⁷ cells/ml at 25°C for 15 h. For the
immunoprecipitation of Rec8-3×Flag with Psm1 (Figure S5G), ChIP assay (Figure S6B),
detection of phosphorylated Rec11 (Figure 7B), and Hi-C analyses, we used h+/h− diploid
cells with pat1-as2 and MDR-sup alleles. Cells grown in YE liquid medium at a density
of 5–10×10⁶ cells/ml at 25°C were collected by centrifugation and washed twice with
EMM2 (1% glucose) medium lacking NH₄Cl (EMM2-N). Cells were then resuspended
in EMM2-N with 50 mg/ml leucine at a density of 5×10⁶ cells/ml and incubated at 25°C
for 6.5 h to induce G1 arrest. By adding the same volume of EMM2-N with 50 mg/ml
leucine and 15 µM of 1-NM-PP1 (Toronto Research Chemicals Inc.) (5–8×10⁶ cells/ml),
cells can enter meiosis. They were further incubated for 2.5 h or more. Before harvesting
the meiotic cells, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) was
added to reduce protein degradation.

**Rec8 mutant screening**

The rec8⁺ cDNA mutant library used for the screening was constructed as follows. The
DNA region encoding 3×HA-ura4⁺ of the plasmid VP068 (pUC119 base, Padh1-
rec8⁺ cDNA-3×HA-ura4⁺-Trec8) (Yokobayashi et al., 2003) was replaced by the DNA
region encoding GFP-BsdR to produce VP068-GFP-bsd. Error-prone PCR of rec8⁺
cDNA in VP068-GFP-BsdR by Ex taq (TaKaRa Bio) was performed using several
concentrations of MnCl₂ (final 0.05 mM, 0.15 mM, 0.25 mM, and 0.3 mM) and an
additional 1 mM dGTP or dATP with 0.2 mM of dNTP mix (total 8 conditions). By using
the NEBuilder system, amplified rec8⁺ cDNA pools (from eight conditions) were then
cloned into the fragment, which was amplified by inverse PCR of VP068-GFP-BsdR without the rec8+ cDNA region. In total, approximately 5300 Escherichia coli transformants (representing library size) were generated. All colonies were recovered from the plates, and plasmids (pRec8 library) were extracted. The pRec8 library was estimated to contain approximately six mutations/kb. Using the pRec8 library as a template, the region from 620 bp upstream of the adh1+ promoter to 600 bp downstream of the rec8+ terminator was amplified by PCR. These fragments were used to transform the screening host strains. More than 95% of blasticidin-resistant colonies obtained after transformation showed correct integration, as confirmed by PCR. Rec8-GFP signals during meiotic prophase were observed among the blasticidin-resistant colonies, which showed relatively normal growth.

Fluorescence microscopy

Fluorescence images were obtained using an Axioplan2 microscope (Carl Zeiss) equipped with a Quantix cooled CCD camera (Photometrics) and a DeltaVision microscope system (GE Healthcare Inc.) equipped with a CoolSNAP HQ2 cooled CCD camera (Photometrics) and a 60× Plan-ApoN SC oil immersion objective lens (NA=1.40; Olympus). The brightness of the images was adjusted using Fiji software (Schindelin et al., 2012) without changing the gamma settings. The pairing frequency was quantified as described previously (Ding et al., 2004).

For time-lapse live-cell imaging of Rec8-GFP and H2B-mCherry, cells were cultured overnight in liquid EMM2 at 26°C to reach stationary phase, and cells were spotted onto SPA plates to induce meiosis, and incubated at 26°C for 4–5 h. Zygotes suspended in 1% glucose EMM2 (1% glucose) without nitrogen were sonicated briefly
and immobilized on a coverslip coated with lectin in a 35 mm glass-bottomed culture dish (MatTek) and observed at 26°C in liquid EMM2 (1% glucose) without nitrogen using a DeltaVision microscope in an air-conditioned room. Images were collected at approximately nine focal sections with 0.45 µm interval at each time point. The images were collected every 5 min at three focal sections to minimize the excitation light during long-term observation. Images were subsequently acquired using Z-sectioning and projected according to the SoftWoRx software program (Applied Precision).

