Doctoral Thesis

Importance of centromere chromatin in regulation of homologous recombination in fission yeast

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Contents

Abstract

Eukaryotic centromeres viewed as a constriction on mitotic chromosomes are indispensable for faithful segregation of chromosomes. Errors in centromere function result in aneuploidy that may lead to genetic diseases, and cancer. A group of proteins including histone H3 variant CENP-A and histone fold containing CENP-TWSX, both of which are essential for proper microtubule attachment during mitosis, specifically localize to the centromere forming unique chromatin structure called kinetochore. This chromatin is flanked by the pericentric heterochromatin that is marked by the methylation of histone H3 on 9th lysine by the Clr4/Suv39 methyltransferase. It provides a platform for Swi6/HP1 that stabilizes cohesin proteins that is important for sister chromatid attachment and bi-polar attachment of kinetochore to microtubules. Another conserved feature of the centromere is presence of repeats sequences that are prone to rearrangement. Interestingly, gross chromosomal rearrangement (GCR) mediated by the centromere repeats is increased by a deletion of *rad51* in fission yeast, showing that homologous recombination (HR) is important for maintaining the structural integrity of centromere. However, the precise regulatory mechanism of recombination in centromeres remains elusive.

To gain insight of recombination in centromere, I determined the spontaneous recombination that occurs between the *ade6B/ade6X* heteroalleles integrated at the inverted repeats of centromere 1 (*cen1*) and compared it with a non-centromeric *ura4* locus in fission yeast, *Schizosaccharomyces pombe*. In the centromere, Rad51-dependent HR that requires Rad51, Rad54, and Rad52 was predominant, whereas Rad51-independent HR that requires Rad52 also occurred in the non-centromeric region. Moreover, crossovers (CO) between inverted repeats were suppressed in the centromere as compared to the non-centromeric region. Thus, the mechanism of HR is differently regulated in centromere from that of non-centromeric *ura4* locus.

Remarkably, the choice of recombination pathway is important to maintain integrity of centromeres. To see if the centromere chromatin is responsible for the specific regulation of recombination, I examined the effect of several factors of heterochromatin and kinetochore on recombination. I found that the deletion of *clr4*, *swi6* and a temperature sensitive mutation in *rad21* subunit of cohesin increased the spontaneous rate of recombination. However, *clr4*Δ did not increase the proportion of COs. These results suggest that the heterochromatin affects the initial events of recombination but does not play a role in the formation of recombination products. A mutation in CENP-A and several other kinetochore factors did not change the proportion of COs in centromere, suggesting that they do not play a role in regulation of HR in centromere. CENP-S, CENP-X histone-fold proteins, form CENP-TWSX and also CENP-SXSX complex, which can bind Fml1/FANCM helicase that is involved in DNA repair. Mhf1/CENP-S, Mhf2/CENP-X and Fml1 were required to suppress COs. Interestingly, a mutation in Mhf1, *mhf1-LR* that disrupts the tetramer complex is mildly sensitive to genotoxins such as MMS, CPT and HU unlike *mhf1*Δ and *fml1*Δ. However, *mhf1-LR* suppressed COs and GCRs in the centromere, similar to *fml1*Δ. Thus, it is likely that MHF tetramers are particularly important in the CO suppression in centromere. When replication forks stall in centromere, the unique chromatin may prevent excessive branch migration of joint molecules that can lead to COs. Instead, Mhf tetramer in concert with Fml1 bind such branched DNA structures and dissociate the joint molecules to drive synthesis-dependent strand annealing (SDSA) pathway of HR that always results in noncrossovers (NCOs). These data for the first time uncovered the regulation of mitotic recombination between DNA repeats in centromeres and its physiological role in maintaining genome integrity.

General Introduction

Centromere is composed of repeat sequences and unique chromatin

Viewed as a constriction on a condensed chromosome, a centromere is a unique region of a eukaryotic genome. Centromeres are responsible for maintaining sister chromatid attachment and providing proper spindle microtubule attachment that is required for faithful pairing and segregation of chromosomes during cell division. Efficient centromere function leading to inheritance of complete copies of the genome by daughter cells is critical for cell proliferation. Defects in this segregation machinery may produce aneuploids (cells containing abnormal number of chromosomes), a situation which contributes to cancer progression and genetic diseases (Figure 1). Correct centromere structure and function are therefore crucial for maintaining genome stability.

A vital function of the centromere is to assemble the chromatin that facilitates microtubule attachment and sister chromatid attachment. Fission yeast, *Schizosaccharomyces pombe* is an excellent model organism for analyzing centromere features as it is very facile to the genetic analysis. *S. pombe* centromeres also assemble highly specialized and conserved centromere chromatin among the eukaryotes. Centromere chromatin is epigenetically inherited and it results from centric kinetochore flanked by pericentric heterochromatin, together conferring a unique chromatin environment to centromeres (Figure 2). Kinetochore is a large complex consisting of a pool of proteins. The key determinant of kinetochore identity is the histone H3 variant CENP-A (CENtromere Protein-A)/Cnp1 that also acts as an epigenetic landmark of active centromeres. pRab46/48/Mis16 and Mis18α/β/Mis18 and a CENP-A-specific chaperone, HJURP/Scm3, are involved in CENP-A localization, which is conserved among many organisms (Hayashi, Fujita et al., 2004).

Figure 1

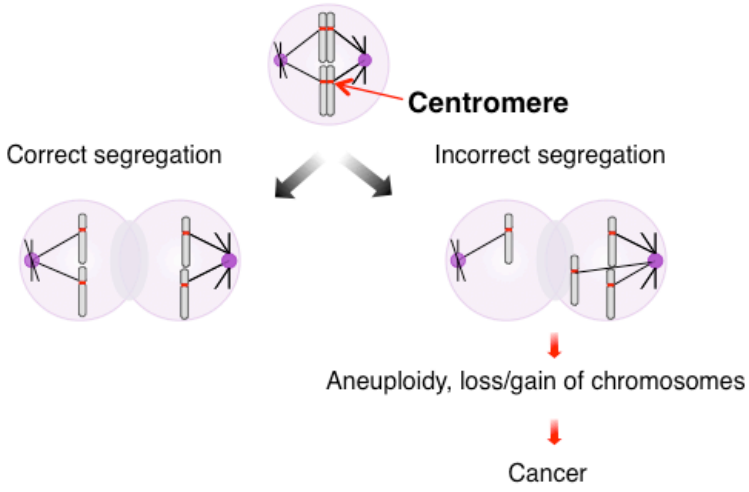


Figure 1. Centromere is essential for correct segregation of chromosomes.
Centromere is essential for sister chromatid attachment and providing microtubule attachment site during cell division. Correct segregation and equal division of chromosomes is important for cell proliferation while incorrect segregation may lead to aneuploidy and cancer.

In humans, pRab46/48 or Mis18α/β is required for centromere incorporation of CENP-A. CENP-A binds CENP-C/Cnp3 that serves as a scaffold for the Mis12-Mis14 complex that binds to the spindle microtubules (Tanaka, Chang et al., 2009). In chicken DT40 cells, a fully functional artificial kinetochore could be successfully constructed by tethering CENP-C to a *lac*-operator array, after deletion of the authentic centromere of the same chromosome (Hori, Shang et al., 2013). Apart from CENP-As, analyses aimed at isolating other constitutive centromere-associated network (CCAN) of proteins isolated CENP-T/Cnp20, CENP-W, CENP-S/Mhf1, and CENP-X/Mhf2 (Amano, Suzuki et al., 2009a, Hori, Amano et al., 2008). These proteins have histone fold domains, which allows them to form a stable tetrameric CENP-TWSX complex (Nishino, Takeuchi et al., 2012). While the C-terminal of CENP-T binds centromeric DNA (Nishino et al., 2012), the N-terminal tail directly interacts with Ndc80 that binds microtubules (Gascoigne, Takeuchi et al., 2011, Nishino, Rago et al., 2013). Indeed, tethering of N terminal tail of CENP-T at non-centromeric locus resulted in an artificial kinetochore that efficiently drove chromosome segregation after the deletion of the authentic centromere in chicken DT40 cells (Gascoigne et al., 2011, Hori et al., 2013). Recruiting the Ndc80 complex therefore is a critical role of centromere chromatin, which is fulfilled in parallel by CENP-A, CENP-C, Mis12-Mis14 complex and through the N- terminal tail of CENP-T of the CENP-TWSX tetramer complex. Interestingly, CENP-S/Mhf1 and CENP-X/Mhf2 also form the complex (CENP-SXSX) that is able to recruit Fanconi anemia M (FANCM/Fml1) DNA helicase (Singh, Saro et al., 2010, Yan, Delannoy et al., 2010) to branched DNA structure during homologous recombination, implicating their role in DNA repair.

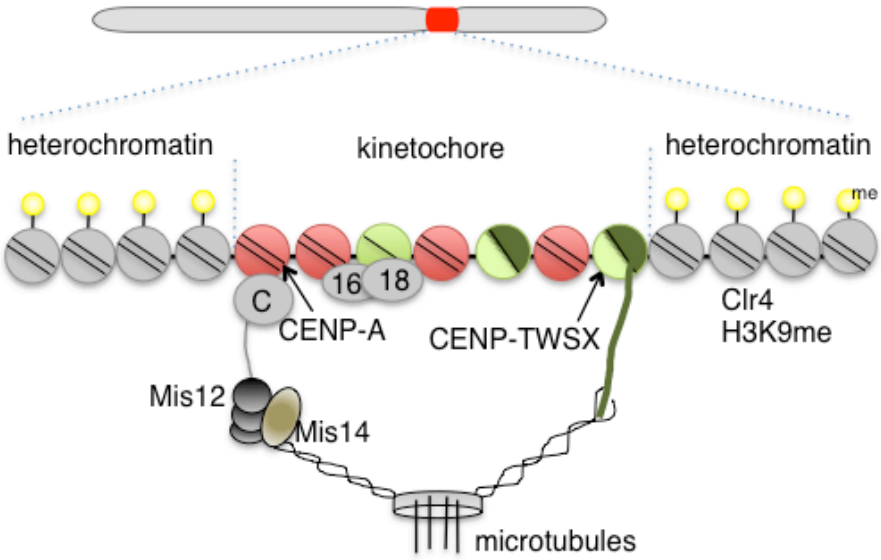


Figure 2. Unique chromatin assembles on centromere repeat sequence
The centromere chromatin assembles as pericentromeric heterochromatin marked by H3K9 methylation and Clr4 and the centric kinetochore marked by CENP-A. Mis16-18 complex are essential for CENP-A localization to kinetochores. CENP-A binds CENP-C which interact with Mis12-14 complex to facilitate microtubule attachment. On the other hand, The N-terminal tail of CENP-T of the CENP-T-W-S-X complex provides microtubule binding. This heterochromatin and kinetochore assemble on repeat sequence of centromere.

The kinetochore is flanked by pericentric heterochromatin that is characterized by methylation of the 9th lysine of histone H3 (H3K9) by Clr4/Suv39 methyltransferase. Methylated H3K9 is bound by Swi6/HP1, which recruits and stabilizes cohesins to this region (Nonaka, Kitajima et al., 2002). Localization of cohesins at heterochromatin generates cohesion between sister chromatids that allows back-to-back orientation of the kinetochore that is crucial for proper microtubule attachment at centromeres (Verdaasdonk & Bloom, 2011). Recruitment of cohesin to pericentromere repeats is a crucial role of Swi6 as artificially tethering of cohesin to the centromere bypasses the requirement of Swi6 in centromere function (Yamagishi, Sakuno et al., 2008). Sister kinetochores must be attached by spindle microtubules from opposite spindle pole bodies to segregate accurately. Merotelic attachment or multiple attachment of kinetochore by microtubules leads to lagging chromosomes, aneuploidy and loss/gain of chromosomes. Therefore, the kinetochore and heterochromatin that assembles on centromere repeat sequences are important for the structure and function of the centromeres, to maintain the genome stability.

Another conserved feature of centromeres is the presence of repeat sequences in many eukaryotes. Centromeres of fission yeast (*S.pombe*) resemble those of higher eukaryotes more closely than budding yeast, *Saccharomyces cerevisiae,* which has a point centromere of only 120-200 bp (Verdaasdonk & Bloom, 2011). In humans, centromere DNA consists of α-satellite repeats with 171 bp consensus sequence that spans from 0.2 to 5 Mb in length (Aldrup-Macdonald & Sullivan, 2014). In *S. pombe,* the centromere spans over 40-110 kb in length, and is composed of sets of inverted repeats: *irc*, the outer repeats (*otr*), and the innermost repeats (*imr*), flanking the central unique sequence (*cnt*) of 4-7 kb (Takahashi, Murakami et al., 1992) (Figure 3).

In this respect, *S. pombe* centromeres are similar to the centromeres of humans. Repetitive sequence being one of the most conserved features of a eukaryotic centromere may contribute to important functions of segregation in concert with the unique chromatin that assembles in the centromere.

Figure 3

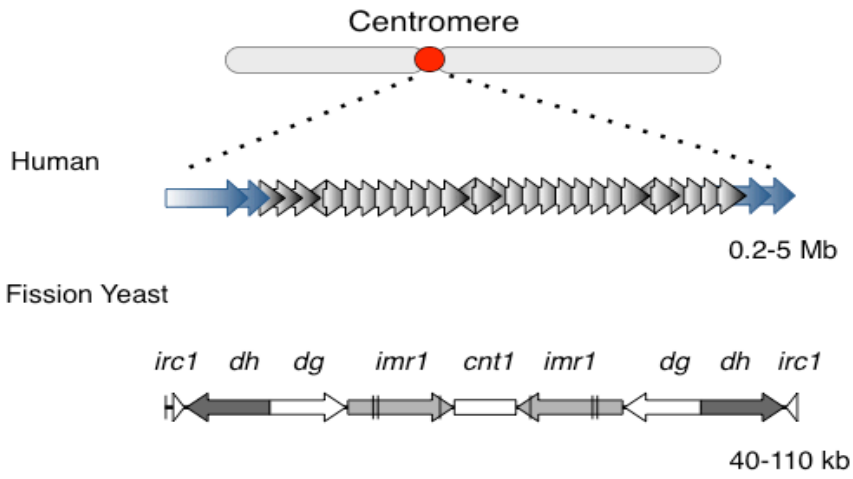


Figure 3. Centromeres comprise of repetitive DNA sequence.
Eukaryotic centromeres contain repeat sequences. Human centromeres are large, about 0.2-5 Mb in length consisting of α-satellite repeats. Fission yeast centromeres are about 40-110 kb in length. They consist of sets of inverted repeat sequences, *irc*, the outer repeats (*otr*), and the innermost repeats (*imr*), flanking the central unique sequence (*cnt*).

Gross chromosomal rearrangements occur in repeat sequences of centromeres.

Rearrangements are a hallmark of genetic disorders. Genetic diseases resulting from rearrangements often lead to gain or loss of genes. Loss of heterogeneity (LOH), loss of one of the two alleles of a gene having tumor suppression functions, is a causal event in cancer, frequently encompasses multiple genetic loci and whole arm chromosomes. DNA rearrangement frequently occurs in repetitive regions such as segmental duplications, transposons, rDNA, telomere and centromere (Padeken, Zeller et al., 2015). Although the exact role of the centromere repeats is not clear, they are susceptible to rearrangement. Centromere repeats are intrinsically unstable as increased levels of H2AX phosphorylation (a conserved characteristic of DNA damage response) have been reported (Rozenzhak, Mejia-Ramirez et al., 2010). Moreover, repetitive DNA sequences undergo replication problems such as fork stalling and collapse, which initiate homologous recombination (HR) between the repeats leading to gross chromosomal rearrangements (GCR)s. Repeat sequences can underlie gross chromosomal rearrangements such as deletions, duplications, translocations through homologous recombination. HR is the mechanism that not only plays an important role in maintenance of genomic integrity but also molecular evolution, gene diversification, and chromosome segregation during cell division. Contrasting with its role in genome maintenance, non-conservative recombination such as break-induced replication and crossovers in repeat sequences lead to rearrangements. The most common mechanism causing disease associated genome rearrangement is non-allelic homologous recombination (NAHR). NAHR between direct repeats on the same chromatid results in reciprocal deletions and duplications,

whereas NAHR between inverted repeats on the same chromatid results in inversions. Reciprocal translocations are common when NAHR occurs between the repeats located on different chromosomes. Deletions, duplications and chromosomal translocations resulting from recombination events in repeat sequences have been implicated in several diseases in humans including cancers (Argueso, Westmoreland et al., 2008, Campbell, Gambin et al., 2014, Deininger & Batzer, 1999).

Homologous recombination suppresses rearrangements in centromere

Homologous recombination (HR) can be explained as a process where DNA is exchanged or copied between two chromosomes or different regions of the same chromosome. The process requires homology between the exchanging DNA regions. Homologous recombination repairs DNA breaks, especially double stranded breaks (DSBs), stabilizes and repairs stalled forks. HR consists of a series of inter related pathways that function in repair of DNA breaks (Figure 4). Initially, stretches of single stranded DNA (ssDNA) are resected at the stalled forks or DSB ends which are quickly bound by replication protein A (RPA). Rad51 replaces RPA and binds to these ssDNA with the aid of the Rad52 mediator function (New, Sugiyama et al., 1998, Shinohara & Ogawa, 1998). Rad51 form a nucleoprotein filament, which can then engage in homology search by strand invasion forming a homologous DNA duplex. Rad51 nucleofilament is stabilized by a Swi/Snf-type of motor protein Rad54, which also takes part in the strand invasion process. Rad54 binds Rad51 and facilitates DNA strand exchange, DNA synthesis from the 3' end of the invading strand, and branch migration (Bugreev, Mazina et al., 2006, Petukhova, Stratton et al., 1998, Wright & Heyer, 2014). When the DNA duplex is paired with the homologous strand, the complementary strand is displaced to produce recombination intermediates

called the displacement loop (D loop) (Murayama, Kurokawa et al., 2008). Second end capture by annealing to the displaced strand forms a double Holliday junction (dHJ), which is a crucial intermediate of the double strand break repair (DSBR). In DSBR pathway, the HJ is resolved either into a crossover (CO), which switches the flanking sequences or a non-crossover (NCO) that maintains the original sequence (Figure 4). Endonucleotic resolution of joint molecules such as D-loops and double Holliday junctions results in crossovers depending on the way they are cleaved (Manhart & Alani, 2016). On the other hand, in synthesis dependent strand annealing (SDSA) the elongating invading strand is displaced from the D loop to anneal to the original strand, always resulting into a non-crossover, which maintains the original linkages of sequence (Nassif, Penney et al., 1994). Rad54, Fml1/Mph1/FANCM helicase are implicated in the disassembly of D-loops (Krejci, Altmannova et al., 2012). Rad51 and Rad54 promote non-crossover recombination between the inverted repeats of the centromere thereby suppressing crossovers resulting in inversion and isochromosome formation (Nakamura, Okamoto et al., 2008, Onaka, Toyofuku et al., 2016b). In meiotic recombination, crossover is important and required for correct segregation of chromosomes. However, crossovers in the vicinity of a centromere may interfere with equal division of genetic material. Reports in several species implicate that crossovers occur infrequently in centromere region as compared to the arm regions of the chromosomes (Lynn, Ashley et al., 2004, Nakaseko, Adachi et al., 1986). However, the specific regulation of recombination in centromere during mitosis and whether it is different from the arm region remains unknown. It also remains to be elucidated, how the unique chromatin and the repeat sequences contribute in the mechanism of GCR suppression and maintenance of genomic integrity of the centromeres.

