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Smc5-Smc6 complex suppresses gross chromosomal rearrangements mediated by break-induced replications

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Abstract

Translocations in chromosomes alter genetic information. Although the frequent translocations observed in many tumors suggest the altered genetic information by translocation could promote tumorigenesis, the mechanisms for how translocations are suppressed and produced are poorly understood. The *smc6-9* mutation increased the translocation class gross chromosomal rearrangement (GCR). Translocations produced in the *smc6-9* strain are unique because they are non-reciprocal and dependent on break-induced replication (BIR) and independent of non-homologous end joining. The high incidence of translocations near repetitive sequences such as δ sequences, ARS, tRNA genes, and telomeres in the *smc6-9* strain indicates that Smc5-Smc6 suppresses translocations by reducing DNA damage at repetitive sequences. Synergistic enhancements of translocations in strains defective in DNA damage checkpoints by the *smc6-9* mutation without affecting *de novo* telomere addition class GCR suggest that Smc5-Smc6 defines a new pathway to suppress GCR formation.

Keywords

Smc5-6; Gross chromosomal rearrangement; Break-induced replication; checkpoints; translocations

1. Introduction

Cancer is a genetic disorder requiring multiple mutations across the entire genome. Widespread genomic mutations can be facilitated by a mutation that affects key processes related to DNA metabolisms such as DNA replication, repair, and recombination. Such mutations are sometimes referred to as “mutator mutations” to reflect their ability to enhance mutation rates [1,2]. One of the most common alterations in the genomes of cancer cells is the alteration of

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normal chromosome size, often referred to as gross chromosomal rearrangements (GCRs). GCR arises through defects in the repair of DNA damage caused either by internal sources, such as defects in DNA replication and telomere erosion, or by exposure to exogenous toxic agents [1]. Recent progress in identification of genes responsible for cancer susceptible syndromes and studies of animal models has begun to reveal the importance of GCR during carcinogenesis [1,3].

To study GCR in more tractable way, the chromosome V GCR assay that can detect interstitial deletions or non-reciprocal translocations with micro-homology, non-homology, or divergent homology (referred as homeology) at the rearrangement breakpoint, chromosome fusions as well as deletion of a chromosome arm with addition of a new telomere referred to *de novo* telomere addition was developed in *Saccharomyces cerevisiae* [4-6]. Studies using this GCR assay have begun to elucidate mechanisms underlying GCR formation [1,5]. Several studies using this approach have demonstrated that there are eight pathways for suppressing these chromosomal aberrations, while six pathways promote GCR formation. The suppression mechanisms include cell cycle checkpoints [7-12], post-replication [13,14] and mismatch repair [15,16], recombination pathways, an anti-*de novo* telomere addition mechanism [17, 18], chromatin assembly factors [11,19], mechanisms that prevent end-to-end chromosome fusions [17,18,20] and a pathway detoxifying reactive oxygen species [14,21,22]. In contrast, the promoters of GCRs include telomerase-related factors [17,23], a mitotic checkpoint network [24], the Rad1-Rad10 endonuclease [25], non-homologous end-joining proteins including Lig4 and Nej1 [17], a pathway generating inappropriate recombination via sumoylation and the Srs2 helicase [13] and the Bre1 ubiquitin ligase [13].

Eukaryotic cells have three protein complexes containing chromosomal ATPases of the Structural Maintenance of Chromosomes (SMC) family that function in various aspects of chromosome metabolism [26-29]. SMC complexes are characterized by the presence of a heterodimer of Smc proteins at the core with additional non-Smc subunits. The Smc complexes include cohesin, with the Smc1-Smc3 dimer, to provide sister-chromatid cohesion, condensin, with the Smc2-Smc4 dimer, important to mediate mitotic chromosome condensation, and the Smc5-Smc6 complex, with roles in DNA repair [27,30]. Although the main role of the Smc5-Smc6 complex is still unclear, recent studies have shown that this complex is recruited to the induced DNA double-strand-breaks (DSBs) [31,32] to enhance their repair by sister-chromatid recombination [31,32]. Smc5-6 heterodimer makes a multi-protein complex with six additional subunits in budding yeast (Nse1-6) [28]. Nse2 also known as Mms21 is an E3 SUMO ligase and several studies suggested that Mms21 links Smc5-6 to DNA repair role through sumoylation [33,34].

Temperature-sensitive mutants of Smc6 have been shown to affect the stability of repetitive sequences in budding yeast by influencing their repair [35,36]. These observations have been recently extended to human cells, where the Smc5-Smc6 complex has been shown to play a role in alternative lengthening of telomeres ALT pathways, which are mediated by homologous recombination events [37].

Recently, we reported that a conditional allele of *SMC6* in budding yeast, *smc6-9* increased GCR rate in a HR dependent manner [31]. In the present study, we further examined how GCR formation by the *smc6-9* mutation interacts with other known GCR pathways. Smc5-Smc6 complex suppresses translocation class GCRs dependent on break-induced replication (BIR) that is different from other non-homologous end joining dependent translocations observed in other GCR mutator strains. Furthermore, GCR rates in the *smc6-9* strain were synergistically increased with mutations causing defects in cell cycle checkpoints and telomerase inhibition suggesting that the Smc5-Smc6 complex defines a novel pathway for suppression of translocation class GCRs. Interestingly, some of the GCR events in the *smc6-9* strain occurred

in close proximity to repetitive sequences, consistent with the known role of Smc5-Smc6 in the stability of repetitive genomic region.

2. Materials and Methods

2.1. General genetic methods

Methods for the construction and propagation of gene-disrupted strains were described previously [10,14]. The sequences of primers used to generate gene-knockout cassettes and to confirm correct disruption are available upon request. All *S. cerevisiae* strains used in this study were derived from the S288c strain RDKY3615 [*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, hxt13::URA3*]. Genotypes of each strain used for this study are listed in Table 1.