**Immunoprecipitation**

The cell extracts (~8×10⁷ cells) were prepared by bead-beating cells in IP buffer (50 mM HEPES-KOH at pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 2.5 mM MgCl₂, 20% glycerol, 1 mM PMSF, 0.8% Triton X-100, 0.1% sodium deoxycholate, and 5× protease inhibitor cocktail (P8215; Sigma-Aldrich). Fifty units of DNase I (TaKaRa Bio) were added to the cell extract and sonicated (Branson Inc.). After collecting the supernatant as whole-cell extracts by centrifugation at 15,000 rpm for 30 min, immunoprecipitation was performed by incubating these extracts for 1 h at 4°C with anti-GFP polyclonal antibodies (Rockland) or anti-Flag polyclonal antibodies (anti-DDDDK, MBL), and an additional 2 h at 4°C with 20 µl of a slurry of IgG-conjugated dynabeads (Invitrogen). After washing the beads five times with IP buffer, the whole-cell extracts and immunoprecipitates were subjected to western blotting using monoclonal anti-GFP (JL-8, Roche), Flag-M2 monoclonal antibody (Sigma-Aldrich), monoclonal anti-HA (3F10, Roche), and monoclonal anti-tubulin (DM1A, Abcam) antibodies.

**ChIP assay**
The procedures were carried out as described previously (Yokobayashi and Watanabe, 2005). Anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) and IgG conjugated dynabeads (Invitrogen) were used for immunoprecipitation. DNA prepared from the whole-cell extracts or immunoprecipitated fractions was analyzed via quantitative PCR (qPCR) with the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) using Power SYBR Green PCR Master Mix"® Green I Master (Thermo Fisher Scientific). The primers used for PCR have been described previously (Sakuno and Watanabe, 2015). Immunoprecipitation with control mouse IgG antibody (Abcam) was performed in each experiment to account for nonspecific binding in the ChIP fractions and verify the significance of immunoprecipitation. The percent IP was quantified using three independent qPCR experiments.

Recombination assay

For intergenic recombination, haploid strains containing appropriate markers were mated and sporulated at 26°C on SSA plates for 30 h. The spores were isolated using β-glucuronidase solution (Wako) and plated on YE. The colonies were then replicated in selective media [EMM2-uracil, EMM2-lysine, EMM2-lysine, YE5S containing hygromycin B (FUJIFILM) or YE5S containing clonNAT (WERNER BioAgents)] and YE5S containing phloxine B (Wako) to select haploid cells. In the ura1-lys3 interval, the degree of lysine auxotrophy among the uracil prototrophic colonies (n>700, replicated three times) was assessed. In the cox7+-vps38+ interval, the degree of hygromycin B-resistant (hygR-containing) colonies among the clonNAT-resistant (natR-containing) colonies was assessed (n>500, and replicated three times). In the vps38+-leu1+ interval, the degree of leucine prototrophic colonies among the clonNAT-resistant (natR-
containing) colonies was assessed (n>500, and replicated three times). For intragenic recombination, \(h^{90}\) haploid strains containing the \(ade6-M26\) allele and plasmids harboring the \(ade6-469\) allele according to the \(ura4^+\) marker (Ponticelli and Smith, 1992) were sporulated at 26°C on SPA plates for 30 h. The spores were isolated by treatment with gluculase and plated on YE5S containing phloxine B (to count all viable haploid spores) and EMM2-adenine plates. The frequency of intragenic recombination was quantified as the degree of adenine prototrophic colonies among viable haploid spores (n>7400, and replicated three times).

Flow cytometry

A total of \(1\times10^7\) cells were fixed with 70% ethanol and incubated with 50 µg/ml RNase A in 500 µl of 50 mM sodium citrate (pH 7.5) for 4 h at 37°C, and then stained with 0.5 µg/ml propidium iodide. DNA content of \(1.5\times10^5\) cells was measured on a FACS Calibur instrument (BD Biosciences).