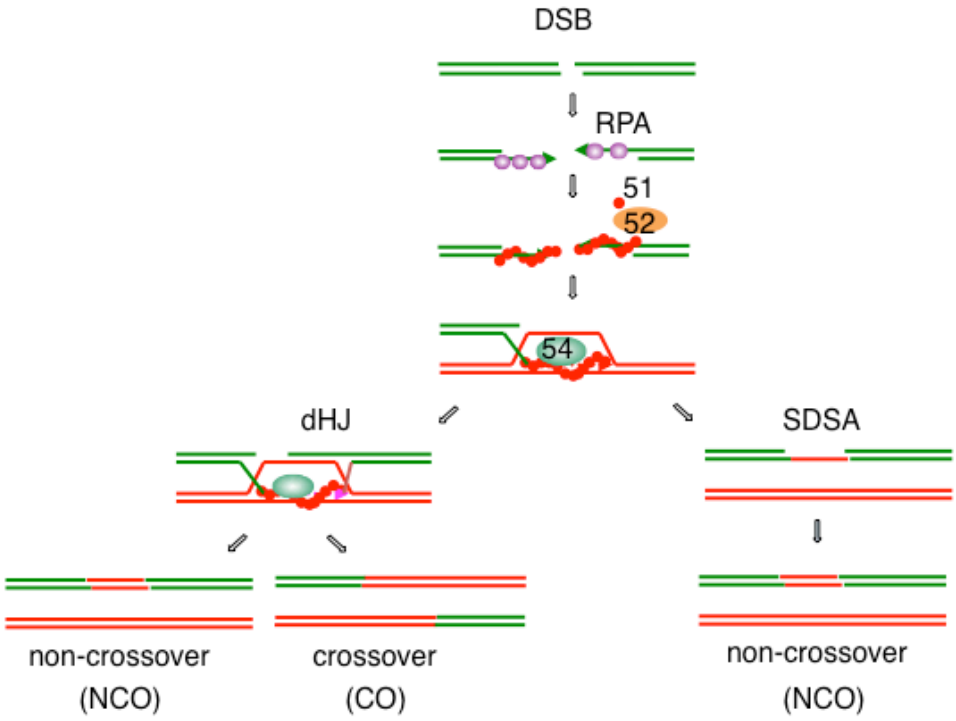


Figure 4. Homologous Recombination is important to repair DNA.
At the site of double strand break (DSB), DNA is resected to generate single stranded DNA (ssDNA) that is bound by RPA. Rad52 mediator function facilitates Rad51 nucleofilament formation and Rad54 facilitates strand invasion and D-loop formation. Second end capture leads to formation of double Holiday Junction (dHJ) which can be resolved into a crossover or a non-crossover. Alternatively, the invading strand may anneal to the original strand to always form non-crossovers by synthesis dependent strand annealing (SDSA).

Introduction

Precise DNA replication and faithful transmission of genetic materials to daughter cells is essential for proliferation of cells and maintaining genomic stability. However, external stress such as ionizing radiation or internal stress such as stalled forks and collapse can generate insults into the DNA such as double strand breaks (DSBs). An increasing body of evidence shows that repeat sequences are prone to replication fork stalling (Mirkin & Mirkin, 2007). Repetitive DNA sequences of the centromeres have also been reported to stall replication forks. DSBs resulting from stalled forks can be repaired by homologous recombination (HR). HR consists of a series of inter related pathways, which can occur in either Rad51-dependent or Rad51-independent manner. In *S. cerevisiae*, the Rad51-dependent mechanism requires a group of genes, including *RAD51*, *RAD52*, and *RAD54*. On the other hand, Rad51-independent recombination requires Rad52 that carries out single-strand annealing (SSA) reaction between complimentary ssDNA molecules (Ivanov, Sugawara et al., 1996, Mortensen, Bendixen et al., 1996). Recombination between direct repeats results in either gene conversion via strand invasion mechanism or a deletion of repeat sequence. Rearrangement event can result from either crossing over or single strand annealing (SSA) which is Rad51-independent HR. In the absence of Rad51, recombination between inverted repeats leads to crossovers that result in inversion of the intervening region (Rattray & Symington, 1994) and it is carried out by the SSA activity of Rad52 (Bai, Davis et al., 1999). Therefore, Rad51-independent recombination is susceptible to gross chromosomal rearrangements.

Chromosome rearrangements including deletions between tandem repeats and translocations between different chromosomes result from SSA. Yet another category

of translocations is Robertsonian translocations, which are formed due to rearrangements of centromere repeat sequences of two acrocentric chromosomes, forming a single large chromosome (Figure 5). Robertsonian translocation cause several genetic diseases in humans such as Patau syndrome, Down's syndrome (Page, Shin et al., 1996). On the other hand, translocation on the same chromatid gives rise to arms that are mirror images of each other known as isochromosomes. Isochromosomes are frequently observed in specific types of cancer (Putnam, Pennaneach et al., 2005) and those of chromosome X can cause Turner syndrome (Miller, Mukherjee et al., 1963). In *S. pombe*, translocations and isochromosomes were formed by the rearrangement of centromere repeats that were suppressed by HR factor Rad51 (Nakamura et al., 2008). Rad51 and Rad54 promote non-crossover recombination between the inverted repeats of the centromere thereby suppressing crossover recombination resulting in inversion and isochromosome formation (Nakamura et al., 2008, Onaka et al., 2016b). Therefore, HR is crucial for suppressing rearrangements in centromere repeats.

Homologous recombination can form a crossover or a non-crossover by the resolution of the intermediate D-loop structures. During meiotic prophase, crossovers provide physical links between a pair of homologous chromosomes. However, meiotic crossovers are reduced around the centromere in order to prevent premature separation of sister chromatids (Talbert & Henikoff, 2010). Crossovers occurring in and around the centromere can lead to lagging chromosomes due to attachment of the centromere to both spindle bodies leading to aneuploidy. The suppression of meiotic crossovers is explained by reduction of meiosis-specific DNA double-strand breaks (DSBs) around centromeres (Ellermeier, Higuchi et al., 2010, Pan, Sasaki et al., 2011). However, non-crossover recombination occurs around centromeres in maize

and budding yeast (Shi, Wolf et al., 2010, Symington & Petes, 1988), suggesting that

Figure 5

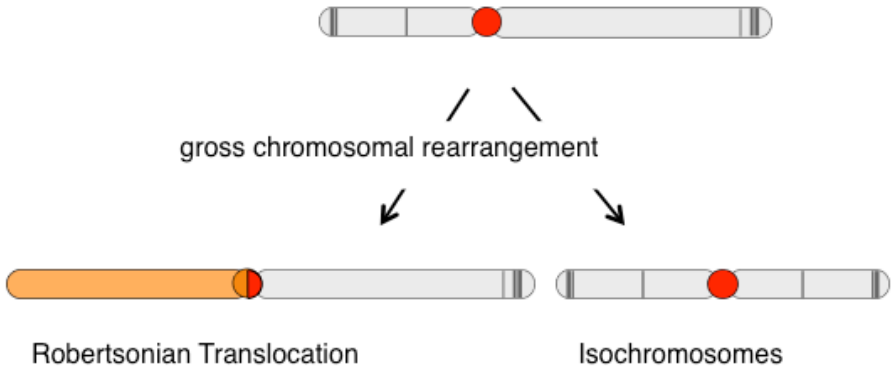


Figure 5. Gross chromosomal rearrangements occur in centromere.
Gross chromosomal rearrangement (GCR) of centromere repeats leads to Robertsonian translocation where whole arms are translocated from another chromosome. GCRs in centromere repeats also lead to isochromosome formation whose arm are mirror images of each other.

crossovers are selectively suppressed even after the formation of meiotic DSBs. However, the regulation of HR and crossover between the centromere repeats in mitotic cells and whether it is differently regulated from a non-centromere region remains unknown. It is suggested that the unique centromere chromatin that ensures faithful segregation of chromosomes may confer important roles in regulation of recombination. However, the precise role of the centromere specific chromatin in context of HR and crossover control to maintain the genomic integrity remains elusive. It also remains to be elucidated how intrinsically unstable repeat sequences of the centromere are maintained to avoid GCRs. A specific regulatory mechanism may be important to maintain the proper structural and functional integrity of one of the most complex and critical regions of the chromosome, the centromere.

Here, using *S pombe*, HR between inverted repeats of the centromere was elucidated. Furthermore, the mitotic recombination between the inverted repeats was compared between centromere and a non-centromeric region. In the centromere, all Rad51, Rad54 and Rad52 were found to be essential for recombination. However, in the non-centromeric *ura4* locus only Rad52 was essential as compared to Rad51 and Rad54 that were only partially required for recombination. Southern hybridization of the recombinant DNAs revealed that crossovers were rare in centromere as compared to non-centromere region. These results suggest that the mechanism of recombination is specifically regulated in the centromere. Although pericentromeric heterochromatin was not essential for regulation of recombination in centromere, Mhf1, Mhf2 and Fml1 suppressed crossovers in centromere that can lead to rearrangements. Indeed, deletion of Mhf1 and Fml1 increased GCRs that were mediated by centromere repeats. These results show that recombination in centromere repeats are highly regulated to maintain the genomic integrity.

Results

Recombination in the centromere occurs exclusively in a Rad51-dependent manner

Homologous recombination (HR) occurs by either Rad51-dependent or -independent pathway. Rad51-depenent HR requires Rad51, Rad54, and Rad52, whereas Rad51-independent HR depends on Rad52. To see which type of HR occurs in the unique region of the chromosome, the centromere, the spontaneous rates of recombination were determined using the cen1-Sn construct, strain where *ade6B* and *ade6X* heteroalleles are integrated at the *Sna*BI restriction enzyme sites in the *imr1* left and right repeats that flank the central unique sequence *cnt*, forming a 5kb interval in centromere 1 (Figure 6A). By a fluctuation analysis, the rates of Ade+ prototroph formation were determined. As compared to the wild type, all the *rad51*Δ, *rad54*Δ, and *rad52*Δ mutants exhibited equally reduced rates of recombination, showing that Rad51-dependent HR is predominant in the centromere. To compare homologous recombination in centromere and non-centromere regions, the *imr1-cnt1-imr1* sequence flanked by *ade6* heteroalleles (the cen1-Sn cassette) was introduced into the *ura4* locus of chromosome 3, and the spontaneous rates of recombination were determined in the same set of strains (Figure 6B). Homologous recombination occurred at a similar rate in the wild type strains of cen1-Sn and ura4-Sn, clearly showing that HR is not suppressed in the centromere. As compared to the wild type, all the *rad51*Δ, *rad54*Δ, and *rad52*Δ mutants showed reduced rate of recombination. Both *rad51*Δ, and *rad54*Δ only partially decreased the recombination rate (about 30% of the wild type level), while *rad52*Δ mutant severely reduced the recombination rate

(about 4% of the wild type level), demonstrating that Rad51-dependent recombination is predominant in centromeres.

Figure 6

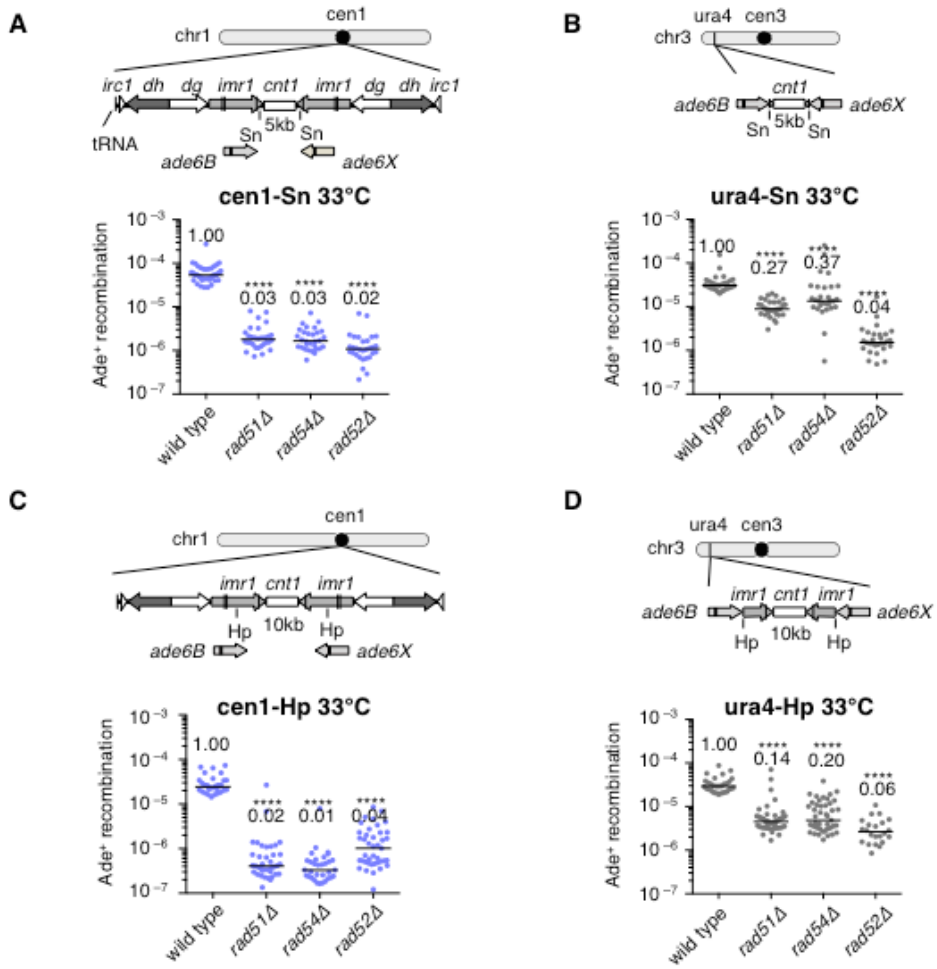


Figure 6. Recombination between *ade6B*/*ade6X* heteroalleles in centromere and arm regions.
(A) Recombination in the cen1-Sn construct. Illustrated are the central sequence *cnt1* and the *imr1*, *dg*, *dh*, and *irc1* inverted repeats in centromere 1 (cen1). *ade6B* and *ade6X* heteroalleles were integrated at the Sn sites in imr1. Spontaneous rates of Ade⁺ prototroph formation were determined in wild type, *rad51Δ*, *rad54Δ*, and *rad52Δ* strains (B) Recombination in the ura4-Sn construct. From the cen1-Sn, the *ade6B/X* flanking the central region of cen1 were amplified and integrated at the *ura4* locus. Recombination rates were determined in the same set of strains. (C) Recombination in the cen1-Hp construct. *ade6B/X* were integrated at the Hp sites in imr1. Recombination rates were determined in wild type, *rad51Δ*, *rad54Δ*, and *rad52Δ* strains. (D) Recombination in the ura4-Hp construct. From the cen1-Hp construct, *ade6B/X* flanking the central region of cen1 were amplified and integrated at the *ura4* locus and Recombination rates were determined in the same set of strains.

Independent experimental values are shown in scatter plots and lines indicate medians. Rates relative to the wild-type value are indicated at the top of each column. *P*-values were determined by the two-tailed Mann-Whitney test. ****$P < 0.0001$; Sn, SnaBI; Hp, HpaI.

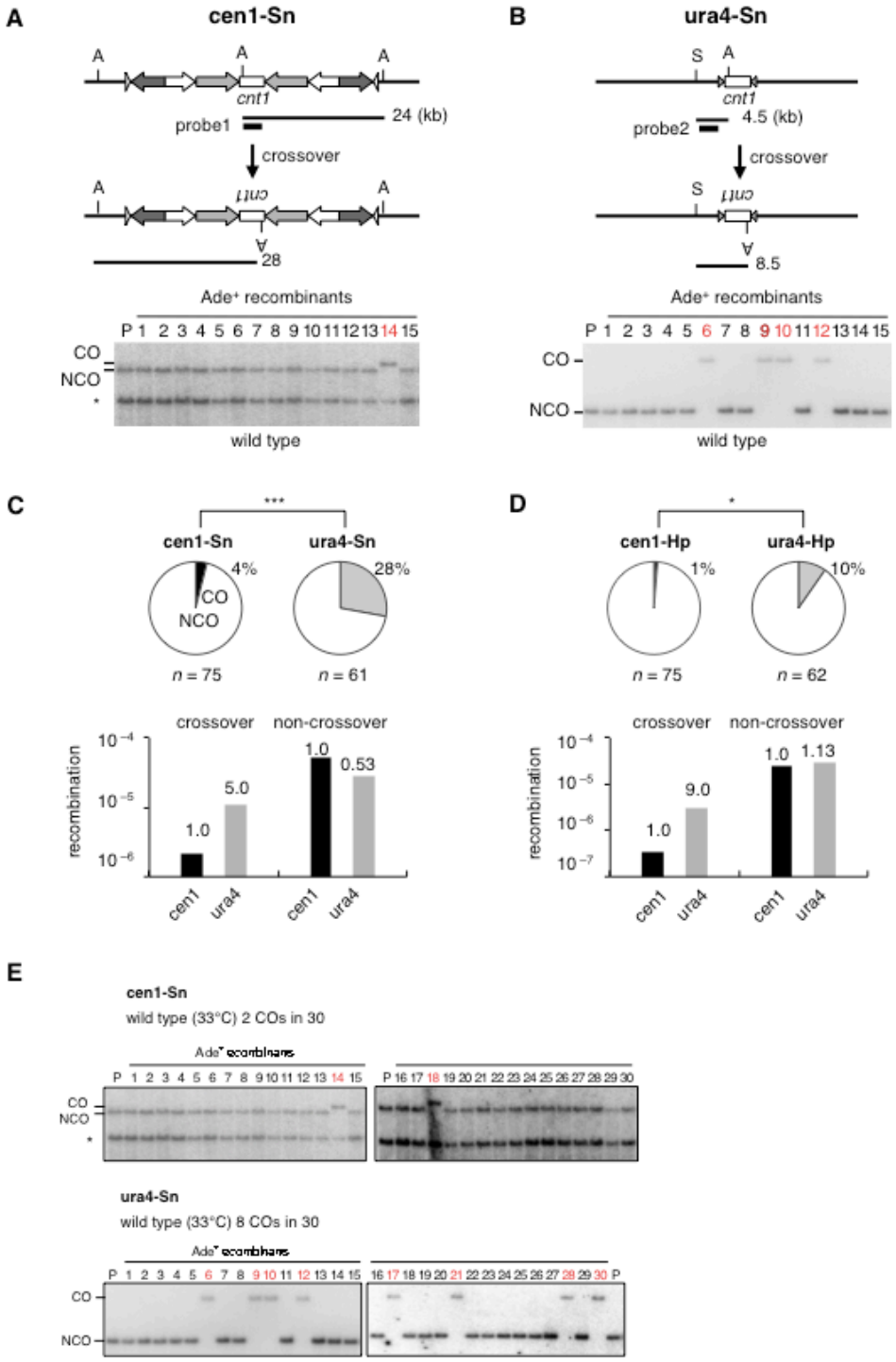
To see whether the difference in HR features is not specific to the Sn site, *ade6* heteroalleles were introduced at the *Hpa*I site on the either side of the *imr1*. The *Hpa*I sites are about 10 kb apart and are present in the middle of the *imr* repeats. Spontaneous rate of HR was determined in the wild type, *rad51*Δ, *rad54*Δ, and *rad52*Δ strains. A similar difference was observed between cen1-Hp and ura4-Hp. All Rad51, Rad54, and Rad52 were essential for HR in cen1-Hp (Figure 6C), whereas Rad51 and Rad54 were only partially required for HR as compared to Rad52 in ura4-Hp (Figure 6D). It clearly shows that the Rad51-dependent recombination predominates not only in a specific region of the centromere, but is a general feature of these repeats. These data show that the requirement of HR proteins is different in the centromere and non-centromere regions. Both Rad51-dependent HR and Rad51-independent SSA take place at *ura4* locus while Rad51-dependent HR is predominant in the centromere.

Crossovers between the centromere repeats are suppressed

Crossovers between non-allelic DNA sequence lead to chromosome rearrangements, while non-crossovers maintain the original linkage of chromosomes. Recombination between inverted repeats in the centromere can be associated with or without crossover of the intervening sequence. To examine whether either crossovers or non-crossovers are generated in the centromere and non-centromere region, DNA from the parental strain and independent Ade^+ recombinants was prepared, digested with the restriction enzyme, separated by pulse field gel electrophoresis (PFGE), and the fragment of interest was detected by Southern hybridization (Figure 7). In cen1-Sn, only 4% of the recombinants were crossovers (Figures 7A and 7C). However, in ura4-

Sn, one fourth of the recombinants (~28%) were crossovers (Figures 7B and 7C). The proportion of crossovers in cen1-Hp was smaller than that in ura4-Hp (Figures 7B and 7D), showing strong suppression of crossovers in the centromere. The net rates of crossovers and non-crossovers were obtained by multiplying the recombination rate (Figure 6) by the proportion of crossovers and non-crossovers, respectively (Figures 7C and 7D). The results show that the crossovers occur ~5 fold more frequently in ura4-Sn as compared to cen1-Sn, while non-crossovers occur at similar levels at *cen1* and *ura4* loci. Similarly, crossovers occur ~9 fold more frequently in ura4-Hp as compared to cen1-Hp, while non-crossovers occur at similar levels at both loci. These results demonstrate that crossovers but not non-crossovers are suppressed in the centromere as compared to the non-centromere region.