2.2. Characterization of spontaneous GCR rates and chromosomal breakpoints

All GCR rates were determined by fluctuation analysis using the method of the median with at least two independent clones [5,38]. The average GCR rates from at least two or more independent experiments using either 5 or 11 cultures for each strain are reported as previously described [10,14]. The sequences of breakpoints from mutants carrying GCR were determined as described [10,14].

2.3. Pulse Field Gel Electrophoresis

Genomic DNA samples were prepared in low melting agarose plugs containing 5×10^7 cells as described [39]. The plugs were inserted in a 1% agarose-TBE gel and chromosomes were separated by pulse field gel electrophoresis (PFGE) CHEF II DR system (BioRad). Electrophoresis was performed for 30 hours at 180V with 120 seconds pulses in 0.5× TBE at 14 °C. The gel was then stained for 30 minutes with 0.5μg/ml ethidium bromide and visualized using a UV illuminator.

2.4. Break-induced replication (BIR) assay

BIR assay was performed following exactly same procedures as previously described [40]. Briefly, exponentially growing yeast cells in yeast-peptone (YP) with 2% succinic acid and 1% glycerol were plated on YP with 2% glucose or YP with 2% galactose and incubated for two days until visible colonies were grown. After counting numbers of colonies, yeast cells were replica plated on plates having canavanine or hygromycin. The percentage of cells repairing by BIR was calculated by the number of colonies sensitive to canavanie and hygromycine on YP-galactose plate divided by the number of colonies resistant to canavanine and hygromycine on YP-glucose plate.

3. Results

3.1. Translocations by break-induced replications (BIR) are preferentially generated by the *smc6-9* mutation

The impaired chromosome segregation of repetitive DNA sequences such as rDNA or telomeres was observed by defects in the Smc5-Smc6 complex [35,41]. Some of these defects due to incorrect repair during the G2/M phases likely cause an increase in spontaneous double strand breaks (DSBs) [36]. To further gain an insight into the mechanisms of GCR formation when the Smc5-Smc6 complex is impaired, we measured GCR rates in strains defective in different subunits of Smc5-6 complex. The *smc6-9, nse3-2,* or *mms21-11* mutation increased the GCR rate 76, 54, or 80 fold compared to wild type, respectively (Table 2). The strain having both *smc6-9* and *mms21-11* mutations increased the GCR rate similar to strains with each mutation (Table 2 footnote).

Breakpoint junctions often leave signatures of GCRs that suggest putative mechanism of GCR formation. We determined breakpoint junction structures of GCRs from fifteen independent clones came from the *smc6-9* strain (Table 3 and Fig. 1). The *smc6-9* mutation mainly generated translocations having micro-homology signatures at the breakpoint junction (67% of total break point junction analyzed) compared to wild-type cells, which preferentially produced terminal deletion with *de novo* telomere addition class GCR in the chromosome V GCR assay used in this study. To determine the nature of translocations generated by the *smc6-9* mutation, we further investigated these rearrangements by PFGE (Fig. 1). Yeast chromosomes VIII and V are very similar in size and difficult to resolve by PFGE making the visualization of chromosome V loss, where the GCR assay resides problematic. Nevertheless, chromosomes from the clones carrying a GCR collected from the *smc6-9* strain exhibited the appearance of a new sized chromosome except in one case, (third mutant clone in Fig. 1A) which had two new sized chromosomes (Fig. 1A).

The chromosome translocated to chromosome V in each case was determined by linker-mediated PCR as previously described [14]. The sizes of the new chromosomes observed by PFGE matched the expected size obtained by adding the size of the broken chromosome V and the size of the translocated chromosome from the breakpoint to its end (Fig. 1B). In addition, even though there was a change in the size of chromosome V, it did not affect the electrophoretic mobility of the donor chromosomes suggesting all translocations were non-reciprocal translocations. The appearance of a new sized chromosome can only be explained through break-induced replication (BIR), where a DSB is repaired by undergoing recombination-dependent DNA replication with the re-establisment of a unidirectional replication fork that proceeds to the end of the chromosome or until it meets a converging fork [42]. Therefore, most major translocations in the *smc6-9* strain seem to be produced by BIR.

3.2. Translocations produced in the *smc6-9* strain are dependent on homologous recombination (HR) and independent of non-homologous end joining (NHEJ)

The *rad52, rfa1-t33,* or *mre11* mutation also enhanced translocation types of GCRs. However, translocations in these mutator strains are largely dependent on the non-homologous end joining (NHEJ) proteins, Lig4-Nej1-yKu70-yKu80 [17]. In contrast, translocations observed in the *smc6-9* strain exhibited different genetic interactions (Table 3 and 4). The *smc6-9 lig4* strain showed more than three-fold increase in GCR rate compared to the *smc6-9* strain (Table 4). When we investigated the class of GCR in the *smc6-9 lig4* strain, the rates of translocations carrying both non- and micro-homology at the breakpoint junction were enhanced (Table 3), thus confirming that the *lig4* mutation did not suppress any types of GCR formation in the *smc6-9* strain. We also investigated the effect of *yku70* mutation on the GCR rate and class in the *smc6-9* strain. Deletion of *yku70* has been shown to inhibit *de novo* telomere addition as well as NHEJ-dependent translocations [17]. Similar to the *lig4* mutation, the *yku70* mutation in the *smc6-9* strain did not change substantially either the rate or the type of GCR events (Table 3 and 4). These results again confirm that translocations in the *smc6-9* strain do not require the NHEJ machinery.

BIR events are dependent on HR [42]. Since the GCRs observed in the *smc6-9* strain are suspected to be BIR-induced translocations, they sould be dependent on the HR machinery. In budding yeast, there are two BIR pathways, both dependent on the recombination protein Rad52, but one of the pathways is Rad51-dependent whereas the other is Rad51-independent but requiring other recombination genes (besides Rad52) such as Rad59 [42]. To investigate whether translocations generated by the *smc6-9* mutation are dependent on BIR, we tested whether GCR events in *smc6-9* mutants indeed require Rad52. The GCR formation rates enhanced by the *smc6-9* mutation was reduced five-folds by the *rad52* mutation (Table 4). In addition, the micro-homology mediated translocations that are the major classes of GCRs found

in the *smc6-9* strain were almost absent in the *smc6-9 rad52* strain (Table 3). Similarly, the *rad51* mutation also suppressed GCR formation in the *smc6-9* mutant background (Table 4). These results confirm that Rad51-dependent BIR is responsible for translocations produced in the *smc6-9* strain. In addition, the mutation of the recombination gene *RAD59* in *smc6-9* mutants did not suppress GCR rates but on the contrary it showed an additive effect (Table 3), consistent with the fact that the Rad51-dependent BIR pathway promoted GCRs in the *smc6-9* strain.