In situ Hi-C

In situ Hi-C experiments were performed as described previously (Tanizawa et al., 2017) with minor modifications. Asynchronous or meiotic cell cultures of \(4\times10^8\) cells were fixed with 3% paraformaldehyde at 26°C for 10 min. The fixed cells were disrupted using glass beads and Mini-Beadbeater-16 (BioSpec Products) in lysis buffer (50 mM HEPES/KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1× Complete [Roche, 11836170001], and 1 mM PMSF). The lysed cells were collected by centrifugation and permeabilized by resuspension with 1.11× NEBuffer 2 (B7002, NEB) containing 0.1% sodium dodecyl sulfate (SDS) at 62°C for 7 min. After
SDS was quenched by adding 1/9 volume of 10% Triton X-100, the permeabilized cells were treated with 25 units of MboI (R0147, NEB) at 37°C overnight. The enzyme was inactivated by incubation at 62°C for 20 min. After centrifugation, the pellet containing the restricted DNA fragments was used in the following procedure. A half volume of fill-in mix [1× NEBuffer 2, 150 μM biotin-14-dATP (NU-835-BIO14, Jena Bioscience), 150 μM dCTP, 150 μM dGTP, 150 μM dTTP, 0.4 units/μL Klenow fragment (M0210, NEB)] was added to the pellet and incubated at 37°C for 45 min. Subsequently, three volumes of ligation mix [1.33× T4 DNA ligase buffer (B0202, NEB), 1.11% Triton X-100, 133 μg/ml bovine serum albumin (B9001, NEB), 5.56 units/μl T4 DNA ligase (M0202, NEB)] was added and incubated at room temperature (~25°C) for 4 h. After ligation, the pellet was collected by centrifugation, resuspended in proteinase K solution [10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K (25530049, Invitrogen)], and incubated at 65°C overnight. DNA was purified by phenol-chloroform treatment followed by ethanol precipitation, and then fragmented by sonication with a model UCD-200 Bioruptor (Diagenode). After the biotin-labeled DNA was pulled down with Dynabeads Streptavidin T1 (65601, Invitrogen), the beads were washed twice with TNB (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween-20) at 55°C for 2 min. To ligate the Illumina sequencing adaptors to the biotinylated DNA fragments, the beads were successively treated with end repair mix [1× T4 DNA ligase buffer, 1 mM dATP, 0.5 mM each dNTP, 0.5 units/μl T4 polynucleotide kinase (M0201, NEB), 0.12 units/μl T4 DNA polymerase (M0203, NEB), 0.05 units/μl Klenow fragment] at 25°C for 30 min, dA-tailing mix [1× NEBuffer 2, 0.5 mM dATP, 0.25 units/μl Klenow exo- (M0212, NEB)] at 37°C for 30 min, and Illumina adaptor ligation mix [0.075x End repair reaction buffer (E6050, NEB), 0.32× Ligation master mix (E7595, NEB), 0.011× Ligation
enhancer, and 45 nM NEBNext adaptor for Illumina] at 20°C for 15 min followed by
0.032 units/µl USER treatment at 37°C for 15 min, with two washes with TNB at 55°C
for 2 min after each reaction. The resultant adaptor-ligated Hi-C library was amplified by
PCR with five to seven cycles with Q5 master mix (M0544, NEB) and index primers
(E7335/E7500/E7710/E7730, NEB) before 37 bp paired-end sequencing using the
NextSeq 500 device (Illumina).

In situ Hi-C data analysis

In situ Hi-C data were processed as described previously (Tanizawa et al., 2017). The
paired reads were separately aligned to the fission yeast genome (version ASM294v2.19)
using Bowtie2 (version 2.2.9) with the iterative alignment strategy. The aligned reads
were assigned to MboI fragments. Redundant paired reads derived from PCR biases,
aligned to repetitive sequences, or with low mapping quality (MapQ < 30) were removed.
Paired reads potentially derived from self-ligation and undigested products were also
discarded.