Figure 7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cen1-Sn
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A
A
cnt1
probe1
24 (kb)
crossover
A
cnt1
A
28
Ade+ recombinants
P 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
CO
NCO
*
wild type
B
ura4-Sn
S
A
cnt1
probe2
4.5 (kb)
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S
cnt1
8.5
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CO
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ura4-Sn
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1.0
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cen1
ura4
cen1
ura4
D
*
cen1-Hp
ura4-Hp
1%
10%
n = 75
n = 62
crossover
non-crossover
recombination
10−4
10−5
10−6
10−7
1.0
9.0
1.0
1.13
cen1
ura4
cen1
ura4
E
cen1-Sn
wild type (33°C) 2 COs in 30
Ade+ recombinants
P 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
P 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
CO
NCO
*
ura4-Sn
wild type (33°C) 8 COs in 30
Ade+ recombinants
P 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 P
CO
NCO

Figure 7

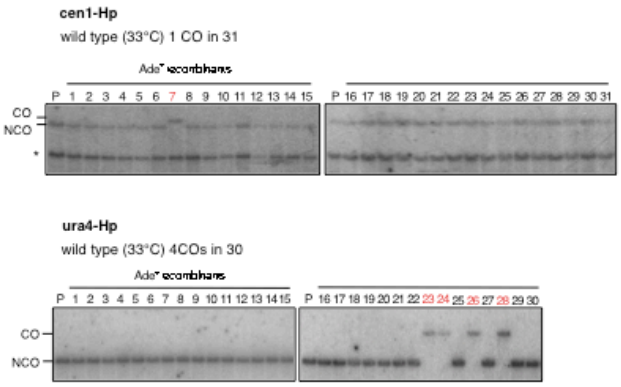


Figure 7. Crossovers (CO) and non-crossovers (NCO) in centromere and arm regions.
(A) CO and NCO recombinants produced in the cen1-Sn construct in wild type. DNA was prepared, digested with AfeI, separated by PFGE, transferred to a nylon membrane, and subjected to Southern hybridization using probe1. An asterisk indicates the band derived from cen3. (B) CO and NCO recombinants produced in the ura4-Sn construct in wild type. DNA was digested with AfeI and SmaI and separated by PFGE. Probe2 was used for Southern hybridization. (C) Proportions of CO in cen1-Sn and ura4-Sn constructs in wild type are indicated in Pie charts. Net rates of CO and NCO recombination are shown in bar graphs. (D) Proportions of CO and net rates of CO and NCO recombination in cen1-Hp and ura4-Hp constructs in wild type. (E) Blot data showing the physical detection of CO and NCO in the wild type strains of cen1-Sn, ura4-Sn, cen1-Hp, and ura4-Hp.

Rates relative to the cen1 value are indicated at the top of each bar. *P*-values were obtained by the two-tailed Fisher's exact test. * $P < 0.05$; *** $P < 0.001$. *n*, sample number; A, AfeI; S, SmaI; CO, crossover; NCO, non-crossover; P, parental. COs are shown in red; *, a band from cnt3.
The blot data shows the actual number of CO-NCO determined by me. The pie graph is the result from my published paper.

Rad51-dependent recombination promotes non-crossovers in centromeres

To understand the roles of Rad51, Rad54, and Rad52 in the suppression of crossovers in centromeres, crossover/non-crossover analysis was done using their mutant strains (Figure 8). In cen1-Sn, *rad51*Δ, *rad54*Δ, and *rad52*Δ strains exhibited increased proportions of crossovers as compared to the wild-type strain (Figure 8A, pie charts), although the net rate of crossovers was decreased in the mutant strains (Figure. 8A, bar graphs). Compared to crossovers, non-crossovers were decreased dramatically in the mutant strains, indicating that Rad51, Rad54, and Rad52 preferentially promote non-crossovers in cen1-Sn. In ura4-Sn, however, none of the mutant strains showed significantly increased proportions of crossovers, and crossovers and non-crossovers were both decreased in the mutant strains (Figure 8B). In cen1-Hp, *rad51*Δ, *rad54*Δ and *rad52*Δ strains dramatically decreased non-crossovers as compared to crossovers (Figure 8C) but not in ura4-Hp (Figure 8D). These data show that the strong preference for non-crossovers in centromeres depends on Rad51-dependent recombination.

Figure 8

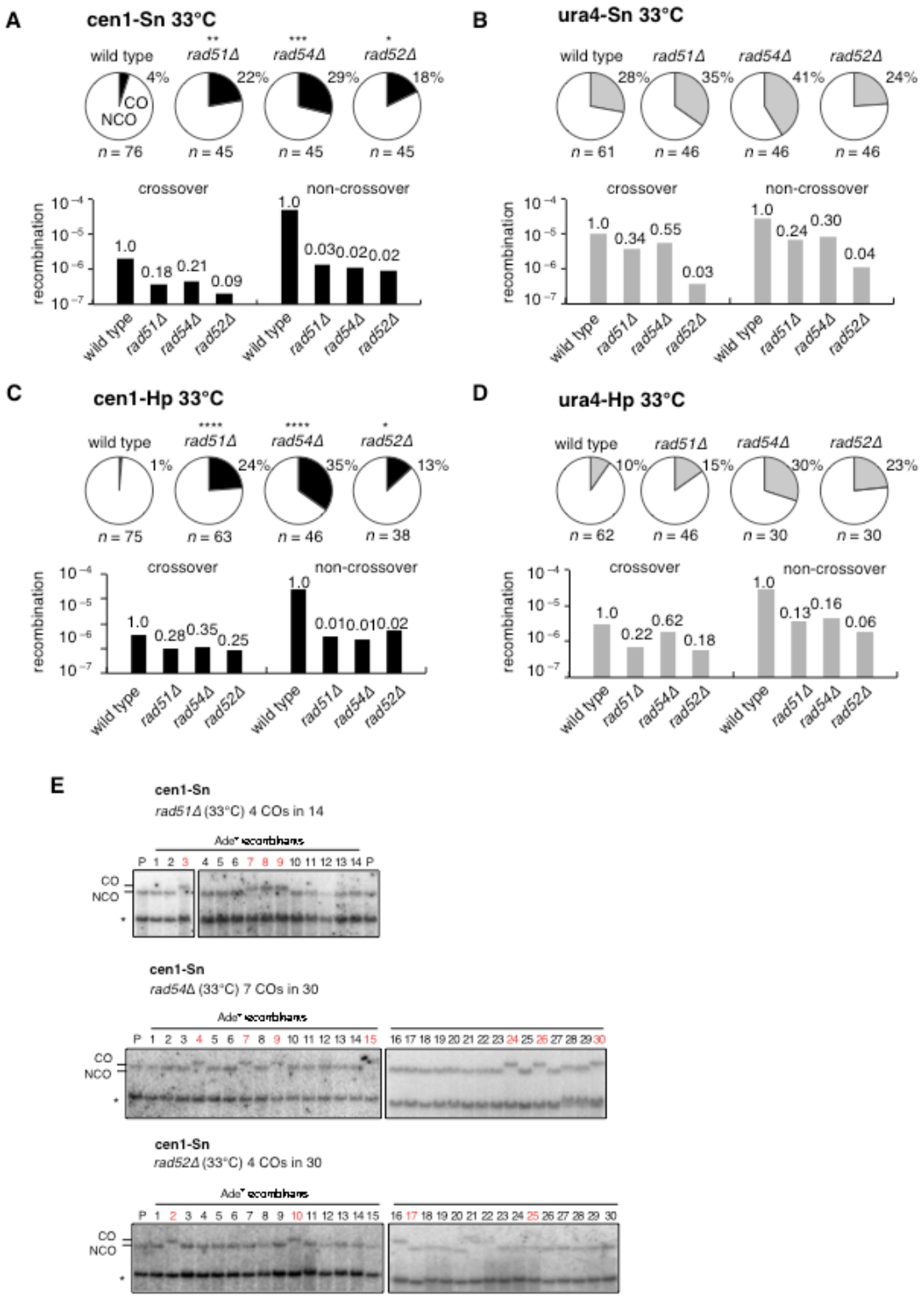

A
cen1-Sn 33°C
wild type
rad51Δ
rad54Δ
rad52Δ
4%
22%
29%
18%
CO
NCO
n = 76
n = 45
n = 45
n = 45
crossover
non-crossover
recombination
1.0
0.18
0.21
0.09
1.0
0.03
0.02
0.02
B
ura4-Sn 33°C
28%
35%
41%
24%
n = 61
n = 46
n = 46
n = 46
1.0
0.34
0.55
0.03
1.0
0.24
0.30
0.04
C
cen1-Hp 33°C
1%
24%
35%
13%
n = 75
n = 63
n = 46
n = 38
1.0
0.28
0.35
0.25
1.0
0.01
0.01
0.02
D
ura4-Hp 33°C
10%
15%
30%
23%
n = 62
n = 46
n = 30
n = 30
1.0
0.22
0.62
0.18
1.0
0.13
0.16
0.06
E
cen1-Sn
rad51Δ (33°C) 4 COs in 14
cen1-Sn
rad54Δ (33°C) 7 COs in 30
cen1-Sn
rad52Δ (33°C) 4 COs in 30

Figure 8

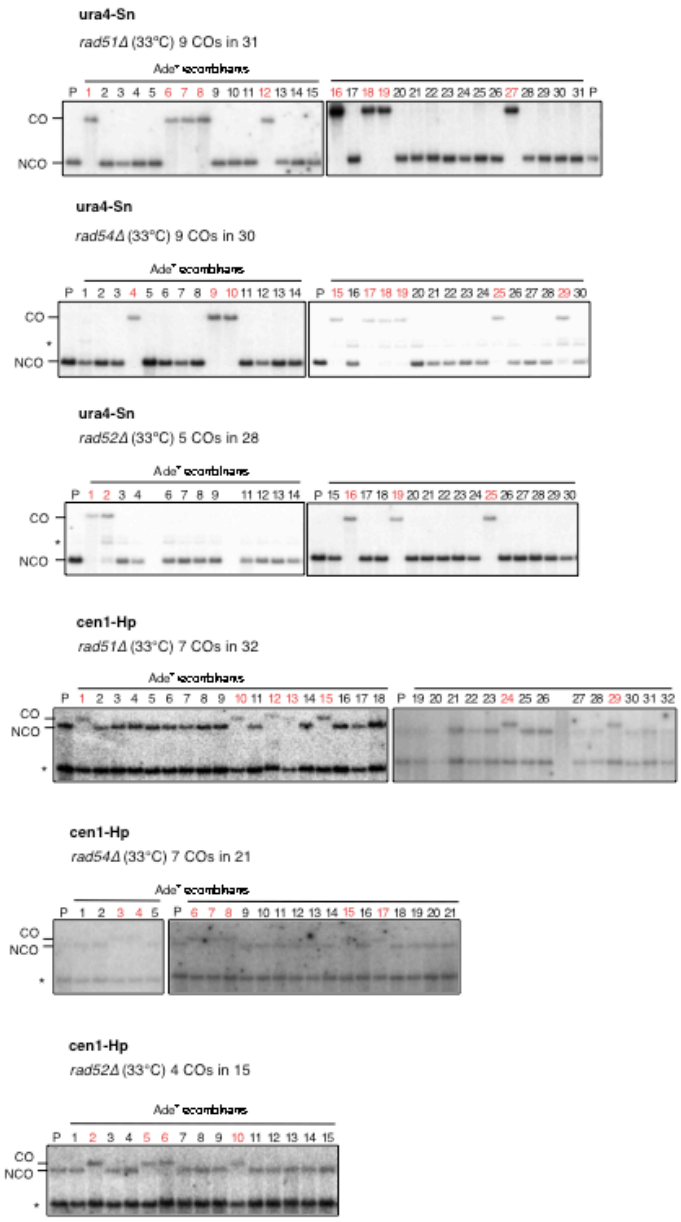

ura4-Sn
rad51Δ (33°C) 9 COs in 31
Ade+ recombinants
P 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 P
CO
NCO
ura4-Sn
rad54Δ (33°C) 9 COs in 30
Ade+ recombinants
P 1 2 3 4 5 6 7 8 9 10 11 12 13 14 P 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
CO
*
NCO
ura4-Sn
rad52Δ (33°C) 5 COs in 28
Ade+ recombinants
P 1 2 3 4 6 7 8 9 11 12 13 14 P 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
CO
*
NCO
cen1-Hp
rad51Δ (33°C) 7 COs in 32
Ade+ recombinants
P 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 P 19 20 21 22 23 24 25 26 27 28 29 30 31 32
CO
NCO
*
cen1-Hp
rad54Δ (33°C) 7 COs in 21
Ade+ recombinants
P 1 2 3 4 5 P 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
CO
NCO
*
cen1-Hp
rad52Δ (33°C) 4 COs in 15
Ade+ recombinants
P 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
CO
NCO
*

Figure 8

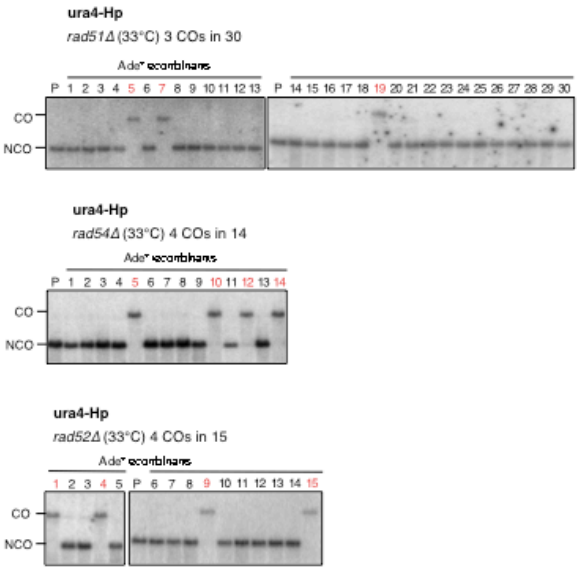


Figure 8. Rad51-dependent recombination preferentially promote non-crossovers in centromeres.
Proportions of COs among recombinants (pie charts) and the net rates of CO and NCO recombination (bar graphs) in wild type, *rad51Δ*, *rad54Δ*, and *rad52Δ* strains of (A) cen1-Sn construct, (B) ura4-Sn construct, (C) cen1-Hp construct and, (D) ura4-Hp construct.

** P <0.01. (E) Blot data showing the physical detection of crossovers and non-crossovers in the wild type, *rad51Δ*, *rad54Δ*, and *rad52Δ* strains of cen1-Sn, ura4-Sn, cen1-Hp, and ura4-Hp.

Heterochromatin is insufficient to suppress crossovers in centromere

The centromere contains a unique sequence *cnt*, flanked by inverted repeats: *imr*, *dg*, *dh* and *irc*. Kinetochore chromatin marked by CENP-A is assembled in the central region, while heterochromatin is formed in pericentromeric repeat region. The heterochromatin marked by the methylation of histone H3K9 by Clr4/Suv39 methyltransferase (Bannister, Zegerman et al., 2001, Rea, Eisenhaber et al., 2000), provides a platform for the assembly of specific proteins such as Swi6/HP1 that define heterochromatin structures. Swi6 is responsible for stabilizing cohesin in this region that is important for proper segregation. The presence of pericentromere repeats and the assembly of heterochromatin might affect gene conversion in the central region through defining high-order chromatin structure and nuclear peripheral localization. To see the effect of heterochromatin on recombination in the centromere, *clr4*$^{+}$ and *swi6*$^{+}$ genes were disrupted and the rates of spontaneous recombination were determined. To see the effect of heterochromatin throughout the kinetochore region, spontaneous recombination was determined using the strains that contain *ade6B* and *ade6X* heteroalleles at *Sna*BI (cen1-Sn), *Hpa*I (cen1-Hp), and *Hin*dIII (cen1-Hi) sites in the *imr1* repeats of centromere 1. Figure 9A shows the position of these restriction enzyme sites, Sn site on either side of *imr1*, is close to *cnt* forming a 5 kb interval, Hp site is almost in the middle of the *imr* repeats forming 10 kb interval and Hi site in between two tRNA genes, at the borderline between kinetochore and the heterochromatin forming a 12 kb interval (Sn and Hp sites are same as in Figure 6 and 7). Spontaneous recombination in the wild type occurred at a similar rate at the Sn and Hp and the Hi sites (Figure 9B). *clr4*Δ increased the rate of recombination at all three sites as compared to the wild type. *swi6*Δ also increased the rate of

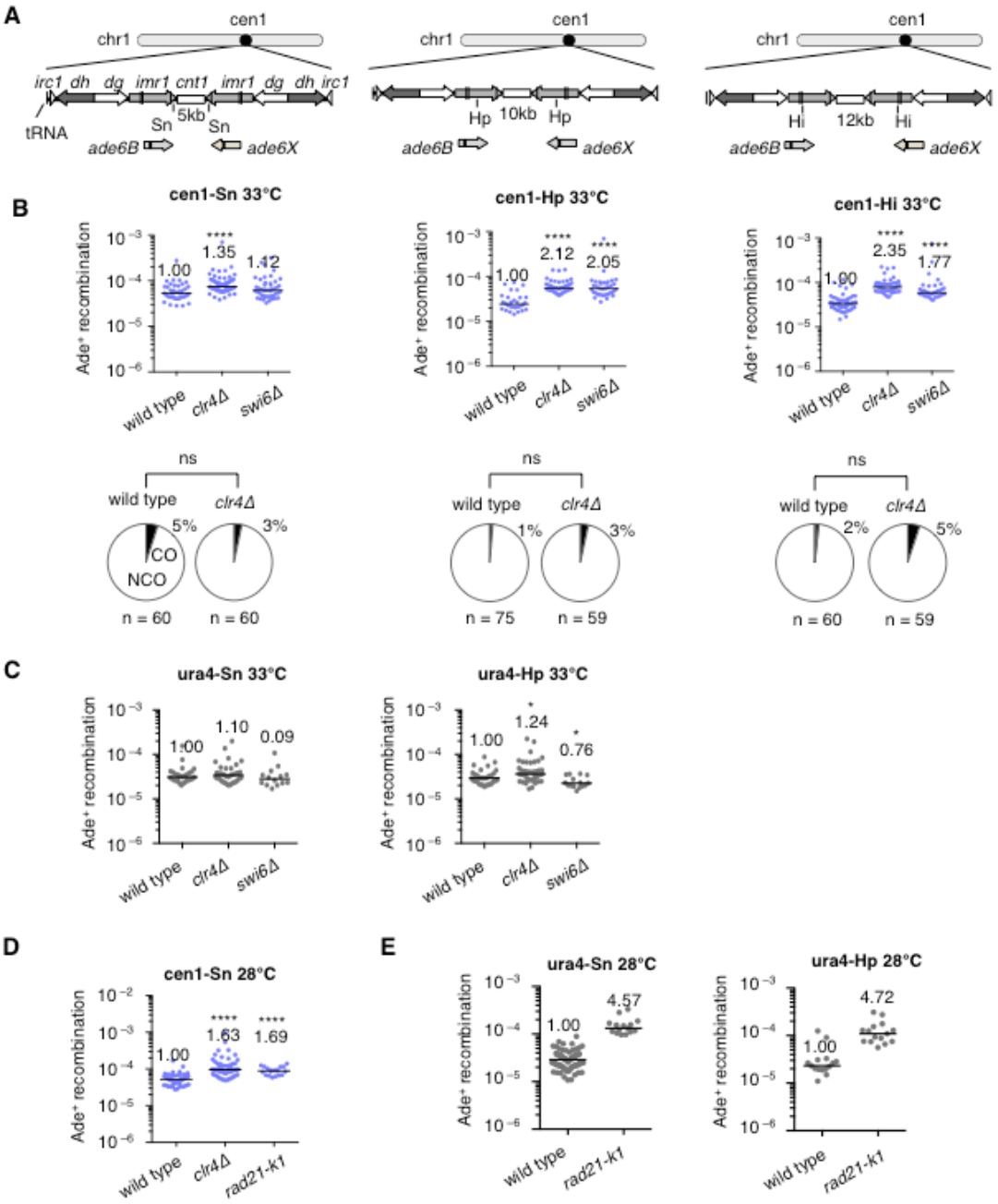
recombination in HP and Hi sites, while the rate remained similar to wild type in the cen1-Sn site. These results suggest that heterochromatin suppresses recombination throughout the kinetochore. As, Swi6 and Clr4 do not localize in the kinetochore, the effect of their mutation indirectly increased the recombination rate at Sn and Hp sites. At Hi site, *swi6*Δ and *clr4*Δ deletion had highest relative values as compared to the wild type, because this site is at the border of the kinetochore and heterochromatin and these proteins are localized up to this region. It also suggests that heterochromatin mainly suppresses recombination at the border of heterochromatin and kinetochore and the effect is limited in the inner kinetochore. To see whether a deletion of *clr4* or *swi6* suppress recombination only in the authentic centromere, or they have an epigenetic regulation in the non-centromere region, rates were determined in the ura4-Sn and ura4-Hp strains. At the ura4-Sn, unlike centromere, both *swi6*Δ and *clr4*Δ did not increase the recombination rate but maintained the rate similar to the wild type (Figure 9C). However, at the ura4-Hp, both *swi6*Δ and *clr4*Δ increased the rate of recombination significantly as compared to the wild type. Deletion of Clr4 may effect the methylation of histones also in non-centromere regions leading to a slight but significant increase in recombination. The deletion of the genes only suppressed recombination in the large ura4-Hp (10kb) probably due to tethering of the repeat regions to the nuclear periphery and silencing.