To determine whether the *smc6-9* mutation affects the BIR efficiency, the BIR efficiency was measured by using the recently developed BIR assay [40]. The *smc6-9* mutation did not show any significant difference in the BIR efficiency (Fig. 2). In addition, the additional *pol32* mutation that abolishes almost all BIR [40] increased the GCR rate synergistically in the *smc6-9* strain (Table 4). We hypothesized that the *smc6-9* mutation would increase the appearance of DNA lesions that could become substrates for BIR-dependent GCR formation. In the absence of BIR, the same DNA lesions could become substrates for other pathway that will produce GCRs having different breakpoint junction signatures. To test this hypothesis, we determined the breakpoint structures of GCRs produced in the *smc6-9 pol32* strain (Table 3). Consistent to our hypothesis, translocations having micro-homology at breakpoint junctions preferentially produced in the *smc6-9* strain were not observed in the *smc6-9 pol32* strain (Table 3). There were high increases of *de novo* telomere addition and translocations having non-homology at breakpoint junctions in GCRs from the *smc6-9 pol32* strain. Therefore, the *smc6-9* mutation seems to increase the appearance of DNA lesions at repetitive DNA sequences and BIR preferentially changes them to translocations with micro-homology signature at breakpoints.

The Rad52 protein is sumoylated largely by Siz2 [43] and PCNA sumoylation by Siz1 is important to promote GCR [13]. We asked whether GCRs enhanced by the *smc6-9* mutation could be affected by the *siz1* or *siz2* mutation (Table 4). The *siz1* or *siz2* mutation synergistically increased GCR rates in the *smc6-9* strain suggesting the Rad52 regulation by sumoylation could be important for suppression of GCR formation.

3.3. Inactivation of DNA damage checkpoints in the *smc6-9* strain synergistically enhances GCR formation

The inactivation of the Smc5-Smc6 complex does not activate the DNA damage checkpoint [41]. Nevertheless, we questioned whether lack of checkpoint activity in *smc6-9* mutants affects GCR formation. To test this question, different cell cycle checkpoint genes were disrupted in the *smc6-9* strain and GCR rates were monitored. The *smc6-9* strains carrying an additional mutation in sensors of the *RAD24* branch DNA damage checkpoint such as *rad24*, *rad17*, or *ddc1* [44], all increased the GCR rates synergistically compared to strains carrying each mutation (Table 5). An additional *smc6-9* mutation in the *rfc5-1* strain, which is defective in the DNA replication checkpoint [45,46] showed GCR formation rate comparable to the one caused by the *rfc5-1* single mutation (Table 5). The replication defects of *rfc5-1* could be suppressed by multicopy PCNA expression [47]. We hypothesized that the epistatic interaction between *rfc5-1* and *smc6-9* could be due to their link to PCNA ubiquitination. To test this hypothesis, we measured the GCR rate of the *rad5 smc6-9* strain. Consistent with our hypothesis, the GCR rate of *rad5 smc6-9* was comparable to strains having each mutation (Table 5). Lastly, an additional mutation of *elg1*, which activates DNA damage checkpoint and enhances GCR [7,48], in the *smc6-9* strain, synergistically increased the GCR rate (Table 5).

In *Saccharomyces cerevisiae*, Mec1 and Tel1, the yeast orthologues of ATR and ATM in higher eukaryotes, respectively, phosphorylate target proteins, including the Chk1 and Rad53 kinases, on Ser/Thr-Gln (S/T-Q) motifs and regulate several aspects of the cellular response to DNA damage and stalled replication forks [49,50]. Several studies in yeast have shown that the ends

of DSBs are subjected to nucleolytic degradation to generate 3′-ended single-stranded DNA that recruits Mec1 and activates DNA damage checkpoint. The mutation of *MEC1* or its downstream kinase, *RAD53* in the *smc6-9* strain synergistically increased the GCR formation rates (Table 5). We need to point out that both strains having *mec1* or *rad53* mutations had the suppressor of lethality mutation, *sml1*. We obtained similar synergistic increases of GCR rates when downstream targets of Mec1 such as Rad9 and Dun1 were mutated (Table 5). In contrast, the mutation in *CHK1*, which encodes another downstream kinase of Mec1 [51], did not show any synergistic interaction with the *smc6-9* mutation (Table 5). Therefore, GCR formation in the *smc6-9* strain is mainly suppressed by the Rad24-Mec1-Rad53/Rad9-Dun1 checkpoint. The inactivation of Pds1 that is a direct downstream target of Chk1, in the *smc6-9* strain synergistically increased the GCR rate (Table 5). Because Rad53 also regulates Pds1 stability, the synergistic interaction of the *pds1* mutation with *smc6-9* could possibly be due to a role of Pds1 independent from the Chk1 regulation [52].

Another DNA damage checkpoint suppressing GCR formation is the Tel1-dependent checkpoint [53]. Like Mec1, the mutation of *TEL1* in the *smc6-9* strain also induced GCR rate considerably (Table 5). The Mre11 complex is required for the enrichment of the Mec1 kinase to DSB sites and the recruitment of Tel1 kinase and Mec1 interacting protein, Ddc2 to DSB sites [54-56]. Consistently, the *mre11* mutation in the *smc6-9* strain caused a synergistic increase in GCR rates similarly to the *mec1* or *tel1* mutation (Table 5). The synergistic effect between the Mec1- and Tel1-dependent branch of the DNA damage checkpoint and the *smc6-9* mutation suggests that aberrant HR would initiate more GCRs in the *smc6-9* strain if the DNA damage checkpoints were nonfunctional.