The fission yeast genome was divided into non-overlapped 20-kb, 5-kb, or 2-kb bins. Raw contact matrices were constructed by counting paired reads assigned to two
bins, and Hi-C biases in the contact matrices were corrected using the ICE method
(Imakaev et al., 2012). The ICE normalization was repeated 30 times. ICE-normalized
contact scores were used for all heat maps, where the maximum intensity corresponded
to the top 5% score. For subtraction heat maps, the top 5% absolute values of difference
scores (T) were extracted. The T and -T scores were drawn as the maximum and minimum
intensity.
Loop detection

Knight and Ruiz (KR) normalized matrices (Rao et al., 2014) in .hic format was calculated from raw contact matrices using the 'pre' function of Juicer_tools (version 1.22.01) (Durand et al., 2016). Loop calling was performed for KR-normalized matrices using HiCCUPS ('hiccups' function of Juicer_tools) with the following parameters: r 2000 -k KR -f .01 -p 3 -i 7 -d 10000".
References


4795. 10.1038/s41467-019-12629-0.


Winters, T., McNicoll, F., and Jessberger, R. (2014). Meiotic cohesin STAG3 is required for chromosome axis formation and sister chromatid cohesion. The EMBO journal 33,


Figure 1. Hi-C analysis firmly recapitulates the structure of meiotic bouquet chromosomes.
Figure 2. Rec8 cohesin shapes chromosomes into a structure suitable for its alignment.

**A**

Meiosis 0 hr

Meiosis 2.5 hr

**B**

- rec10Δ
- rec12Δ
- rec8Δ

Distance (Mb)

Distance (Mb)

Distance (Mb)

Position in Chr 1 (Mb)

Position in Chr 1 (Mb)

Position in Chr 1 (Mb)
Figure 3. Rec8 cohesin is crucial to construct the meiosis-specific chromatin loops.
Figure 4. Wpl1 regulates chromatin loop length during meiotic prophase in *S. pombe.*

A. Rec8-GFP in meiotic prophase nucleus

```
wt

wp1Δ
```

Z section (0.3µm each) 1µm

B. VG

```
wpl1Δ

wp1Δ - wt
```

Meiosis 0 hr

Meiosis 2.5 hr

C. Chr 1, 4.05–4.25Mb, 2kb bins

```
wt

wp1Δ
```

D. *P(s)*

```
Read per million
```

E. Ratio of loop numbers

```
Distance (bp)
```

Distance (bp)

Log2 (wp1Δ/wt)

Distance (bp)
Figure 5. Isolation of rec8 mutant showing defect in axis formation with intact cohesion function

- **A** shows a comparison between mitotic and meiotic conditions with the presence and absence of Rec8 and Rec11 proteins.
- **B** illustrates meiotic prophase with images of rec8-wt and rec8-F204S mutants.
- **C** presents a graph showing hetero cut3-GFP patterns with statistical analysis.
- **D** depicts the binding sites of various proteins (Smc3, Scc2) in Rad21.
- **E** demonstrates the meiotic prophase with H2B-mCherry localization.
- **F** illustrates the distance from the telomere to the ade8 locus for different rec8 mutants.
Figure 6. rec8-F204S mutant displays defective chromatin loop formation

A

Meiosis 0 hr
Meiosis 2.5 hr

rec8-wt
rec8-F204S

B

Meiosis 0 hr
Meiosis 2.5 hr

rec8-wt
rec8-F204S

C

Meiosis 0 hr
Meiosis 2.5 hr

D

Meiosis 0 hr
Meiosis 2.5 hr

E

Loop numbers in each length

Meiosis 0 hr
Meiosis 2.5 hr
Figure 7. Rec8-dependent chromatin loop formation and meiotic recombination

**A**

Rec10-mCherry

- wt
- rec8-F204S
- rec8Δ

**B**

<table>
<thead>
<tr>
<th>Rec8</th>
<th>wt</th>
<th>F204S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psm1 (HA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rec8 (Flag)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRec11 (pT7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubulin</td>
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</tbody>
</table>

**C**

recombination frequency

<table>
<thead>
<tr>
<th>rec8: wt F204S rec8Δ</th>
<th>wt F204S rec8Δ</th>
<th>wt F204S rec8Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ura1-lys3 ~210kbp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cox7-vps38 ~100kbp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vps38-leu1 ~170kbp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D**

commitment of meiosis

- rec8 wild type
- rec8-F204S

- G1 phase
- Pre-meiotic S-phase
- Prophase I

Meiotic recombination

LinE aggregates