Cohesin has role in chromosome segregation, DNA repair, regulation of recombination (Schmidt, Brookes et al., 2009). Clr4 methyltransferase methylates histones to allow Swi6 to stabilize cohesin in the pericentromere repeats (Nonaka et al., 2002). Clr4 suppressed recombination in centromere, partially dependent on Swi6, while the effect of Swi6 on recombination could be due to the enrichment of cohesin. To see whether cohesin mutation also increases the recombination in kinetochore,

rates were determined in wild type and a temperature sensitive (ts) mutant, *rad21-K1*, in cohesin subunit Rad21 at 28°C, at cen1-Sn site (Figure 9D). *Rad21-K1* shows sensitivity to UV and gamma radiation. *Rad21-K1* exhibits loss of cohesion, defects in precise segregation (stretched and scattered chromosomes, unequal segregation, chromosomes displaced to one end of the cell, cut like phenotype) of chromosomes (Tatebayashi, Kato et al., 1998). As compared to the wild type, the *rad21-K1* mutation increased the recombination rate significantly and similar to *clr4*Δ, showing that cohesin suppresses recombination between the repeats of the kinetochore. Cohesin is richly bound to the repeats of the centromere, but it also localizes to the euchromatin regions. To see whether the effect of cohesin on recombination is specific to the centromere or a general feature, rates were determined in the ura4-Sn and ura4-Hp strains of wild type and *rad21-K1* (Figure 9E). As compared to the wild type *rad21-K1* mutation –like centromere- increased the rate of recombination in both constructs. These results suggest that the suppression of recombination is a general feature of cohesin be it centromere or non-centromere region.

Interestingly, however, the proportion of crossovers in *clr4*Δ was very similar to that in wild type, although Clr4 suppresses recombination in centromere (Figure 9B, lower panels pie graphs). Essentially the same phenotypes were observed in the cen1-Hp and cen1-Hi strains. These results show that the heterochromatin suppresses recombination but does not suppress crossovers in the centromere. Therefore, the heterochromatin plays no role in centromere specific recombination that is suppression of Rad51-independent HR and crossovers.

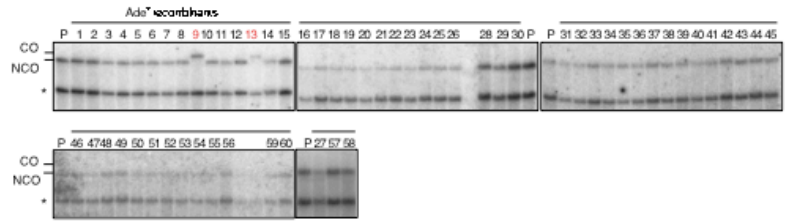
Figure 9


A
cen1
chr1
irc1 dh dg imr1 cnt1 imr1 dg dh irc1
tRNA
Sn 5kb Sn
ade6B
ade6X
Hp 10kb Hp
Hi 12kb Hi
B
cen1-Sn 33°C
cen1-Hp 33°C
cen1-Hi 33°C
Ade+ recombination
1.00
1.35
1.42
2.12
2.05
2.35
1.77
wild type
clr4Δ
swi6Δ
ns
5%
3%
CO
NCO
1%
2%
n = 60
n = 75
n = 59
C
ura4-Sn 33°C
ura4-Hp 33°C
1.10
0.09
1.24
0.76
D
cen1-Sn 28°C
1.63
1.69
rad21-k1
E
ura4-Sn 28°C
ura4-Hp 28°C
4.57
4.72

F

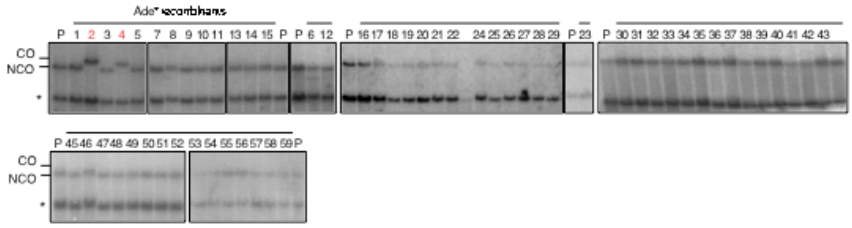
cen1-Sn

clr4Δ (33°C) 2 COs in 60



cen1-Hp

clr4Δ (33°C) 2 COs in 59



cen1-Hi

wild type (33°C) 1 CO in 60

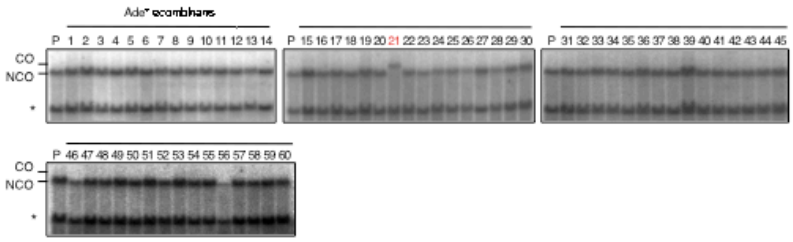


Figure 9

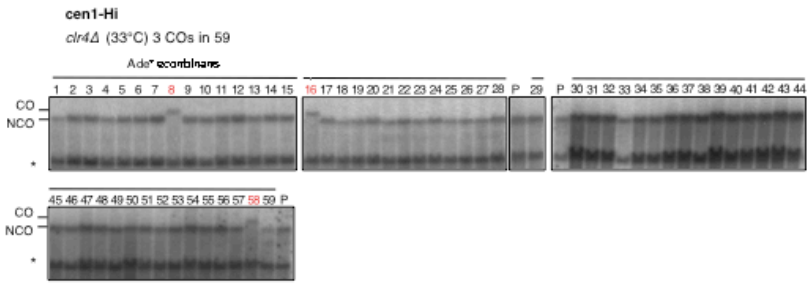


Figure 9. Effect of heterochromatin on recombination.
(A) Illustrated are the cen1-Sn, cen1-Hp and cen1-Hi constructs. In the cen1-Sn construct, the ade6 heteroalleles are integrated at the Sn site in the *imr1* repeats close to the *cnt* sequence at an interval of 5kb. In the cen1-Hp construct, the ade6 heteroalleles are integrated at the Hp site in the middle of the imr1 repeats at an interval of 10kb. In the cen1-Hi construct, the ade6 heteroalleles are integrated at the Hi site in the imr1 repeats close to the heterochromatin at an interval of 12kb. (B) Recombination in the heterochromatin mutants in centromere. Spontaneous rates of Ade$^+$ prototroph formation were determined in wild type, *clr4Δ*, and *swi6Δ* strains in the cen1-Sn, cen1-Hp, and cen1-Hi strains. Proportions of CO in cen1-Sn, cen1-Hp and cen1-Hi constructs in wild type and *clr4Δ* are indicated in Pie charts. (C) Recombination in the heterochromatin mutants in ura4 locus. Spontaneous rates of Ade$^+$ prototroph formation were determined in wild type, *clr4Δ*, and *swi6Δ* strains in the ura4-Sn, and ura4-Hp strains. (D) Recombination in the cohesin mutant in centromere. Spontaneous rates of Ade$^+$ prototroph formation were determined in wild type, *clr4Δ*, and *rad21-K1* strains in the cen1-Sn construct at 28°C. (E) Recombination in the cohesin mutant in ura4 locus. Spontaneous rates of Ade$^+$ prototroph formation were determined in wild type, *clr4Δ*, and *rad21-K1* strains in the ura4-Sn and ura4-Hp construct at 28°C. (F) Blot data showing the physical detection of crossovers and non-crossovers in the wild type, and *clr4Δ* strains of cen1-Sn, cen1-Hp, and cen1-Hi constructs.

Effect of kinetochore factors on recombination

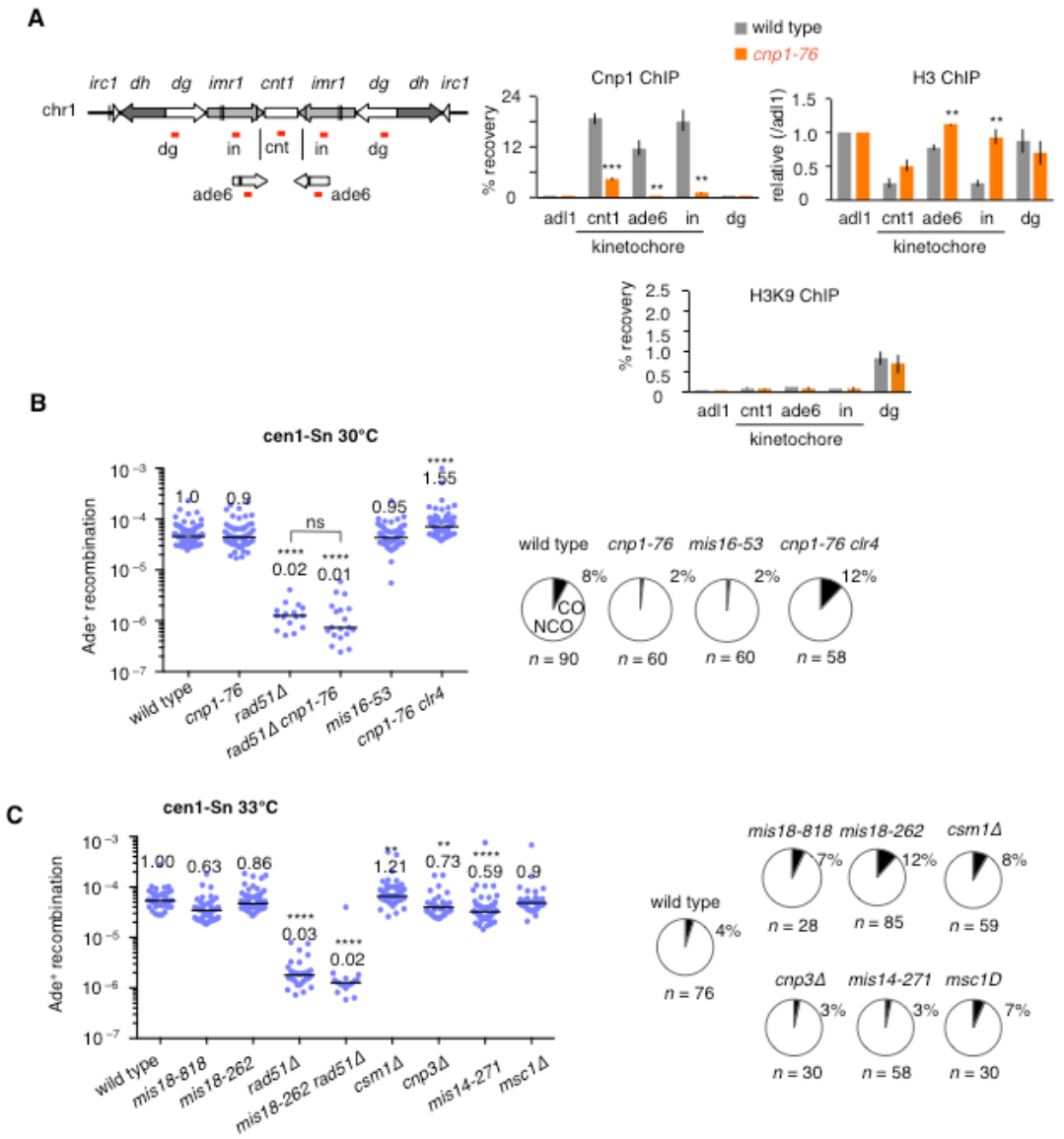
Pericentromeric heterochromatin is not required for centromere-specific recombination, suggesting that the factors related to the central domain (kinetochore) may be responsible for regulation of recombination in centromeres. Therefore, the roles of the proteins that localize to the kinetochore region were examined (Figure 10). Incorporation of histone H3 variant CENP-A is one of the hallmarks of kinetochore chromatin. The *cnp1-76* temperature-sensitive mutation changes threonine at position 76 to methionine of CENP-A (Castillo, Mellone et al., 2007). As expected, ChIP analysis showed that *cnp1-76* dramatically decreases CENP-A level in the *cnt*, the *imr* repeats and the *ade6* heteroalleles in the *imr* repeats at 30°C (Figure 10A). This decrease in CENP-A leads to increase in histone H3 level as reported previously (Castillo et al., 2007). However, the H3K9 methylation remained at the wild type levels. Although CENP-A localization was severely decreased, *cnp1-76* did not change the rate of recombination in the presence or absence of Rad51 (Figure 10B). *cnp1-76* also did not change the proportion of crossovers (Figure 10B, pie graph), suggesting that CENP-A is not essential for centromere-specific HR. However, there is a possibility that both CENP-A and Clr4 redundantly regulate centromere specific recombination. To address this issue, spontaneous rate of recombination was determined in the *cnp1-76 clr4*Δ double mutant (Figure 10B). As compared to the wild type, the double mutant also did not increase the number of crossovers significantly. Therefore, it seems that both CENP-A and Clr4 are not responsible for suppression of crossovers in centromere.

Mis16 and Mis18 form a complex and they are one of the most upstream factors in kinetochore assembly (Hayashi et al 2004). Mis16-18 complex is also required for the association of Mis6 with kinetochore and they together are required

for stabilizing CENP-A to this region. They maintain the de-acetylated state of histones (histone H4K16, H3K9) in the kinetochore to silence the repeat sequences to prevent damage due to replication fork blockage (Mikel et al 2011). *mis16-53*, *mis18-818* and *mis18-262* show high frequency of unequal segregation generating anueploidy and loss of viability (Hayashi et al., 2004): hallmark of mutation in the authentic kinetochore. Since these Mis proteins are the most basal factors that bind to the chromatin and provide the platform for kinetochore assembly, recombination rates were determined in their temperature sensitive mutants at semi-permissive temperatures (Figures 10B and 10C). As compared to their respective wild type, none of the mutations changed the ratio of crossovers significantly in the cen1-Sn strain. Therefore, it seems unlikely that these factors are involved in the centromere specific regulation of recombination.

CENP-A is escorted by Sim3, which is then handed over to a CENP-A receptor Scm3, which in co-ordination with Mis16-18 complex assembles the CENP-A at the kinetochore to ensure proper and complete chromatin assembly. CENP-A is assembled throughout the kinetochore, ie *cnt* and *imr* regions. *mis18-262* increased the crossovers up to 12% as compared to 4% in wild type (P value is insignificant) in cen1-Sn strain, where the *ade6* heteroalleles are close to the *cnt* sequence. There is a possibility that *mis18-262* may increase crossovers at the cen1-Hp construct where the *ade6* heteroalleles are in the middle of the *imr* repeats. To this end, spontaneous rates of recombination were determined in the cen1-Hp construct of wild type, *mis18-262,* and *scm3* strains (Figure 10D). None of these mutations increased the number of crossovers suggesting that they do not play a role in suppression of crossovers in centromere.

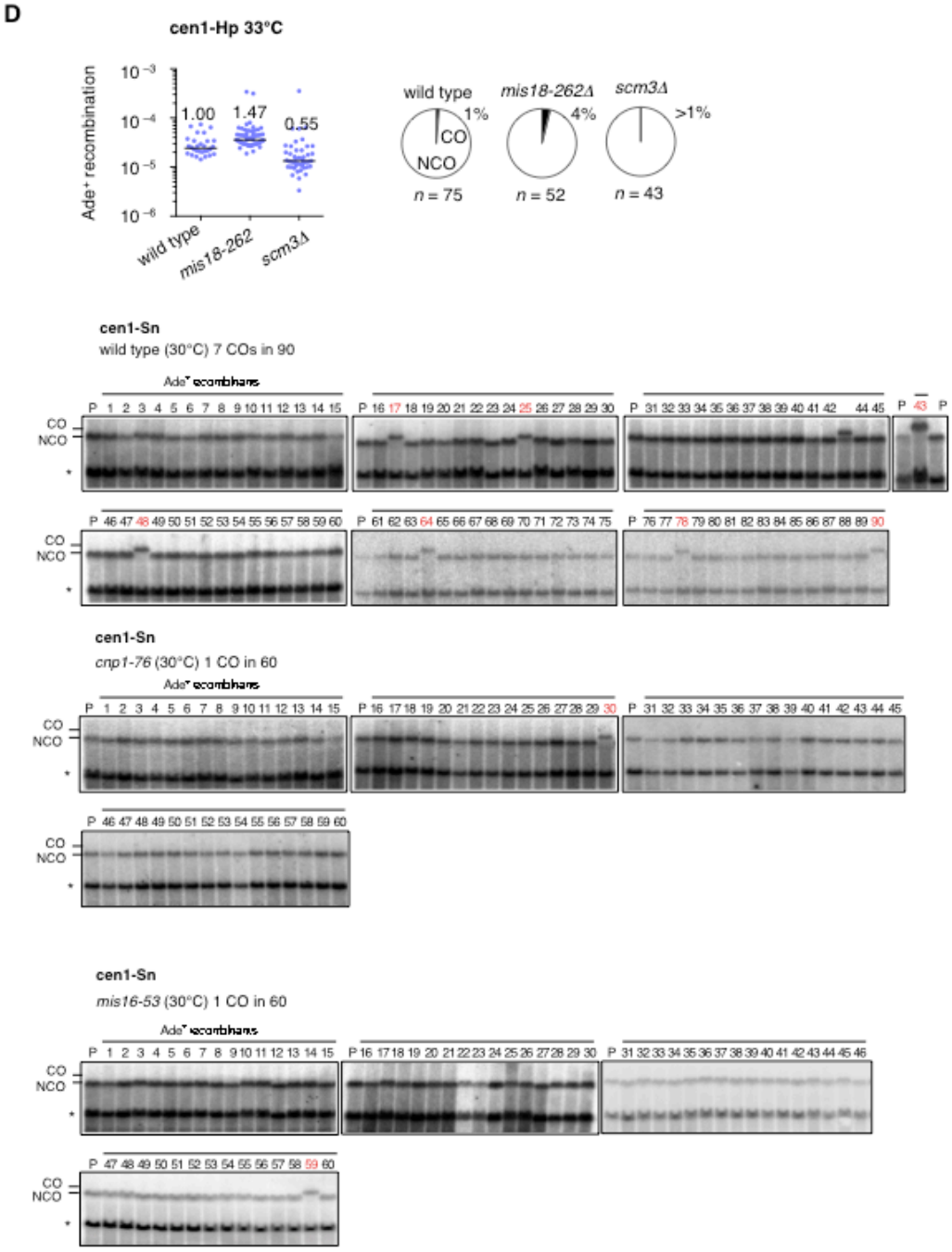
Figure 10


A
irc1 dh dg imr1 cnt1 imr1 dg dh irc1
chr1
dg in cnt in dg
ade6 ade6
wild type
cnp1-76
Cnp1 ChIP
% recovery
adl1 cnt1 ade6 in dg
kinetochore
H3 ChIP
relative (/adl1)
H3K9 ChIP
B
cen1-Sn 30°C
Ade+ recombination
1.0 0.9 0.02 0.01 0.95 1.55
ns
wild type cnp1-76 rad51Δ rad51Δ cnp1-76 mis16-53 cnp1-76 clr4
wild type cnp1-76 mis16-53 cnp1-76 clr4
8% 2% 2% 12%
CO
NCO
n = 90 n = 60 n = 60 n = 58
C
cen1-Sn 33°C
Ade+ recombination
1.00 0.63 0.86 0.03 0.02 1.21 0.73 0.59 0.9
wild type mis18-818 mis18-262 rad51Δ mis18-262 rad51Δ csm1Δ cnp3Δ mis14-271 msc1Δ
wild type
4%
n = 76
mis18-818 mis18-262 csm1Δ
7% 12% 8%
n = 28 n = 85 n = 59
cnp3Δ mis14-271 msc1D
3% 3% 7%
n = 30 n = 58 n = 30

Csm1 acts at kinetochores as a molecular clamp to lock together microtubule attachment sites to prevent merotelic attachment (error in which a single kinetochore is attached to microtubules emanating from both spindle poles) (Gregan, Riedel et al., 2007). Csm1 recruits condensin to the kinetochores which is also important to prevent merotely, aneuploidy and lagging chromosomes (Tada, Susumu et al., 2011). Condensin that binds the regions of the same chromatid are enriched in the kinetochore, therefore, to see the effect of condensin on recombination between the repeats of the same chromatid spontaneous rate of recombination was determined in the *csm1* deletion mutant. As compared to the wild type, *csm1Δ* only slightly increased the rate of recombination but the ratio of crossovers was not changed significantly (Figure 10C). Thus, it is clear from the result that Csm1 or rather condensin does not have a significant role in regulation of crossover in centromere.