The inactivation of Mms21, a protein in the Smc5-Smc6 complex results in the accumulation of X molecules [33] that resemble pseudo-double Holliday junctions (dHJs) or hemicatenane-like molecules, and they occur specifically when forks encounter a damaged template [57]. Cells lacking the helicase Sgs1 or Topoisomerase III also accumulate these pseudo-dHJs. Sgs1 and Top3 mutants suffer from GCR [15]. Deletion of either *SGS1* or *TOP3* in the *smc6-9* mutant synergistically increased the GCR rate (Table 5), raising the possibility that such hemicatenane-like molecules might generate breaks or be substrates for the generation of GCR.

The inactivation of the Mec1 kinase preferentially increases *de novo* telomere addition class GCRs [10,58]. In contrast, the *smc6-9* mutation generated preferentially BIR-dependent translocation class GCRs (Table 3). To investigate whether the *smc6-9* mutation specifically enhances translocation class GCRs even in strains defective in cell cycle checkpoints, we analyzed the breakpoint junction structures of GCRs from the *smc6-9 mec1* strain. In contrast to 100% *de novo* telomere addition class GCR observed in the *mec1* strain, the *smc6-9 mec1* strain produced *de novo* telomere addition and translocations with micro-homology at breakpoints, 44% and 56%, respectively (Table 3). When the GCR rate of *smc6-9 mec1* is divided by different GCR structures, the *de novo* telomere addition rate of *smc6-9 mec1* (5.3×10^{-8}) was not significantly different from that of *mec1* (4.6×10^{-8}). Therefore, the major enhancement of GCR in *smc6-9 mec1* was due to the increase in the rate of translocation with micro-homology at breakpoints as observed in 6.7×10^{-8} of the *smc6-9 mec1* strain compared to no translocation in the *mec1* strain. Similarly, the *tel1* mutation synergistically increased translocation class GCRs in the *smc6-9* strain (Table 3). It should be pointed out that less homology at the breakpoint junctions seems to be required in the *smc6-9 tel1* strain because there was a large increase of non-homology mediated translocations in the *smc6-9 tel1* strain compared to the *smc6-9* strain. These results are consistent with observations that the Smc5-Smc6 complex specifically suppresses BIR-mediated translocations.

3.4. Defects in telomere maintenance enhance GCR formation synergistically in the *smc6-9* strain

The Smc5-Smc6 complex binds to telomeric regions [35] and it is important to regulate the maintenance of telomeres [37,59]. We have shown that despite *de novo* telomere addition being the preferred choice of GCR in yeast chromosome V GCR assay [10,17], most GCRs in the *smc6-9* mutant occur through BIR-mediated translocations (Table 3). One possibility is that the Smc5-Smc6 complex plays a direct role in the *de novo* telomere GCR pathway. The *PIF1* gene encodes a transcript that translates into two helicases by alternative initiations [60]. In contrast to the unique function to mitochondria by one translated Pif1, the nuclear Pif1 is a telomerase inhibitor that blocks the recruitment of telomerase to the telomere [61]. The *pif1-m2* mutation specifically inactivates nuclear Pif1 and increases *de novo* telomere addition class GCRs [17].

The GCR rate of the *smc6-9 pif1-m2* strain showed a synergistic enhancement compared to strains having each mutation (Table 6). A mutation in a non-Smc subunit of the Smc5-Smc6 complex, *nse3-2* showed a similar effect (Table 6, footnote). Next we characterized the nature of the GCR events in these strains. We found that while chromosomal translocations were still present in the *smc6-9 pif1-m2* strain, a high increase in *de novo* telomere addition class GCRs was also observed (Table 3). Conversely, when the *de novo* telomere addition pathway was inactivated in the *smc6-9* strain by deletion of *TLC1* or *EST2*, which encode the RNA subunit and catalytic subunit of telomerase, respectively [62], the translocation class GCRs were synergistically increased (Table 3 and 6). These translocations showed both non-homology and micro-homology at the breakpoint junctions. We also detected chromosome fusion events likely mediated by NHEJ. Interestingly we found one case of *de novo* telomere addition GCR in the *smc6-9 tlc1* mutant. It is not statistically significant (p=0.27) and was most likely generated by a BIR event where the broken chromosome invaded very close to the telomere sequence of another chromosome. Even though there was strong genetic interaction between the *smc6-9* mutation and mutations in telomere maintenance gene, the *smc6-9* mutation did not affect telomere length (Supplement Fig. 1).

Our results on the genetic interactions of *smc6-9* and mutations in different telomere maintenance genes demonstrate that the pathways for *de novo* telomere addition are functional in *smc6-9* cells, and confirm that the BIR-dependent translocations (Fig. 1) are likely due to a defect of a specific role of the Smc5-Smc6 complex. Therefore, the Smc5-Smc6 complex defines a new pathway for the suppression of GCRs mediated by BIR.

4. Discussion

Defects in Smc5-Smc6 cause delayed DNA replication of repetitive sequences, such as rDNA and lead to mitosis before the completion of replication in these regions [41]. It would cause an increase in DNA DSBs during mitosis [35], which could provide potential substrates for GCR. Indeed, we have found that the *smc6-9* strain preferentially enhanced translocations type GCR (Table 2 and 3). Translocations observed in the *smc6-9* strain have unique features. All translocations seem to be produced by Rad51-Rad52 dependent BIR (Table 4 and Fig. 1) and are independent of NHEJ (Table 4). In addition, translocations seem to be generated close to repetitive sequences including δ sequences, autonomously replication sequences (ARS), tRNA genes and other sequences that are repeated many times in genome (Fig. 1B). The δ sequences are Ty recombination hot spot and were found as a putative hot spot for DSB induced non-reciprocal translocations [63,64]. ARS are used as a DNA replication origin and the deletion of ARS sequence in meiotic recombination showed the reduction of both gene conversions and reciprocal crossovers in the hotspot region [65]. In additioin, these sequences could cause pausing of DNA replication. Therefore, higher instability of these repetitive sequences in the *smc6-9* strain could result in translocations.

Because the Smc5-Smc6 complex has many roles during DNA replication and chromosome segregation [35,41], it is difficult to ascertain which defects by the *smc6-9* mutation cause DNA damage to translocations. For example, the cohesion defect impairs sister chromatid recombination [31,66], increasing intra-chromatid recombination in the tandem array of ribosomal repeats [36]. Recent work even suggested that Smc5-Smc6 would be required for the efficient loading of cohesin to DSB sites [67,68]. Smc5-Smc6 could restrain the initiation of strand invasions between different chromosomes that lead to BIR-mediated translocation events. Furthermore, the fact that Smc5-Smc6 interacting proteins possess enzymatic activity, such as the E3 SUMO ligase Mms21 [59] raises the possibility that DSBs are generated by defects in the regulation of Mms21 during DNA repair and replication. To support this, the *mms21-11* mutation increased the GCR rate similar to *smc6-9* (Table 2). Further analysis of Smc5-Smc6 protein functions is necessary to reveal a solid mechanism for how DNA damage is generated to produce translocations.

Many mutations affecting GCR rates preferentially increased *de novo* telomere addition in the chromosome V GCR assay [1,5]. In contrast, the *smc6-9* mutation preferentially enhanced translocation (Table 3). Furthermore, the translocations in the *smc6-9* strain depend on the HR machinery proteins, Rad51 and Rad52 (Table 4) that also block telomerase access to DSBs [69]. The *smc6-9* mutation affects certain types of HR during the G2/M phase. It is possible that the *smc6-9* mutation allows Rad51 and/or Rad52 to associate or bind with higher affinity to DSBs at the G2/M phase thus blocking telomerase access. Prolonged Rad51 association with DSB would eventually initiate BIR with other chromosomes to produce a translocation event. Intriguingly, the Rad52 protein and its *S. pombe* homolog Rad22 protein are sumoylated [43,70]. Although it is still unclear whether the sumoylation of Rad52 affects HR efficiency, it could at least affect the kinetics of the removal of Rad51 from DSB. Consistent with this, the *siz1* or *siz2* mutation that would affect Rad52 sumoylation synergistically increased GCR rate in the *smc6-9* strain (Table 4). The defect in the Smc5-Smc6 complex could result in a longer association of Rad51 with the DSB and allow initiation of BIR.

There are three different cell cycle checkpoints redundantly suppressing GCR formation. Even though a mutation of the DNA replication checkpoint by *rfc5-1* was epistatic with the *smc6-9* mutation in GCR rates (Table 5), the GCRs produced by these two mutations were different. In contrast to *de novo* telomere addition class GCRs by the *rfc5-1* mutation [10], the *smc6-9* mutation preferentially produced translocations (Table 3). Therefore, there is a complex genetic interaction between *rfc5-1* and *smc6-9* that is currently not clearly understood. Genetic interactions regarding GCR suppression between defects in DNA damage checkpoint and the *smc6-9* mutation imply that both DNA damage checkpoints, Rad24-Mec1-Rad53/Rad9-Dun1 and Mre11-Tel1 pathways function to suppress GCRs from DNA damage caused in the *smc6-9* strain.

The helicase Sgs1 and its binding partner Top3 are known to be crucial in the maintenance of genomic stability. In addition to the loss of cell cycle checkpoint function [71], the loss of Sgs1 or Top3 increases sister chromatid exchange rate and HR [72]. Similar to the *mms21-11* mutation, the *sgs1* mutation accumulates pseudo-dHJ like structures upon exposure to the DNA damaging agent methylmethane sulfonate [33,57]. We found synergistic increases of GCR rates by either the *sgs1* or *top3* mutation with the *smc6-9* mutation (Table 5). Thus, one possibility is that an increased level of hemicatenane-like molecules causes the observed increment in GCR rates seen in the double mutants.

High enhancement of GCR formation in the *smc6-9* strain by an additional mutation in telomerase subunits (*TLC1* or *EST2*), *TEL1*, or *yKU70* suggested that unprotected or unusual telomeres could be a good substrate for GCR formation. Although we did not see the telomere size change in the *smc6-9* strain (Supplement Fig. 1), it is possible that mutations in the *SMC5*-

SMC6 complex genes and one of the telomere maintenance genes could produce more DNA damage at telomeres to generate GCR formation. Intriguingly, human SMC5-SMC6 functions in telomere maintenance, especially in the alternative telomere length mechanism through the sumoylation of telomere component such as TRF1 and RAP1 [37].

One interesting observation in GCR structures identified among these strains was chromosome fusions observed only when telomerase subunits or yKu70 was inactivated (Table 3). Previously, we observed chromosome fusions when the *TLC1* gene is mutated together with checkpoint genes (*rfc5-1, mec1,* or *tel1*) [17]. In this study, it was not clear whether further decrease of telomere size by the *tlc1* mutation in the checkpoint defective strains facilitated chromosome fusions. However, the presence of chromosome fusion in the *smc6-9 tlc1* and *smc6-9 est2* strains suggests that decreased telomere size is not a major cause of chromosome fusion. Furthermore, because the *smc6-9* mutation did not cause any cell cycle checkpoint defect, checkpoint inactivation is not an absolute requirement for chromosome fusion. Lastly, there were no chromosome fusions in the *smc6-9 tel1* strain despite a marked enhancement of GCR formation (Table 3 and 5). Therefore, telomerase itself or recruitment of telomerase to DNA damage by yKu are extremely important for the protection of chromosome ends from fusion to other broken chromosomes.