CENP-C/Cnp3 is necessary for chromosome stability and it functions in an architectural role, perhaps by assembling a platform upon which other components of the kinetochore assemble (Saitoh, Tomkiel et al., 1992, Tomkiel, Cooke et al., 1994). Mis14-Mis12 complex localize to the kinetochore with interaction with CENP-C and they are essential for segregation of chromosomes. CENP-C can bypass the requirement of CENP-A to induce ectopic kinetochore assembly (Gascoigne et al., 2011). One possibility for CENP-A not showing any effect on regulation of HR could be that the function of CENP-A could have been rescued by CENP-C. To address this possibility, spontaneous rate of recombination was determined in CENP-C and Mis14 mutants (Figure. 10C). *cnp3Δ* and *mis14-271* did not increase the ratio of crossovers as compared to the wild type. Thus it is evident that these factors do not regulate crossover suppression in centromere.

Figure 10


D
cen1-Hp 33°C
Ade+ recombination
1.00
1.47
0.55
wild type
mis18-262
scm3Δ
wild type
mis18-262Δ
scm3Δ
1%
4%
>1%
CO
NCO
n = 75
n = 52
n = 43
cen1-Sn
wild type (30°C) 7 COs in 90
cen1-Sn
cnp1-76 (30°C) 1 CO in 60
cen1-Sn
mis16-53 (30°C) 1 CO in 60

It has been reported that in euchromatin, histone H2A.Z is exchanged onto nucleosomes at DSB sites, creating open, relaxed chromatin domains. H2A.Zs are essential for acetylation and ubiquitination of the chromatin for employing DNA damage response factors (Xu et al 2012). Centromere chromatin is unique as compared to the euchromatin. H2A.Z is absent from all centromeric regions but is present in the euchromatin (Buchanan et al 2009). Thus it is possible that the absence of H2A.Z in the centromere accounts for the Rad51-dependent HR and suppression of COs in the centromere. To test this possibility, I deleted *msc1* gene, the product of which is the negative regulator of H2A.Z incorporation at centromeres. Chromatin binding profiles for H2A.Z show that, in the absence of Msc1, H2A.Z became incorporated specifically in the inner centromere (Buchanan et al 2009). To see whether the presence of H2A.Z suppresses crossovers in centromere, in centromere, recombination was determined in *msc1Δ* strain (Figure 10C). The *msc1* deletion did not significantly change the proportion of crossovers as compared to wild type, suggesting that the increased level of H2AZ does not promote Rad51-independent recombination in centromere. This also suggests that some centromere specific factor may be important to regulate Rad51-dependent HR in centromere.

Figure 10

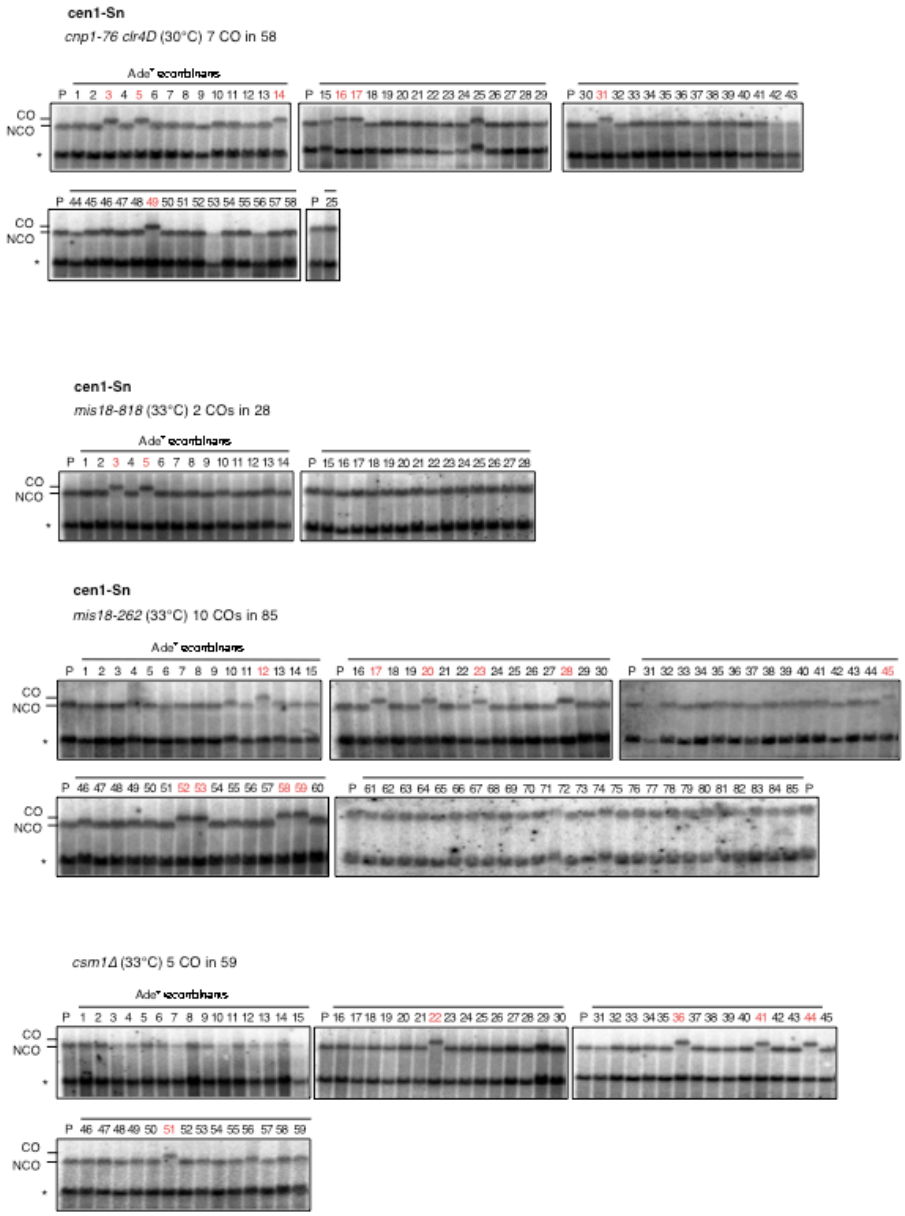

cen1-Sn
cnp1-76 clr4D (30°C) 7 CO in 58
Ade+ recombinants
CO
NCO
*
cen1-Sn
mis18-818 (33°C) 2 COs in 28
Ade+ recombinants
CO
NCO
*
cen1-Sn
mis18-262 (33°C) 10 COs in 85
Ade+ recombinants
CO
NCO
*
csm1Δ (33°C) 5 CO in 59
Ade+ recombinants
CO
NCO
*

Figure 10

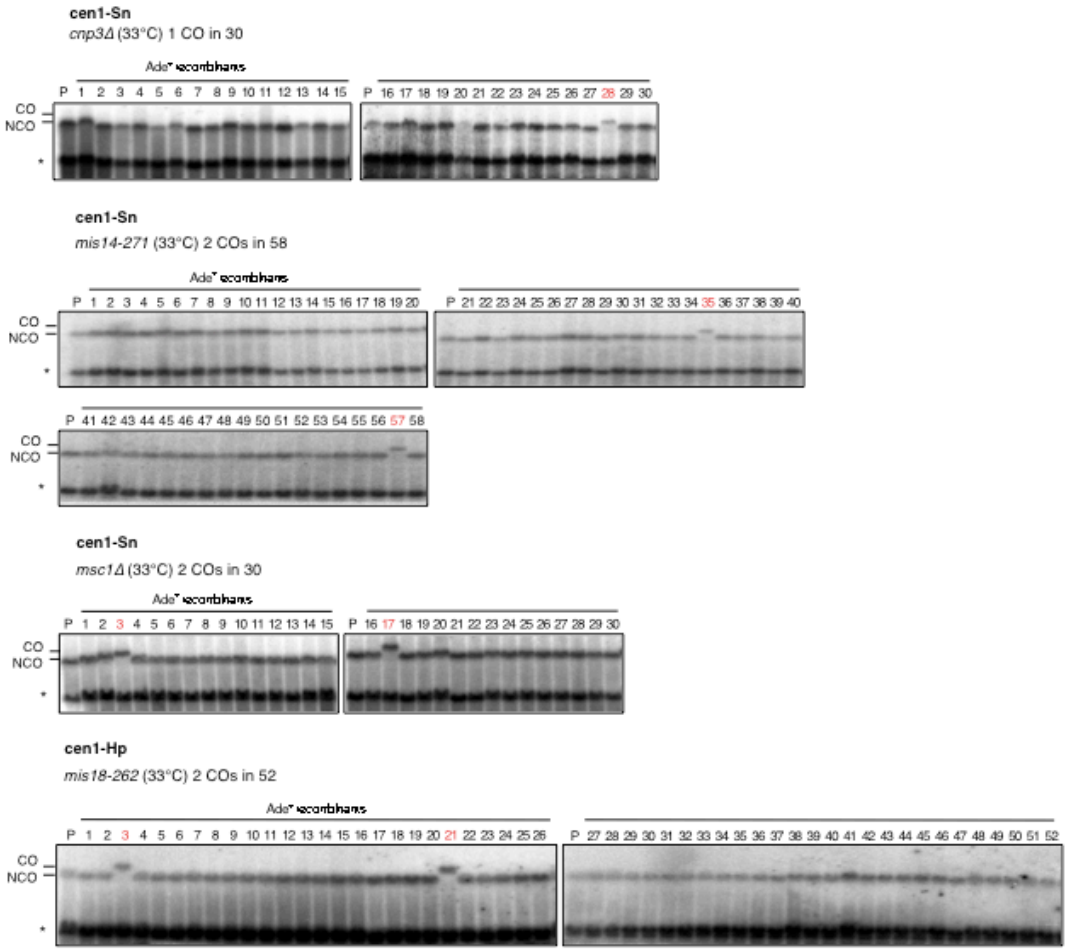


Figure 10. The effect of kinetochore factors on recombination and crossover suppression.
(A) Illustrated is the cen1-Sn construct. Positions of centromere repeats, *ade6B/X*, and the regions amplified by real-time PCR are shown. The *adl1* gene is present on the arm of chr2. (B) ChIP experiments were carried out using wild type and *cnp1-76* mutant strains grown at a semi-permissive temperature of 30°C. Mean ± SEM from three independent experiments are shown. $^{**}P < 0.01$; $^{***}P < 0.001$. *P*-values were determined by the two-tailed student T-test. (B) Spontaneous rate of recombination (scatter plot) and proportions of COs and NCOs (pie graphs) was determined in the cen1-Sn construct of wild type, *cnp1-76*, *cnp1-76 rad51Δ*, *mis16-53* and *cnp1-76 clr4Δ* at 30°C. (C) Spontaneous rate of recombination and proportions of COs and NCOs was determined in the cen1-Sn construct of wild type, *mis18-818*, *mis18-262*, *csm1Δ*, *cnp3Δ*, *mis14-271*, and *msc1Δ* strains at 33°C. (D) Spontaneous rate of recombination and proportions of COs and NCOs was determined in the cen1-Hp construct of wild type, *mis18-262*, and *scm3Δ* strains at 33°C. (E) Blot data showing the physical detection of COs and NCOs in all the mentioned strains.

Mhf1/CENP-S, Mhf2/CENP-X, and Fml1/FANCM suppress crossovers in the centromere

Histone-fold proteins Mhf1/CENP-S and Mhf2/CENP-X localize at centromeres in fission yeast and humans (Amano, Suzuki et al., 2009b, Bhattacharjee, Osman et al., 2013). Mhf1 is important for proper centromere chromatin as a deletion of the gene leads to shorter kinetochore plate, which hinders segregation. More importantly, the Mhf1 and Mhf2 show preference for branched DNA structures such as DNA repair intermediates and displacement loops (D-loops) indicating their role in DNA repair. Because deletion of *mhf1* or *mhf2* resulted in growth defects at high temperatures, their effects on recombination were examined at 28°C and found that *mhf1*Δ and *mhf2*Δ increased the proportion of crossovers (Figure 11A, pie graphs), showing that Mhf1 and Mhf2 are required to suppress crossovers in centromeres. Mhf1-Mhf2 complexes bind to cruciform DNA, recruit Fml1/FANCM to DNA, and stimulate its helicase activity (Singh et al., 2010, Yan et al., 2010, Zhao, Saro et al., 2014). It appears that Mhf1-Mhf2 and Fml1 function in the same pathway to suppress crossovers in centromeres as *fml1*Δ also increased the crossover ratio and it did not further increase crossovers in *mhf1*Δ cells (Figure 11A). *mhf1*Δ and *mhf2*Δ slightly but significantly increased the rate of recombination either in the presence or absence of Rad51. In contrast, *fml1*Δ increased the recombination rate only in the presence of Rad51, suggesting that Mhf1-Mhf2 has Fml1-independnet role in centromere recombination. Nonetheless, Mhf1-Mhf2 and Fml1 suppress crossovers in the centromere.

Figure 11

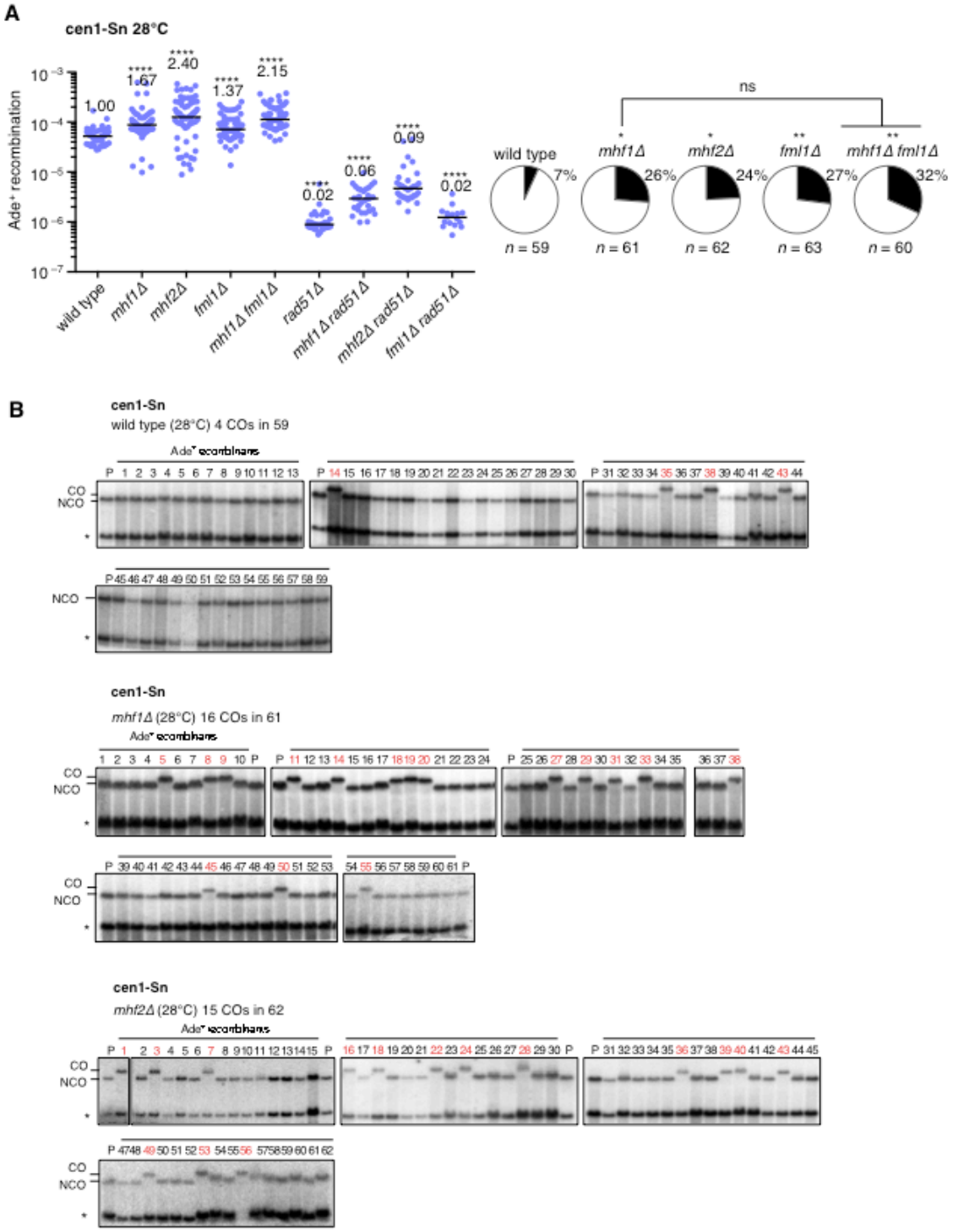

A
cen1-Sn 28°C
Ade+ recombination
1.00
1.67
2.40
1.37
2.15
0.02
0.06
0.09
0.02
wild type
mhf1Δ
mhf2Δ
fml1Δ
mhf1Δ fml1Δ
rad51Δ
mhf1Δ rad51Δ
mhf2Δ rad51Δ
fml1Δ rad51Δ
ns
7%
26%
24%
27%
32%
n = 59
n = 61
n = 62
n = 63
n = 60
B
cen1-Sn
wild type (28°C) 4 COs in 59
cen1-Sn
mhf1Δ (28°C) 16 COs in 61
cen1-Sn
mhf2Δ (28°C) 15 COs in 62
CO
NCO

Figure 11

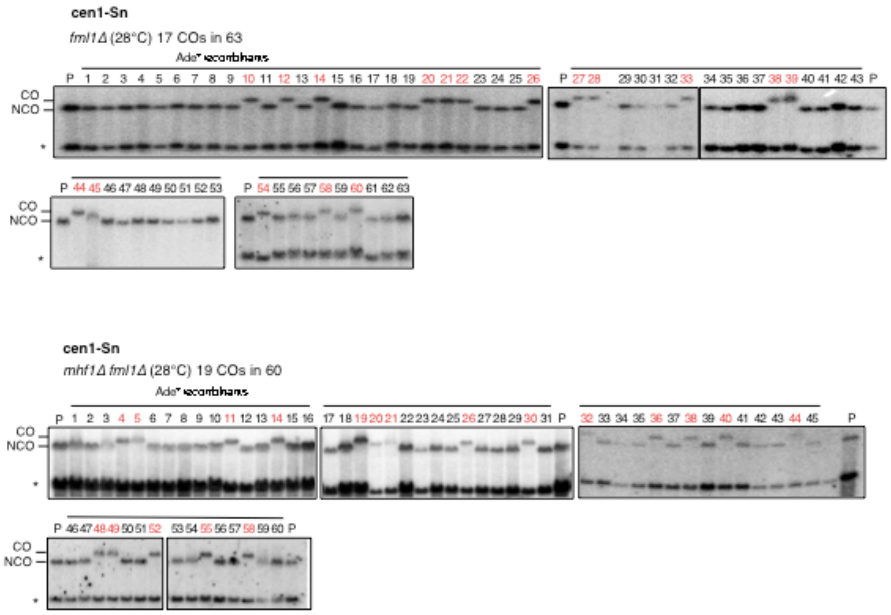


Figure 11. Mhf1-Mhf2 and Fml1 suppress crossovers in centromere
(A) Spontaneous rate of recombination and proportions of COs and NCOs were determined in the cen1-Sn construct of wild type, *mhf1Δ*, *mhf2Δ*, *fml1Δ*, *mhf1Δ fml1Δ*, *mhf1Δ rad51Δ*, *mhf2Δ rad51Δ* and *fml1Δ rad51Δ* strains at 28°C. (B) Blot data showing the physical detection of COs and NCOs in the strains mentioned above.