The studies presented here have extended the identification of GCR suppression pathways by defining the role of the Smc5-Smc6 complex. In addition, the detailed analysis of GCRs produced by the defect in this pathway revealed Smc5-Smc6's putative roles to prevent DNA damage from repetitive sequences to initiate BIR-dependent translocation. Lastly, high incidences of translocations in the δ sequence, ARS, tRNA gene, and other repetitive sequences strongly argue that some genomic sequences are more prone to becoming substrates for GCR formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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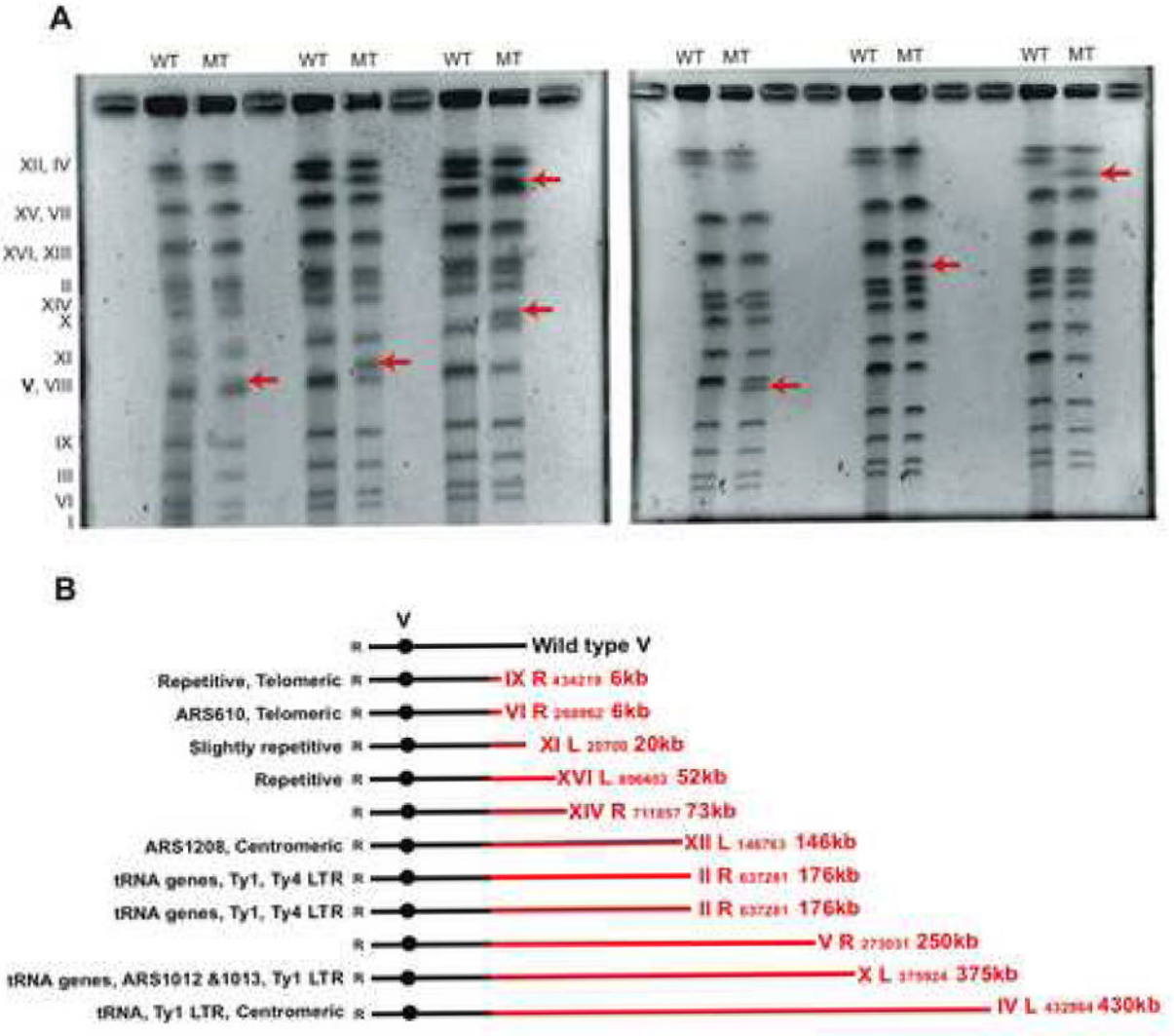

Fig. 1.

GCRs in the *smc6-9* strain are mainly non-reciprocal translocations through the break-induced replication mechanism. (A) Chromosomes from six independent clones were separated by PFGE. Arrows indicate new size chromosomes only observed in mutant clones carrying GCR. Roman numbers in the left side of gel indicate *Saccharomyces cerevisiae* chromosome numbers. WT and MT are wild type and mutant clone carrying GCR, respectively. (B) Graphic presentations of translocated chromosomes observed in the *smc6-9* strain. Red colored line represents chromosomes translocated into broken chromosome V, drawn as black line. Black circle represents the centromere of chromosome V. Roman numbers represent the number of chromosome translocated. Following R and L represent right and left arm of chromosome translocated. The numbers followed represent the starting nucleotide position of translocated chromosome and size (kb) increased after translocation. The coordinates of nucleotide positions were based on the *Saccharomyces* Genome Database (SGD: http://www.yeastgenome.org/).

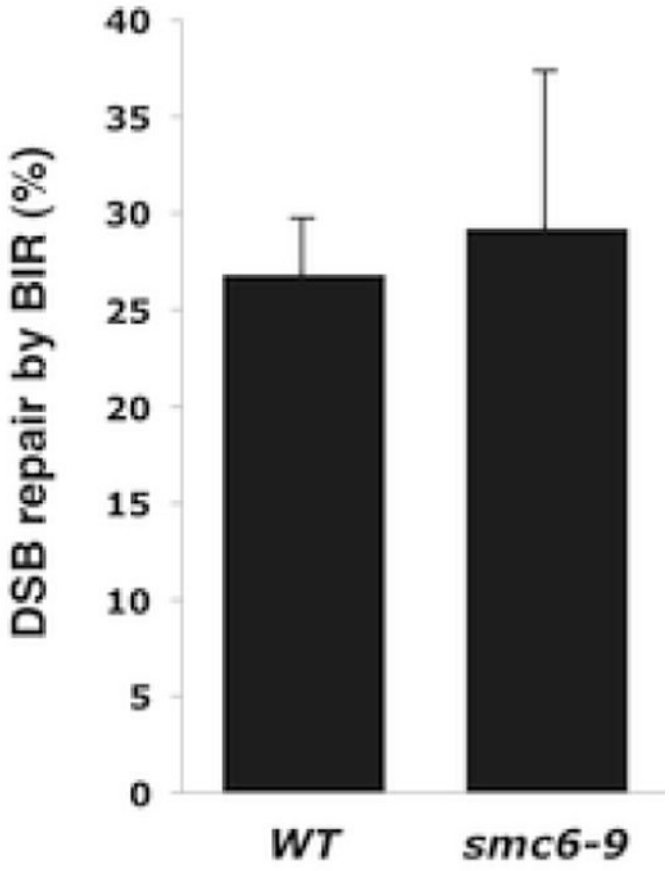

Fig. 2.
Efficiency of BIR is not affected by the *smc6-9* mutation. The BIR efficiency was measured by viability following a DSB.

Table 1

Saccharomyces cerevisiae strains used in this study.

Strains	Relevant Genotype	Source
RDKY3615	Wild type	[4]
RDKY3633	*mre11::TRP1*	[4]
RDKY3636	*rad51::HIS3*	[4]
RDKY3639	*yku70::TRP1*	[4]
RDKY3641	*lig4::HIS3*	[17]
RDKY3719	*rad9::HIS3*	[10]
RDKY3721	*rad17::HIS3*	[10]
RDKY3723	*rad24::HIS3*	[10]
RDKY3727	*rfc5-1*	[10]
RDKY3729	*pds1::TRP1*	[10]
RDKY3731	*tel1::HIS3*	[10]
RDKY3735	*sml1::KAN, mec1::HIS3*	[10]
RDKY3739	*dun1::HIS3*	[10]
RDKY3745	*chk1::HIS3*	[10]
RDKY3749	*sml1::KAN rad53::HIS3*	[10]
RDKY3814	*sgs1::HIS3*	[15]
RDKY3815	*top3::KAN*	[15]
RDKY4224	*tlc1::TRP1*	[17]
RDKY4343	*pif1-m2*	[17]
RDKY4347	*est2::TRP1*	[17]
RDKY4421	*rad52::HIS3*	[17]
RDKY4423	*rad59::TRP1*	[17]
YKJM1385	*rad5::HIS3*	[14]
YKJM1405	*elg1::HIS3*	[14]
YKJM1560	*pol32::TRP1*	[13]
YKJM2179	*siz1::TRP1*	[13]
YKJM3088	*smc6-9*	This study
YKJM3539	*nse3-2*	This study
YKJM3091	*pif1-m2 smc6-9*	This study
YKJM3115	*smc6-9 elg1::TRP1*	This study
YKJM3117	*smc6-9 pds1::TRP1*	This study
YKJM3118	*smc6-9 lig4::KAN*	This study
YKJM3134	*smc6-9 chk1::TRP1*	This study
YKJM3136	*smc6-9 dun1::TRP1*	This study
YKJM3787	*smc6-9 est1::TRP1*	This study
YKJM3137	*smc6-9 est2::TRP1*	This study
YKJM3788	*smc6-9 est3::TRP1*	This study
YKJM3139	*smc6-9 rad5::TRP1*	This study
YKJM3141	*smc6-9 rad51::TRP1*	This study
YKJM3143	*smc6-9 tlc1::TRP1*	This study
YKJM3146	*smc6-9 sml1::KAN mec1::TRP1*	This study
YKJM3203	*smc6-9 mre11::KAN*	This study
YKJM3207	*smc6-9 rad52::TRP1*	This study
YKJM3575	*smc6-9 ddc1::TRP1*	This study
YKJM3576	*smc6-9 yku70::TRP1*	This study
YKJM3611	*smc6-9 tel1::TRP1*	This study
YKJM3612	*smc6-9 sgs1::TRP1*	This study
YKJM3622	*smc6-9 siz1::TRP1*	This study
YKJM3626	*smc6-9 rad24::TRP1*	This study
YKJM3758	*pif1-m2 nse3-2*	This study
YKJM3760	*ddc1::TRP1*	This study
YKJM3767	*smc6-9 rad59::TRP1*	This study
YKJM3796	*smc6-9 rad9::KAN*	This study
YKJM3816	*smc6-9 sml1::KAN rad53::TRP1*	This study
YKJM3819	*smc6-9 rfc5-1*	This study
YKJM3825	*smc6-9 rad17::TRP1*	This study
YKJM3833	*smc6-9 top3::TRP1*	This study
YKJM5039	*siz2::TRP1*	This study
YKJM5041	*smc6-9 siz2::TRP1*	This study
YKJM5057	*mms21-11*	This study
YKJM5061	*smc6-9 mms21-11*	This study
YKJM5063	*smc6-9 pol32::KAN*	This study
YJL092	Wild type for BIR assay	[40]
YKJM5044	*smc6-9* for BIR assay	This study

All strains used for GCR assay are isogenic to RDKY3615 [*ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069::URA3*]. Strains used for BIR assay are isogenic to JL092 [mata::HOcsΔ::hisG ura3Δ851 trp1Δ63 sup53Δ::leu2ΔKANMX hmlΔ::hisG HMRa-stk ade3::GAL10::HO can1, 1-1446::HOcs::HPH::ΔAVT2 ykl215c::LEU2::can1Δ1-289].

Table 2

The impairment of Smc5-Smc6 increased the GCR rates

Relevant genotype	Wild type	
	Strain	Mutation rate (Can^r $5\text{-}FOA^r$)
Wild type	RDKY3615	3.5×10^{-10} (1)
smc6-9	YKJM3088	2.7×10^{-8} (76)
nse3-2	YKJM3539	1.9×10^{-8} (54)
mms21-11	YKJM5057	2.8×10^{-8} (80)

All strains are isogenic with the wild type strain, RDKY3615 [*ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069::URA3*] with the exception of the indicated mutations. The GCR rate of strain having both *smc6-9* and *mms21-11* (YKJM5061) was 2.9×10^{-8} (83). The numbers in parenthesis indicate the fold induction of GCR rate relative to wild type GCR rate. Can^r $5\text{-}FOA^r$ indicates resistant to canavanine and 5-FOA.