Mhf1-Mhf2 tetramers suppress crossovers in centromeres

Mhf1/CENP-S and Mhf2/CENP-X have histone fold domains like histone H3 and H4. Mhf1/CENP-S and Mhf2/CENP-X form hetero-tetramers called MHF tetramers through their histone fold domains, and they also form different complexes with CENP-T and CENP-W called CENP-T-W-S-X complex. (Nishino et al., 2012). Through the N-terminal portion of CENP-T, the CENP-T-W-S-X complex binds to the Ndc80 complex that interact to mitotic spindle tubules (Gascoigne et al., 2011). The MHF complex recruits FANCM/Fml1 to specific DNA substrates such as cruciform DNA and stimulates the helicase activity of Fml1 (Singh et al., 2010, Zhao et al., 2014). Mutations in Mhf1 that disrupt the interaction between Mhf1/2 dimers and that between Mhf1/2 and CENP-T/W dimers increase sensitivity to DNA damage (Yang, Zhang et al., 2012), and decrease the localization of Mhf1/2 and FANCM to centromeres (Nishino et al., 2012, Tao, Jin et al., 2012). To see if the formation of MHF tetramers is required for crossover suppression in centromeres, a conserved leucine residue of Mhf1 was changed to arginine (*mhf1-L78R*), because corresponding leucine is specifically involved in tetramer but not in dimer formation (Yang et al., 2012). The *mhf1-LR* mutation slightly increased the recombination rate as compared to the wild type in the cen1-Sn strain background at 33°C (Figure 12A), as was observed for *mhf1*Δ at 28°C (Figure 11A). *mhf1-LR* increased the proportion of crossovers similar to *fml1*Δ as compared to the wild type, suggesting that the MHF tetramer formation is important for suppression of COs in centromeres (Figure A). (P-values calculated by Fisher exact test; *mhf1-LR* (0.00098); *fml1*Δ (0.000006); (Figure 12B) suggesting that the MHF tetramer formation is essential for suppression of crossovers in centromeres.

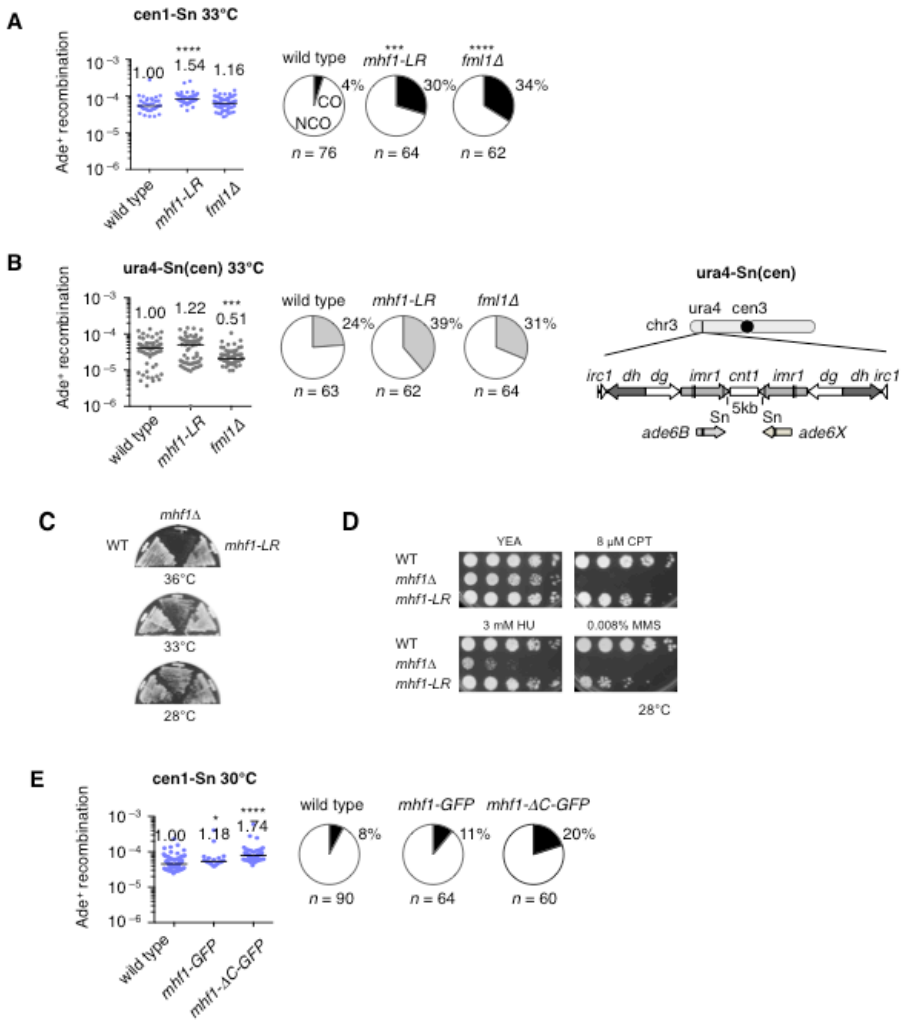
To see whether MHF tetramers and Fml1 suppress crossovers in the non-centromere region, spontaneous rates of recombination were determined in the wild type, *mhf1-LR* and *fml1*Δ in the ura4-Sn(cen) construct. This construct contains an entire region (~40 kb) of *cen1* including pericentromeric sequences into the *ura4* locus of chromosome 3. The heterochromatin assembles on the pericentromere repeats but no kinetochore assembles in the *imr* repeats. Using this construct I found that neither *mfh1-LR* nor *fml1*Δ increased crossovers significantly (Figure 12B), (P-values calculated by Fisher exact test; *mhf1-LR* (0.084); *fml1*Δ (0.428), suggesting that MHF tetramers and Fml1 have predominant role in the context of centromere chromatin.

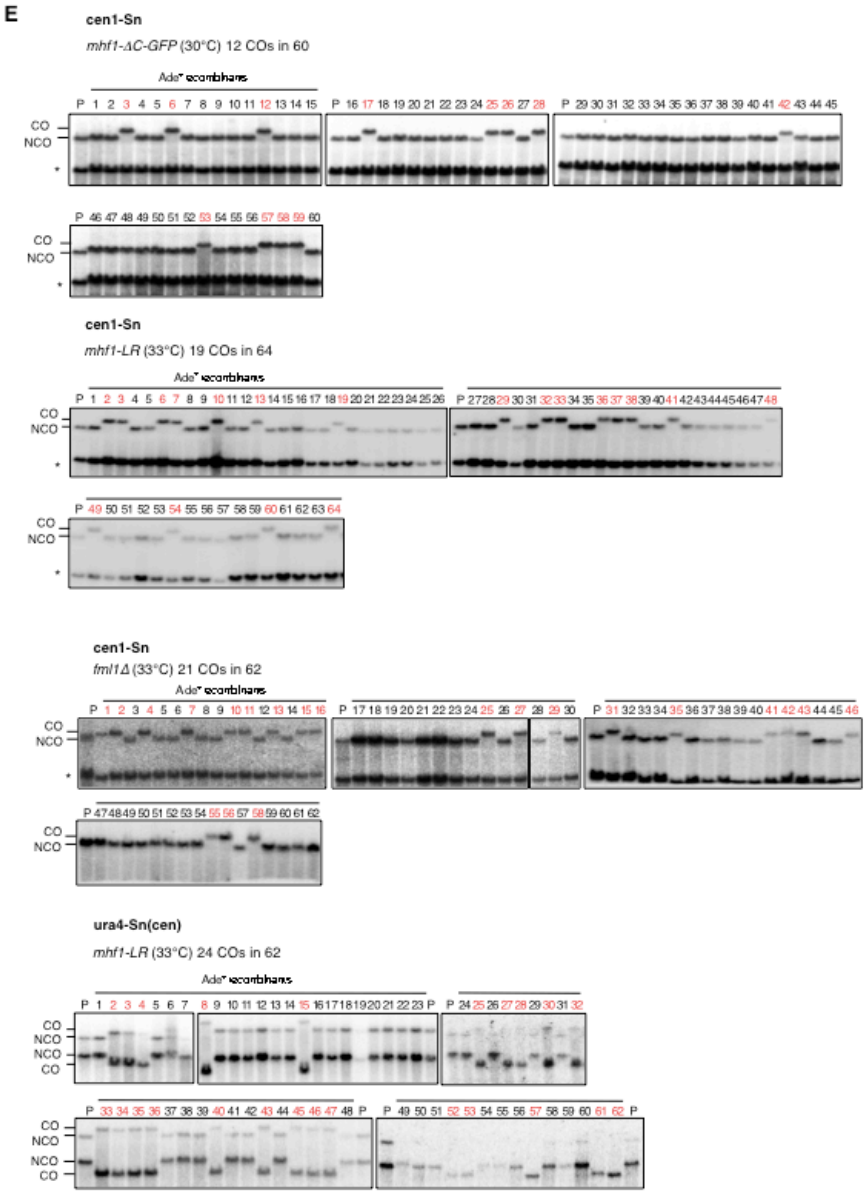
To gain insight into the role of *mhf1-LR* mutation on growth and repair, the following experiments were done. Wild type, *mhf1Δ* and *mhf1-LR* strains were streaked and incubated at 36, 33, and 28°C to compare the growth of these strains. *mhf1Δ* was sensitive to higher temperatures, while *mhf1-LR* could grow at all temperatures similar to wild-type (Figure 12C). *mhf1-LR* was also found to be mildly sensitive to genotoxins, MMS, CPT and HU as compared to wild type although *mhf1Δ* was severely sensitive to these drugs (Figure 12D). These results suggest that Mhf1 is essential for DNA repair and it has a function independent of the MHF tetramer formation. There is also a possibility that mhf1-LR mutation retains residual activity in DNA repair as this is not a null mutation.

To see whether the centromere specific suppression of crossovers by the *mhf1* mutation is limited to the defect in Mhf tetramer formation, spontaneous rate of recombination were determined in the *mhf1-ΔC*-GFP whose C-terminal tail is deleted. X-ray crystallographic studies in human CENP-S revealed that the C-terminal tail binds to branched DNA structures (Zhao et al 2013). As compared to wild type and a

control GFP tagged mhf1, *mhf1-ΔC*-GFP did increase the rate of spontaneous recombination –like *mfh1-LR*- however it did not change the ratio of crossovers in the cen1-Sn construct (Figure 12E). These results clearly indicate the specific role of MHF tetramer in crossover suppression in centromere.

Figure 12




E
cen1-Sn
mhf1-ΔC-GFP (30°C) 12 COs in 60
Ade+ recombinants
CO
NCO
*
cen1-Sn
mhf1-LR (33°C) 19 COs in 64
Ade+ recombinants
CO
NCO
*
cen1-Sn
fml1Δ (33°C) 21 COs in 62
Ade+ recombinants
CO
NCO
*
ura4-Sn(cen)
mhf1-LR (33°C) 24 COs in 62
Ade+ recombinants
CO
NCO
NCO
CO

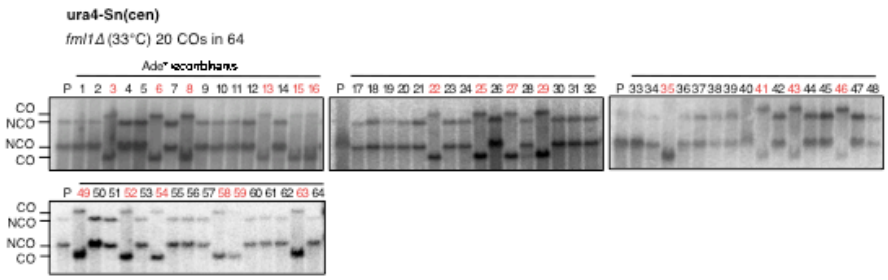


Figure 12. Mhf1-Mhf2 tetramers suppress crossovers in centromeres
(A) Recombination rates and the proportion of COs were determined using the cen1-Sn strain of wild type, *mhf1-LR, fml1Δ* grown at 33°C. (B) Recombination rates and the proportion of COs were determined using the ura4-Sn(cen) strain of wild type, *mhf1-LR,* and *fml1Δ* grown at 33°C. The ura4-Sn(cen) construct is illustrated, where cen1 sequence is introducing at ura4 locus of chromosome 3. (C) Recombination rates and the proportion of COs were determined using the cen1-Sn strain of wild type, *mhf1-GFP, mhf1-ΔC-GFP* grown at 30°C.(D) Blot data showing the physical detection of COs and NCOs of the above mentioned strains.

Mhf1 and Fml1 suppress the gross chromosomal rearrangements in centromeres

Mhf1 and Mhf2 have been implicated in the repair of DNA damage (Bhattacharjee et al., 2013, Sun, Nandi et al., 2008, Yan et al., 2010). To see whether the *mhf1-LR* mutation affects the growth and DNA repair in a similar manner, a serial dilution assay was performed using the medium supplemented with an alkylating agent methyl methanesulphonate (MMS), or a topoisomerase I inhibitor camptothecin (CPT) in wild-type, *mhf1*Δ, *mhf1-LR* and *fml1*Δ strains (Figure 13A, B). Interestingly, *mhf1*Δ

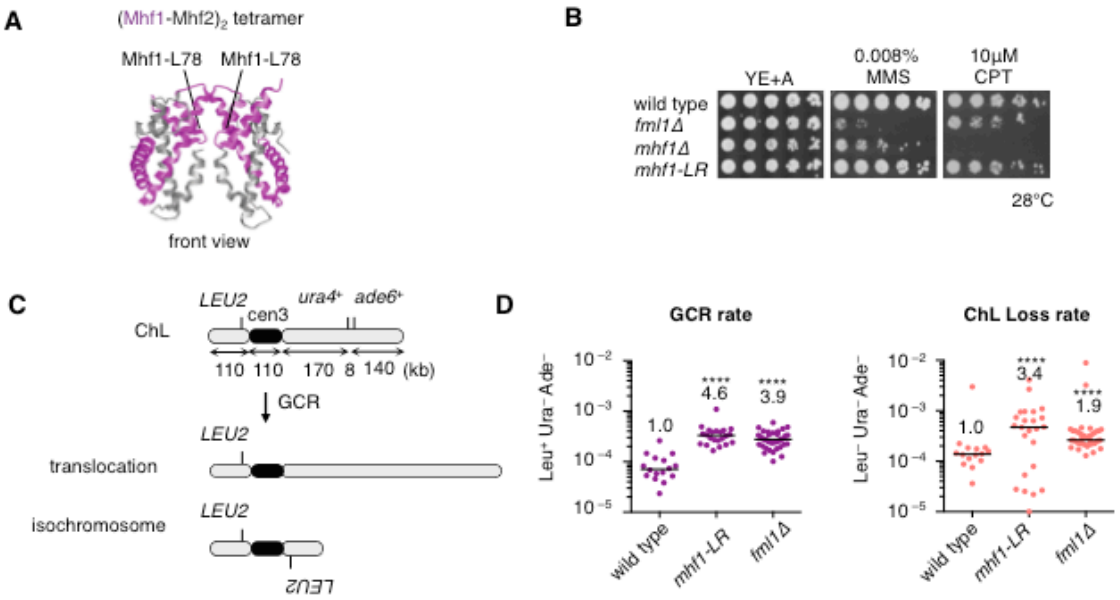
cells exhibited hypersensitivity to CPT than *fml1*Δ cells, whereas *fml1*Δ cells showed hypersensitivity to MMS than *mhf1*Δ cells (Sun et al., 2008), suggestive of non-overlapping as well as overlapping functions of Mhf1 and Fml1. Importantly, *mhf1-LR* cells were no more sensitive to MMS or CPT than wild-type cells, suggesting that the tetramer formation is not essential for DNA repair. Together, these results suggest that the formation of the tetramers that contain Mhf1 and Mhf2 is required for the crossover suppression in centromeres but has a limited role in DNA damage repair as compared to Mhf1.

Non-allelic recombination between inverted repeats in the centromere results in either gene conversion or gross chromosomal rearrangements (GCRs). Crossing over between centromere repeats causes the isochromosome formation in the *rad51*Δ mutant (Onaka, Toyofuku et al., 2016a). Because mutations in *mhf1*, *mhf2*, and *fml1* increased crossovers in centromeres, it is possible that Mhf1, Mhf2, and Fml1 are important to prevent GCRs in centromeres. To test this, the rate of spontaneous GCRs were determined using an extra-minichromosome ChL (Figure 13C), that is derived from chromosome 3 and contains a complete set of *cen3* and telomere repeats at their ends (Matsumoto, Fukui et al., 1987). Taking advantage of ChL the otherwise lethal GCR events in haploid cells can be detected. Colonies formed on YE3S were suspended in distilled water and plated onto YE plates, on which *ade6*$^{-}$ cells produce red colonies. Inspection of the red colonies using the minimum medium supplemented with amino acids identified the Leu^{+} Ade^{-} Ura^{-} clones that suffer GCRs and the Leu^{-} Ade^{-} clones that have lost ChL (Figure. 13D). To see a role of Mhf1, the *mhf1-LR* strain that contain ChL was constructed. *mhf1*Δ cells could not retain the extra-chromosome probably due to a defect in the kinetochore function (Amano et al., 2009a). By fluctuation test, I found that both *fml1*Δ and *mhf1-LR* mutation increase

the rate of spontaneous GCRs and ChL loss as compared to the wild type. These results suggest that Mhf1 and Fml1 play important role in maintaining the genome integrity by suppressing GCRs.

To characterize GCRs that occur in the *mfh1-LR* and *fml1*Δ mutants, chromosomal DNA was prepared from parental and independent GCR clones, separated by pulse field gel electrophoresis (PFGE), and stained with ethidium bromide (EtBr). Figure table 13E shows the summary of the GCR events in wild type, *mhf1-LR* and *fml1*Δ strains. In wild type, half of the GCR products were large (translocations) and the other half was small as compared to the parental ChL (Isochromosomes). In both *mhf1-LR* and *fml1*Δ strains the number of isochromosome formation was increased. PCR amplification of the GCR products recovered from the agarose gel as the template confirmed that the GCR breakpoints are present in the centromere repeats except translocations. GCR breakpoints were also found in the centromere repeats in *mhf1-LR* and *fml1*Δ strains. These data show that Mhf1 and Fml1 suppress GCRs between centromere repeats.

Figure 13



E

Strain	Total	Isochromosome	Translocation
wild type	16	8	8
mhf1-LR	16	11	5
fml1Δ	17	14	3

Figure 13. Mhf1 and Fml1 suppress gross chromosomal rearrangements (GCRs) in centromeres. (A) Ribbon diagram of the crystal structure of chicken $(Mhf1\text{-}Mhf2)_2$ tetramers. The position of the conserved leucine residue in α3 helix, which corresponds to the Mhf1-L78 of fission yeast is indicated. (B) CPT, and MMS sensitivities. Exponentially growing cells of wild type, *mhf1Δ*, and *mhf1-LR* strains were five-fold serially diluted with distilled water and spotted on YE+A plates supplemented with the indicated concentrations of CPT, and MMS. Plates were incubated for 3–5 days at 28°C. (C) GCR assay using the extra chromosome ChL. GCRs associated with loss of the right arm of ChL result in Leu^+ Ura^- Ade^-. The GCR product can be translocation, isochromosome, and truncate of different lengths. (D) Spontaneous GCR rates in wild type, *mhf1-LR,* and *fml1Δ* strains. ChL. loss result in Leu^- Ura^- Ade^-. Spontaneous loss rates in wild type, *mhf1-LR,* and *fml1Δ* strains are shown in scatter plot. Rates relative to wild-type values are indicated at the top of each column. *P*-values were determined by the two-tailed Mann-Whitney test. (E) Table showing the total, isochromosomes and translocations in wild type, *mhf1-LR,* and *fml1Δ* strains.

Discussion

Here, I found that DNA recombination in centromeres occurs differently from that in the arm region. In the arm region, both Rad51-dependent and Rad51-indepenent recombination can occur. In contrast, only Rad51-dependent HR occurs in the centromeres. Analyses of the recombinant DNAs showed that crossover between inverted repeats of centromere are infrequent, compared to the arm region. Although, pericentromeric heterochromatin did not prevent Rad51-independent HR and crossovers, mutations of Mhf1 and Mhf2 histone-fold proteins that localize to the kinetochore region of centromeres and Fml1 DNA helicase increased crossovers. The mutation on the interface of Mhf1-Mhf2 dimers, *mhf1-L78R*, increased the formation of crossovers and GCRs in centromeres. These data show that the centromere-specific regulation of DNA recombination, in part mediated by Mhf1-Mhf2 and Fml1, suppress gross chromosomal rearrangements in the centromere.

Centromere recombination occurs exclusively in a Rad51-dependent manner

Homologous recombination (HR) is an important mechanism implicated in the repair of DBSs and restart or repair stalled replication forks. HR occurs in either a Rad51-dependent or a Rad51-independent manner. As seen in budding yeast (Rattray & Symington, 1994), it was observed at the ura4 locus in fission yeast that *rad51*Δ and *rad54*Δ only partially decrease the rate of spontaneous recombination between inverted repeats as compared to *rad52*Δ, suggesting that recombination can happen through both Rad51-dependent and Rad51-independent manner in the arm region. In wild type, recombination occurs at comparable rates in the arm and the centromere

regions, showing that HR is not suppressed in centromeres during mitosis. However, in the centromere, *rad51*Δ and *rad54*Δ decrease the recombination rate to the same level as *rad52*Δ, indicating that Rad51-dependent recombination predominantly occurs in this region. Thus, the mechanism of HR is different between arm and centromere regions, and this was observed in both Sn- and Hp-intervals. The requirement of Rad51 and Rad54 was increased at Hp sites (10 kb) as compared to Sn sites (5 kb), probably due to the distance between the heteroalleles (Mott & Symington, 2011), which showed that extending the distance increased the dependence on Rad51. Recombination in centromere is therefore differently regulated from the arm region. The specific mechanism of recombination in centromere is regulated predominantly in a Rad51-dependent manner.

Rad51-independent recombination is specifically suppressed in centromere. Rad51-independent HR requires Rad52 that facilitates annealing of two complementary ssDNA independently of Rad51 and Rad54: the reaction called single-strand annealing (SSA). The SSA might be suppressed in the centromere due to dense kinetochore chromatin or lack of ssDNA, as very limited amount of RPA has been reported to bind centromeres of Xenopus egg extracts during replication (Aze, Sannino et al., 2016). SSA between tandem repeats of the centromere leads to deletion or loss of repeats resulting in inactivation of centromere in mammals (Stimpson, Song et al., 2010). Therefore, suppression of Rad52 mediated SSA in centromere repeats is important for maintaining the integrity of centromere repeats and function.

Crossovers are suppressed in the centromere

Homologous recombination is important for repairing DNA breaks and maintaining

genomic integrity. However, a crossover product of HR in and around the centromere interferes with chromosome segregation during cell division. Failure of this repression, can result in occasional meiotic missegregation in *S. pombe* (Hall, Noma et al., 2003). Repression of centromeric recombination in humans (Lynn et al., 2004), is important to prevent several genetic diseases including Down's syndrome, and miscarriages (Lamb, Freeman et al., 1996). Crossovers are generally suppressed in mitotic cells as compared to meiotic cells, because they give rise to gross chromosomal rearrangements (GCRs) or loss of heterozygosity (LOH). Crossovers between inverted repeats on the same chromatid result in inversions of the central sequence (Mizuno, Miyabe et al., 2013, Nakamura et al., 2008, VanHulle, Lemoine et al., 2007). In both Sn- and Hp-intervals, the crossovers (i.e. inversions) happen infrequently in the centromere than in the arm region, demonstrating the suppression of crossovers in centromeres. Meiotic crossovers are underrepresented around centromeres due to suppression of meiosis-specific DNA double-strand breaks in the vicinity of centromeres (Buhler, Borde et al., 2007, Ellermeier et al., 2010). Suppression of crossovers near centromeres seems important for the proper alignment of homologous chromosomes in meiotic prophase (Rockmill, Voelkel-Meiman et al., 2006). While both crossovers and non-crossovers are underrepresented in meiotic cells (Chen, Tsubouchi et al., 2008), the crossovers but not non-crossovers are specifically suppressed in mitotic cells. Rad51, Rad54, and Rad52 are involved in the promotion of non-crossovers as deletion of them severely decreased the net rate of non-crossovers among the residual recombinants in the centromere (Figure 2). In the arm region, however, *rad51*Δ, *rad54*Δ, and *rad52*Δ decreased the proportion of crossovers and the non-crossovers almost equally. This might be due to a partial contribution of Rad51-dependent HR that occurs in the arm region. Some centromere-

specific factor is also required to cause strong suppression of crossovers in centromeres.

Centromeres are composed of the kinetochore and its flanking heterochromatin regions. Heterochromatin plays an important role in suppressing excessive recombination events that would otherwise hinder centromere function during segregation. H3K9 methylation by Clr4 methyltransferase enables Swi6 to bind and stabilize cohesin to centromeric heterochromatin. An increase in the net rate of recombination observed in the *clr4*Δ mutant is probably due to a defect in sister chromatid cohesion as a similar increase was seen by the cohesin mutation. Cohesin is known to bind the sister chromatids together, which would facilitate HR between the sister chromatids. The increase in recombination in *clr4*Δ and *rad21-K1* would be due to loss of cohesion leading to recombination between the repeats of the same chromatid (Figure 14). Similarly, deletion of cohesin in the ura4 region also increased recombinant rate as cohesin is not a centromere specific factor and it localizes in the euchromatin at sites of DNA repair, transcription. Interestingly, deletion of *clr4*Δ also increased recombination rate in the arm region. There is a possibility that the deletion of Clr4 affects the methylation of histones in the repeats of the arm region. Nevertheless, it appears that heterochromatin is dispensable for the centromere-specific regulation of HR, as Clr4 histone H3K9 methyltransferase was not required for the suppression of crossovers in the centromere. These data suggest that the unique features of centromere recombination; suppression of Rad51-indepenedent HR and crossovers are brought about by an epigenetic system of the kinetochore chromatin. Surprisingly, CENP-A and its related factors were not found to be essential for the suppression of crossovers in centromere. One possibility is that the

residual activity of the temperature sensitive mutant proteins might be sufficient to suppress crossovers at the semi permissive temperatures. There is also a possibility that several factors take part in the regulation of centromere specific regulation in redundant mechanisms.

Mhf1-2/CENP-S-X suppresses centromere crossovers through the Fml1/FANCM helicase

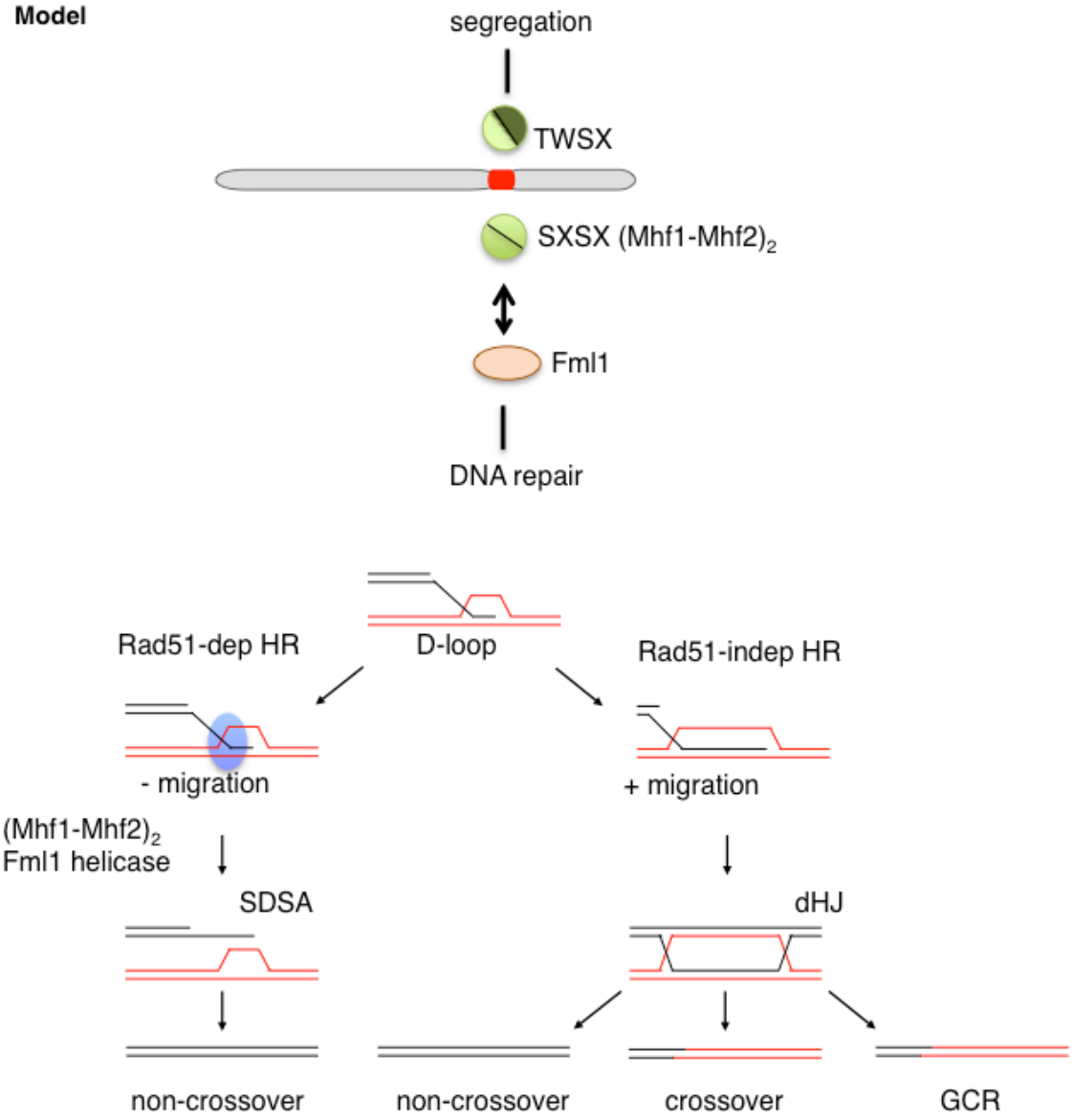
Mhf1 and Mhf2, members of the constitutive centromere-associated network (CCAN) together with Fml1 DNA have role in DNA repair. Mhf1 and Mhf2 in fission yeast, as in vertebrates, have been shown to serve a dual function, in recombination and promoting chromosome segregation (Bhattacharjee et al., 2013). Mhf1 and Mhf2 form hetero tetramers (MHF tetramer) and regulate Fml1 DNA helicase during DNA repair. They also form tetramer with CENP-T and CENP-W (CENP-TWSX) at centromeres that binds to Ndc80 and participate in segregation of chromosomes. At centromeres where Rad51-dependent HR occurs, MHF tetramers bind to cruciform DNA structures to which they reportedly show high affinity. They have been shown to stimulate DNA-binding activity of Fml1, a major meiotic anti-crossover factor, preventing MUS81-dependent crossover formation (Crismani, Girard et al., 2012). Mph1, budding yeast ortholog of Fml1 helicase has been shown to suppress crossovers by dissociating Rad51-made D-loops (Prakash, Satory et al., 2009). In this study, I found that both *mhf1* and *mhf2* increased crossovers in the centromeres. *fml1*Δ increased crossovers -like *mhf1*Δ and *mhf2*Δ, and did not further increase crossovers in *mhf1*Δ cells, suggesting that Mhf1-Mhf2 and Fml1 suppress crossovers in the same pathway. In contrast to *rad51*, *rad54,* and *rad52* mutations, *mhf1, mhf2,* and *fml1* increased the net rate of crossovers, demonstrating that Mhf1-Mhf2 and Fml1 do

suppress crossovers. The *mhf1-LR* was found to be mildly sensitive to high temperatures and genotoxins (MMS, HU, CPT) unlike mhf1*Δ*. *mhf1-LR* and Fml1 mutation increased COs in centromere significantly, and not in the non-centromeric *ura4* locus. Since, the Fisher exact test can clearly show statistical difference between the wild-type strains of centromere and non-centromeric locus, this test does hold value in the case of *mhf1-LR* and Fml1 also. However, such statistical analyses do have their limitations and increasing the sample numbers can affect the P-values. The possibility of CO suppression by these factors in the *ura4* locus cannot be excluded completely. It is worth noting that from the pie chart it is clear that both *mhf1-LR and fml1Δ* increased the percentage of COs in the non-centromeric *ura4* locus, suggesting that they definitely play a role in crossover suppression, but the effect of these factors do not reach to the level of significance. In other words, these factors have a more predominant role in the centromere and a minor role in the *ura4* locus. Collectively, these results suggest that the MHF tetramers are particularly required to suppress crossovers in the authentic centromeres. There is a possibility that at non-centromeric locus, Mhf1-2 dimer may be sufficient to interact with Fml1 for DNA damage repair as MHF1-2 dimers in human can interact with FANCM to stimulate its DNA binding activity (T.R.Singh et al 2010).

I propose a model showing how MHF tetramer and Fml1 suppress crossovers in centromere. In centromeres, CENP-TWSX may mainly function in segregation, however MHF tetramer together with Fml1 functions in DNA repair. In centromere, Rad51-dependent HR mediated by Rad51, Rad54 and Rad52 are initiated at DSB or stalled fork site to form D-loop structures. Unidentified centromere factors (such as protein DNA complex, or the dense chromatin) may inhibit branch migration of joint molecules. Once branched DNA is stabilized, $(Mhf1\text{-}Mhf2)_2$ (MHF tetramer) binds it

and recruit Fml1 helicase so as to disassemble joint molecules, resulting in synthesis-dependent strand annealing (SDSA) that generates only non-crossovers. However, Rad51-independent mechanism, branch migration extends heteroduplex, facilitating the formation of Holliday junctions, resolution of which results either in crossovers, non-crossovers or GCR (model). This mechanism of non-crossover formation by MHF tetramer complex suppresses GCRs as crossing over between inverted repeats of the centromere results in isochromosome formation (Onaka et al, 2016). The concerted action of Rad51, MHF tetramer and Fml1 may destabilize intermediates of homologous recombination that have a potential to form a crossover or GCR to maintain the integrity of centromeres

Figure 15



Model. In the centromere CENP-TWSX functions in segregation of chromosomes, and CENP-SXSX tetramer in concert with Fml1 functions in DNA repair. A DSB in the centromere is repaired by Rad51-dependent HR. Some unknown centromere factor (blue circle) may inhibit branch migration and $(Mhf1\text{-}Mhf2)_2$ with Fml1helicase forms NCO by SDSA pathway. On the other hand Rad51-independent pathway has extensive branch migration to form double Holliday Junctions that can be resolved into a CO, NCO or a GCR.

Materials and Methods

Yeast strains and Media

The yeast strains used in this study are listed in Table 1. To create deletion mutants, the target gene was replaced by the marker genes from pFA6a-kanMX6 (Bahler, Wu et al., 1998), hphMX6, or natMX6 (Van Driessche, Tafforeau et al., 2005). Yeast transformation was carried out using lithium acetate. The transformants were selected on yeast extracts (YE) medium supplemented with G418 (Nacalai Tesque), Hygromycin B (Nacalai Tesque), or Nourseothricin (Nacalai Tesque) at a final concentration of 50–100 μg/ml. Correct integration was confirmed by PCR. Cells were grown on YE or Edinburgh minimal medium (EMM) with appropriate amino acids at a final concentration of 225 μg/ml (Moreno, Klar et al., 1991). 5-fluoroorotic acid (5FOA; 1 mg/ml) (Apollo Science) and uracil (56 μg/ml) were added to Yeast Nitrogen Base (YNB) media containing 1.7 g/l yeast nitrogen base (Difco 233520, BD Biosciences), 5 g/l of ammonium sulphate, and 2% glucose. Solid medium contains 1.5% agarose (Nacalai Tesque). To generate the *mhf1-L78R* mutant strain, the *ura4*$^{+}$ gene was introduced at 303 bp upstream of the *mhf1*coding region after which the Ura^{+} cells were transformed with a 1.9 kb PCR fragment that contains the *mhf1-L78R* mutation and the *ura4*$^{+}$ integration site and were selected on 5FOA plates. Unless otherwise indicated, cells were grown at 33°C.

Recombination Assay

Fission Yeast strains containing *ade6B* and *ade6X* hetroalleles from -80 stock were streaked on YE+Ade plates and incubated at indicated temperatures for 3-5 days. Single colonies from these plates were then inoculated into EMM+Ade liquid media and incubated for 1–2 days. Exponentially growing cells of cultures were washed and

plated on EMM+Ade and EMM+Guanine plates (EMM supplemented with 50 µg/ml of guanine prevents growth of Ade^- cells) and incubated at appropriate temperatures for 3-6 days. The colonies were then counted and total number of viable colonies and total number of viable recombinants that is Ade^+ formation were determined. The rate of recombination was determined by means of fluctuation analysis using the method of medians (Lea & Coulson, 1949, Lin, Chang et al., 1996). To measure the recombination rate of ura4-Sn or ura4-Hp construct, all media were supplemented with uracil.

Crossover and Non-crossover determination assay

Genomic DNA preparation: To prepare yeast DNA, single colonies from EMM+Gua or EMM+Gua+Ura plates (Recombination assay) were inoculated into YE+LUA (YE supplemented with Leucine, Uracil, and Adenine) liquid cultures and incubated for 1-2 days at appropriate temperatures. $7x10^8$ cells were washed with ice-cold 25xTE (10mM Tris-HCL, 25mM EDTA, pH 8.0) and centrifuged at 3000rpm for 2min. The recovered cells were suspended in SP1 (20mM sodium citrate, 20mM di-sodium hydrogenphosphate, 40mM EDTA, pH 5.6) and incubated with 10µl β-Mercaptoethanol for 20min at 30°C. Cells were recovered by centrifugation at 6000rpm for 1 min. The cell pellet was then re-suspended in SP1 and treated with 3.5mg/ml Lyticase (Sigma L4025) for 20-30min at 37°C. The cell spheroplast was then recovered by centrifugation at 3000rpm for 1min and suspended in TE 50:20 (50mM Tris-HCL, 20mM EDTA, pH 8.0) and 10%SDS and incubated for 20min at 65°C. 300 µl of 5MKAc was added and allowed to stand for 10min at 4°C then centrifuged at 15000rpm for 5min. The supernatant containing the DNA was recovered by centrifugation and was precipitated by addition of 750 µl of isopropanol.

DNA was dried and suspended in TE10:1 and treated with 10mg/ml RNAase A.

Restriction Enzyme treatment: In a 100µl reaction, 20µl of DNA+ TE10:1 solution was treated with the enzyme (AfeI for cen1, and ura4-Sn(cen) strains and AfeI and SmaI for ura4 strains) at 37°C for 3 hrs. Phenol chloroform extraction was done to recover the DNA. DNA was dried and suspended in 15µl TE10:1 and 2µl Dye solution (50% glycerol, 0.01%XC, 0.01%BPB).

PFGE & Blotting: The DNA was separated in 0.6% agarose gel (Certified Molecular Biology Agarose, Bio-Rad) in 0.5xTBE buffer and run in CHEF-DRII system. Setting: 6V/cm, switching time 1->6sec, 11-15hrs. After the run, the gel was soaked in milliQ water containing 0.2µg/ml EtBr for an hour, picture of gel was taken by Typhoon FLA9000 (GE Healthcare), irradiated with 300mJ UV light. Gel was then washed with alkali buffer (1.2M NaCl, 0.4M NaOH buffer) for 40min, then soaked in 25mM Na-phosphate (pH6.5) buffer for 10min. DNA was transferred by capillary action to a nylon membrane (Nytran N, pore size 0.45µm, Whatman) for 2 days. The membrane was removed from capillary blotting and the damp membrane was irradiated with 150mJ UV for covalent attachment of DNA to the membrane.

Hybridization: 12µl labeling solution containing ~25ng template DNA was prepared using Random Primer DNA Labeling Kit Ver.2 (TaKaRa). A 2.8 kb HindIII-EcoRI fragment containing cnt1 prepared from pKT110, a 0.5 kb XbaI-HindIII fragment containing the *new25* downstream region from pTN446, and a 1.9 kb BamHI-PstI fragment containing a 1.9 kb DraI-DraI fragment containing the *ade6B* gene were used as DNA templates to prepare probes 1, 2 and 3, respectively. 2µl Random primer

containing 9-mer oligonucleotides was added to labeling solution and boiled at 95°C for 3min. and then cooled at room temperature. 2.5µl of 10X Buffer, 2.5µl of dNTP, 2.5µl of α^{32}P-dCTP (111TBq/mmol), 1.0µl (2U/ µl) of Exo-free Klenow fragment was sequentially added and incubated at 37°C for 15-20min. Gel filtration of the labeling solution was done using Illustra Autoseq G-50 dye Terminator Removal Kit (GE Healthcare). The extracted solution was then boiled at 95°C for 3min, cooled on ice and added to hybridizing tubes containing the membrane and hybridizing buffer. The tubes were rotated at 60°C overnight. Membrane was washed and the bands were detected with BAS2500 phosphoimager (Fuji film) and measured using Image Gauge software (Fuji film) or Typhoon FLA9000.

Chromatin immunoprecipitation (ChIP)

ChIP was performed essentially as described previously (Maki, Inoue et al., 2011). 1.7 $\times 10^8$ cells were fixed in 1% formaldehyde for 15 min at room temperature. After the addition of 2.5 M glycine to a final concentration of 125 mM, cell suspensions were incubated for additional 5 min. Cells were washed twice with cold water and then suspended in 500 µl of 0.1% lysis buffer (100 mM HEPES-KOH (pH7.4), 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% Na-deoxycholate). After centrifugation at 5,800 ×*g* for 1 min at 4°C using MX-201 (TOMY) at 4°C, the buffer was discarded and cells were stored cells at –80°C. Cells were suspended in 200 µl of 0.1% lysis buffer. Glass beads, 2 µl of proteinase inhibitor cocktail (Sigma-Aldrich), 4 µl of 1mM phenylmethylsulfonylfluoride (PMSF) were added to the tube. Cells were disrupted with glass beads in using Micro Smash MS-100 (TOMY). After addition of 200 µl of 0.1% lysis buffer and 10% Triton X-100 to a final concentration of 1%, the extracts were sonicated for 10 sec for four times at 4°C using Sonifier 250 (Branson).

The supernatant was obtained after centrifugation at 20,400 ×*g* for 10 min at 4°C. The extract was incubated at 4°C with rotation in 1% lysis buffer (100 mM HEPES-KOH (pH7.4), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate). Immunoprecipitation was carried out using anti-Cnp1 and anti-H3 rabbit antibodies (ab1791, Abcam) attached to the dynabeads M-280 sheep anti-Rabbit IgG (Invitrogen); anti-H3K9me2 mouse antibodies (Kimura, Hayashi-Takanaka et al., 2008) attached to dynabeads M-280 sheep anti-mouse IgG (Invitrogen). After extensive washing with 1% lysis buffer, 1% lysis buffer supplemented with 500 mM NaCl, Wash buffer (10 mM TrisHCl (ph8.0), 1 mM EDTA, 250 mM LiCl, 0.5% NP40, 0.5% Na-deoxycholate), and TE10:1 (10 mM TrisHCl (pH8.0), 1 mM EDTA), the beads were suspended in 100 µl of elution buffer (10 mM TrisHCl (pH8.0), 1 mM EDTA, 1% SDS) and incubated at 65°C overnight to disrupt crosslinks. After the treatment with protease K (Nacalai Tesque) at a final concentration of 0.3 mg/ml, DNA was purified by phenol/chloroform extraction. Rabbit antibodies were raised against Cnp1: NH_2- MAKKSLMAEPGDPIPRPRKKRC pedtides. The DNA prepared from whole cell extracts and immunoprecipitation fractions were quantified by real-time PCR using Power SYBR green PCR master mix (Applied Biosystems) in a StepOnePlus real-time PCR System (Applied Biosystems). Sequences of the primers used for real-time PCR are listed in table 2.

Spot Test

Fission yeast cen1 strains harboring the *ade6* heteroalleles were streaked on YE+Ade plates and incubated at 28°C for 3days. Single colonies were inoculated in liquid YE+ALU media. Exponentially growing cells were serially diluted ten fold with

distilled water and aliquots of 5µl were spotted on YE+Ade plates supplemented with indicated concentrations of MMS and CPT. Plates were incubated for 5days at 28°C.

GCR assay

Rates of spontaneous GCRs and chromosome loss were determined essentially as described before (Nakamura et al., 2008), with the some modifications. Yeast strains harboring the ChL were streaked on YE+LUA plates and incubated at 30°C for 3–4 days. Single colony was suspended in 1ml of distilled water and the concentration was determined. 400 cells were plated on 20 YE plates (total 8000 cells) and incubated at 30°C for 4–5 days. The total number of colonies and the Ade^- red colonies were counted. All the red colonies were patched on EMM+AU plates, and incubated 30°C for 2–3 days to inspect leucine prototrophs. Leu^+ Ade^- grown on EMM+AU plates were replica plated on EMM+A and EMM+U plates and incubated at 30°C for 2 days to confirm Ade^- and determine uracil prototrophs. Leu^- Ade^- indicative of ChL loss were obtained by subtracting Leu^+ Ade^- from Ade^-. Leu^+ Ura^- Ade^- indicative of GCRs were obtained by subtracting Leu^+ Ura^+ Ade^- from Leu^+ Ade^-. The rates per generation were determined by means of fluctuation test using the method of medians (Lea & Coulson, 1949, Lin et al., 1996). Leu^+ Ade^- Ura^- was then picked from the EMM+AU plate for preparing the chromosomal DNA in agarose plugs for PFGE.

Preparation of Plugs: Leu^+ Ade^- Ura^- clones were inoculated in YE+LUA media and incubated at 25°C for 1-2 days. $1.0x10^8$ cells were collected by centrifugation at 3000 rpm for 1 min and suspended in 2.5ml ice cold 50mM EDTA. Cells were then harvested by centrifugation at 3000rpm for 1 min and then suspended in 1ml CSE buffer (20 mM citrate phosphate, 50mM EDTA, 1 M sorbitol, pH5.6) and 5µl

Zymolyase 20T (Seikagaku) and 5µl Lyzing enzyme 25 mg/ml (Sigma) and incubated at 30°C for 20-50min. The spheroplast was harvested by centrifugation at 700rpm for 10min and suspended in 140 µl CSE buffer. Pre-melted 140µl of 1.6% low melting agarose was mixed to the cell pellet and the mixture was poured into the mold to make plugs. The plugs were then suspended in 1 ml of SDS-EDTA solution and incubated at 60°C for 2 hours. The buffer was then exchanged to 1ml of ESP buffer (0.5 M EDTA pH9.0, 1% N-lauryl sarcosine, 1.5 mMCaAc) supplemented with 1mg/ml proteinase K and incubated at 50°C for 24 hours. Lastly, the buffer was then exchanged to 1ml of ice cold TE10:1.

GCR products were analysed by PFGE, Southern hybridization, and PCR essentially as described previously (Nakamura et al., 2008).

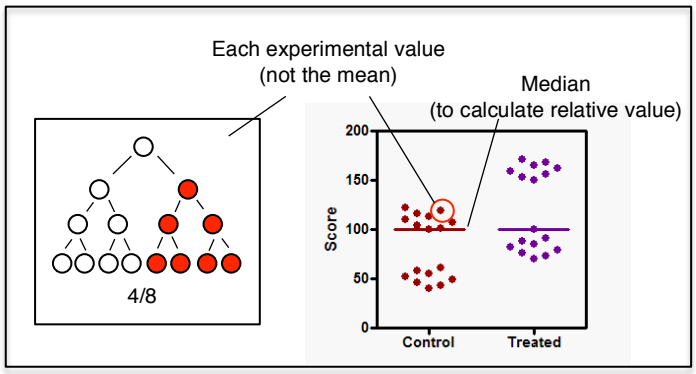
PFGE condition: Gel- 0.55% Certified Megabase agarose gel (Bio-Rad). Buffer- 1xTAE buffer at 10°C. Switching Time: 1st block: 2 V/cm, 45 hrs, 1,800 to 1,000 sec; 2nd block: 2 V/cm, 3 hrs, 70 sec.

Blotting: After PFGE, the gel was soaked in 800ml of milliQ containing 0.2mg/ml EtBr for 1hour and the picture was taken using Typhoon FLA9000 (GE Healthcare). The gel was then irradiated with UV at 300J. Gel was first soaked in 800ml of alkali buffer (1.2M NaCl, 0.4M NaOH) for 40min then in 400 ml of 25 mM Na-phosphate buffer (pH6.5) for 10 min. Nylon membrane (Nytran N, pore size 0.45 µm, Whatman) was used for capillary blotting.

Hybridization was done in essentially the same way as described in Crossover Non-crossover assay using specific probes, also described in (Nakamura et al., 2008).

Statistical analysis The Fisher's exact test and The Mann-Whitney test were performed using GraphPad Prism version 6.0g for Mac, (GraphPad Software, La Jolla CA. USA). The student T-test was performed using Excel (Microsoft).

Mann-Whitney test has been used to determine the differences between the recombination rates of two strains in this study. The experiment for this study was done using a fluctuation test. A single colony was inoculated in liquid culture and allowed to grow till saturation. Spontaneous recombination between heteroalleles occurs during the culture (4 recombinants shown as red circles out of 8). Similarly there were parallel cultures with different number of recombinants. The total number of colonies and the total number of recombinants was counted and the rates were determined. The scatter plot was drawn with each dot representing each experimental value. The line in the scatter represents the median, which is used to calculate the relative value between the Control and Treated. Mann-Whitney test was used to determine the difference between the recombination rate between the two groups (Control & Treated). This test was used because it compares the distribution of individual rates between the two groups. It should be noted that this test does not compare medians. For example, in the graph above, the medians showed by horizontal lines are at identical positions and still the P value shows statistic difference. It is because this test ranks all the individual rates from low to high and then compares them between the groups.



Chi-square and Fishers exact test are statistical analyses used to show relationship

between two groups, using a 2x2 (rows x columns) contingency tables. These tests are used when the outcome is a categorical variable (such as Yes, or No; (+), or (-)) and cannot be used to compare distributions between the two groups. These tests are similar and Chi square test is more accurate with large sample size while Fishers is used for small sample size. Fisher's test is based on assuming that the row and column totals are fixed by the experiment. For example in the crossover (CO) non-crossover (NCO) assay, the P values were determined by the Fishers test, where total number of recombinanats were fixed (for eg 30) and the number of CO (for eg 3) were counted and automatically the NCOs will be 27. Thus, Fishers exact test works with different assumptions and experimental design and does not fit to determine statistics of recombination rate between two groups.

Table 1. Fission yeast strains

strain	genotype	
TNF3347	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X)*	*Lab stock*
TNF3446	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), rad51::kanMX6*	*Lab stock*
TNF3452	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), rad54::kanMX6*	*Lab stock*
TNF3459	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), rad52::kanMX6*	*Lab stock*
TNF3631	*h+, ade6Δ-D, ura4::ade6B-cen1(Sn-Sn)-ade6X*	*Lab stock*
TNF3635	*h+, ade6Δ-D, ura4::ade6B-cen1(Sn-Sn)-ade6X, rad51::kanMX6*	*Lab stock*
TNF3645	*h+, ade6Δ-D, ura4::ade6B-cen1(Sn-Sn)-ade6X, rad54::kanMX6*	*Lab stock*
TNF3643	*h+, ade6Δ-D, ura4::ade6B-cen1(Sn-Sn)-ade6X, rad52::kanMX6*	*Lab stock*
TNF3144	*h+, ade6Δ-D, imr1L(Hp:ade6B), imr1R(Hp:ade6X)*	*Lab stock*
TNF3257	*h+, ade6Δ-D, imr1L(Hp:ade6B), imr1R(Hp:ade6X), rad51::kanMX6*	*Lab stock*
TNF3286	*h+, ade6Δ-D, imr1L(Hp:ade6B), imr1R(Hp:ade6X), rad54::kanMX6*	*Lab stock*
TNF3277	*h+, ade6Δ-D, imr1L(Hp:ade6B), imr1R(Hp:ade6X), rad52::kanMX6*	*Lab stock*
TNF3650	*h+, ade6Δ-D, ura4::ade6B-cen1(Hp-Hp)-ade6X*	*Lab stock*
TNF3664	*h+, ade6Δ-D, ura4::ade6B-cen1(Hp-Hp)-ade6X, rad51::kanMX6*	*Lab stock*
TNF3670	*h+, ade6Δ-D, ura4::ade6B-cen1(Hp-Hp)-ade6X, rad54::kanMX6*	*Lab stock*
TNF3667	*h+, ade6Δ-D, ura4::ade6B-cen1(Hp-Hp)-ade6X, rad52::kanMX6*	*Lab stock*
TNF3734	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), clr4::kanMX6*	*This study*
TNF3550	*h+, ade6Δ-D, imr1L(Hp:ade6B), imr1R(Hp:ade6X), clr4::kanMX6*	*This study*
TNF3256	*h+, ade6Δ-D, imr1L(Hi:ade6B), imr1R(Hi:ade6X)*	*Lab stock*
TNF3627	*h+, ade6Δ-D, imr1L(Hi:ade6B), imr1R(Hi:ade6X), clr4::kanMX6*	*This study*
TNF3710	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), swi6::hphMX6*	*This study*
TNF3699	*h+, ade6Δ-D, imr1L(Hp:ade6B), imr1R(Hp:ade6X), swi6::hphMX6*	*This study*
TNF3708	*h+, ade6Δ-D, imr1L(Hi:ade6B), imr1R(Hi:ade6X), swi6::hphMX6*	*This study*
TNF3739	*h+, ade6Δ-D, ura4::ade6B-cen1(Sn-Sn)-ade6X, rad21-K1*	*This study*
TNF3736	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), cnp1-76*	*This study*
TNF4668	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), cnp1-76, rad51::kanMx6*	*This study*
TNF4644	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), cnp1-76, clr4::kanMx6*	*This study*
TNF4656	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mis16-53*	*This study*
TNF4658	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mis18-818*	*This study*
TNF4657	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mis18-262*	*This study*
TNF4672	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mis18-262, rad51::kanMx6*	*This study*
TNF5376	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mis14-271*	*This study*
TNF4139	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), csm1::kanMX6*	*This study*
TNF4115	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), cnp3::kanMX6*	*This study*
TNF4754	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), msc1::kanMX6*	*This study*
TNF6282	*h+, ade6Δ-D, imr1L(Hp:ade6B), imr1R(Hp:ade6X), mis18-262*	*This study*
TNF3476	*h+, ade6Δ-D, imr1L(Hp:ade6B), imr1R(Hp:ade6X), scm3/sim1-106*	*This study*
TNF4779	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mhf1::hphMX6*	*This study*

TNF5082	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mhf2::hphMX6*	*This study*
TNF5353	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), fml1::hphMX6*	*This study*
TNF5128	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mhf1::hphMX6, fml1::kanMX6*	*This study*
TNF5666	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mhf1::hphMX6, rad51::kanMX6*	*This study*
TNF5698	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mhf2::hphMX6, rad51::kanMX6*	*This study*
TNF4740	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), fml1::hphMX6, rad51::kanMX6*	*This study*
TNF5444	*h-, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mhf1-L78R*	*This study*
TNF4684	*h+, ade6Δ-D, ura4+: cen1(imr1L(Sn:ade6B), imr1R(Sn:ade6X))*	*This study*
TNF5455	*h+, ade6Δ-D, ura4+: cen1(imr1L(Sn:ade6B), imr1R(Sn:ade6X)), mhf1-L78R*	*This study*
TNF4806	*h+, ade6Δ-D, ura4+: cen1(imr1L(Sn:ade6B), imr1R(Sn:ade6X)), fml1::hphMX6*	*This study*
TNF4976	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mhf1-GFP::natMX6*	*This study*
TNF5285	*h+, ade6Δ-D, imr1L(Sn:ade6B), imr1R(Sn:ade6X), mhf1-DC-GFP::natMX6*	*This study*
TNF3896	*h-, smt0, ade6Δ-D, ura4-D18, leu1-32, ChL (ubcp4::LEU2+::chk1, spcc1322::ura4, ade6+)*	*Lab stock*
TNF5477	*h-, smt0, ade6Δ-D, ura4-D18, leu1-32, ChL (ubcp4::LEU2+::chk1, spcc1322::ura4, ade6+), mhf1-L78R*	*This study*
TNF4813	*h-, smt0, ade6Δ-D, ura4-D18, leu1-32, ChL (ubcp4::LEU2+::chk1, spcc1322::ura4, ade6+), fml1::hphMX6*	*This study*
TNF3631	*h+, ade6Δ-D, ura4::ade6B-cen1(Sn-Sn)-ade6X, clr4::kanMX6*	*This study*
TNF3650	*h+, ade6Δ-D, ura4::ade6B-cen1(Hp-Hp)-ade6X, clr4::kanMX6*	*This study*
TNF3762	*h+, ade6Δ-D, ura4::ade6B-cen1(Sn-Sn)-ade6X, swi6::kanMX6*	*This study*
TNF3760	*h+, ade6Δ-D, ura4::ade6B-cen1(Hp-Hp)-ade6X, swi6::kanMX6*	*This study*
TNF3757	*h+, ade6Δ-D, ura4::ade6B-cen1(Sn-Sn)-ade6X, rad21-K1*	*This study*
TNF3759	*h+, ade6Δ-D, ura4::ade6B-cen1(Hp-Hp)-ade6X, rad21-K1*	*This study*

Table 2. List of PCR primers (LAB stock)

Primer	Sequence	Real-time PCR target
ade6-D-D-F	5' -GCTCGTACCGCAGCTTCAAG	ade6
ade6-D-D-R	5' -GCAACCATACCAGGCAAATGA	ade6
imr1-in-F	5' -ATTTCCGCTTACAAAATGCCA	imr-in
imr1-in-R	5' -TTTCTCAACAGCAAAGCCTGAA	imr-in
cnt1/3-F	5' -CAACCACTGAAAGCGAATCTGTA	cnt1
cnt1/3-R	5' -ATTCTGTAAGTTTGCTGTGCTTTATATCA	cnt1
adl1-F	5' -AAATATGGCGATCCAGGAGATG	adl1
adl1-R	5' -GCTTAACGTGCGCACAGACA	adl1
dg-F	5' -TTGCACTCGGTTTCAGCTAT	dg
dg-R	5' -TGCTCTGACTTGGCTTGTCT	dg

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