Table 3

GCR breakpoint spectra in different mutant strains

Relevant genotype	Strain	Telomere addition	Translocation		Chromosome fusion
			Non-homology	Micro-homology	
Wild type	RDKY3615	12 (2.8×10^{-10})	3 (7.0×10^{-11})	0	0
smc6-9	YKJM3088	4 (7.2×10^{-9})	1 (1.8×10^{-9})	10 (1.8×10^{-8})	0
*lig4*Δ[2]	RDKY3641	6 (1.6×10^{-9})	0	0	0
*smc6-9 lig4*Δ	YKJM3118	2 (1.7×10^{-8})	2 (1.7×10^{-8})	7[3] (5.9×10^{-8})	0
*smc6-9 yku70*Δ	YKJM3576	2 (7.0×10^{-9})	1 (3.5×10^{-9})	6 (2.1×10^{-8})	1 (3.5×10^{-9})
*rad52*Δ[2]	RDKY4421	3 (1.1×10^{-8})	0	7 (2.5×10^{-8})	0
*smc6-9 rad52*Δ	YKJM3207	9 (1.1×10^{-9})	0	1 (6.0×10^{-10})	0
*smc6-9 pol32*Δ	YKJM5063	6 (3.2×10^{-8})	4 (2.2×10^{-8})	0	0
*mec1*Δ[1]	RDKY3735	9 (4.6×10^{-8})	0	0	0
*smc6-9 mec1*Δ	YKJM3146	4 (5.3×10^{-8})	0	5 (6.7×10^{-8})	0
*tel1*Δ[1]	RDKY3731	0	0	6 (2.0×10^{-10})	0
*smc6-9 tel1*Δ	YKJM3611	2 (7.5×10^{-8})	4 (1.5×10^{-7})	5 (1.9×10^{-7})	0
pif1-m2[2]	RDKY4343	17 (4.8×10^{-8})	0	0	0
smc6-9 pif1-m2	YKJM3090	12 (5.4×10^{-7})	0	2 (9.0×10^{-9})	0
*tlc1*Δ	YKJM3137	1 (2.8×10^{-11})	0	2 (5.6×10^{-11})	8 (1.1×10^{-10})
*smc6-9 est2*Δ	YKJM3137	0	2 (2.6×10^{-8})	6 (7.8×10^{-8})	2 (2.6×10^{-8})
*smc6-9 tlc1*Δ	YKJM3143	1 (2.9×10^{-8})	2 (5.8×10^{-8})	4 (1.7×10^{-7})	4 (1.7×10^{-7})

The number of individual GCR structures from different strains is presented. The rate in the parenthesis is calculated by dividing the GCR rates observed in Table 2, 4, 5, and 6 with different GCR types observed. [1]. Data from [10]. [2]. Data from [17]. [3]. One translocation event with vector sequences.

Table 4

The GCR formation enhanced by the *smc6-9* mutation requires Rad51-dependent BIR, while it is synergistically increased with defects in NHEJ or Rad59-dependent HR

Relevant genotype	Wild type		*smc6-9*	
	Strain	Mutation rate (Can^r $5\text{-}FOA^r$)	Strain	Mutation rate (Can^r $5\text{-}FOA^r$)
Wild type	RDKY3615	3.5×10^{-10} (1)	YKJM3088	2.7×10^{-8} (76)
lig4Δ	RDKY3641	1.6×10^{-9} (5)	YKJM3118	9.3×10^{-8} (265)
yku70Δ	RDKY3639	4.1×10^{-10} (1)	YKJM3576	3.5×10^{-8} (100)
rad52Δ	RDKY4421	3.5×10^{-8} (100)	YKJM3207	6.0×10^{-9} (17)
rad51Δ	RDKY3636	3.5×10^{-9} (10)	YKJM3141	3.7×10^{-9} (11)
rad59Δ	RDKY4423	7.5×10^{-9} (21)	YKJM3767	5.1×10^{-8} (145)
pol32Δ	RDKY4349	1.5×10^{-10} (0.4)	YKJM3788	1.1×10^{-7} (300)
siz1Δ	RDKY4345	1.5×10^{-10} (0.4)	YKJM3787	6.2×10^{-8} (177)
siz2Δ	RDKY4347	1.2×10^{-10} (0.3)	YKJM3137	1.2×10^{-7} (339)

All strains are isogenic with the wild type strain, RDKY3615 [*ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069::URA3*] with the exception of the indicated mutations. The numbers in parenthesis indicate the fold induction of GCR rate relative to wild type GCR rate. Can^r $5\text{-}FOA^r$ indicates resistant to canavanine and 5-FOA.

Table 5

Mutations of genes functioning in DNA damage checkpoints synergistically increased GCR rates with the *smc6-9* mutation

Relevant genotype	Wild type		*smc6-9*	
	Strain	Mutation rate (Can^r 5-FOA^r)	Strain	Mutation rate (Can^r 5-FOA^r)
Wild type	RDKY3615	3.5×10^{-10} (1)	YKJM3088	2.7×10^{-8} (76)
rad24Δ	RDKY3723	4.0×10^{-9} (11)	YKJM3626	6.7×10^{-8} (191)
rad17Δ	RDKY3721	3.0×10^{-9} (9)	YKJM3825	9.6×10^{-8} (274)
ddc1Δ	YKJM3760	2.0×10^{-9} (6)	YKJM3575	1.3×10^{-7} (378)
*rfc5-1**	RDKY3727	6.6×10^{-8} (189) 25°C	YKJM3819	5.6×10^{-8} (160) 25°C
rad5Δ	RDKY1385	2.4×10^{-8} (68)	YKJM3139	3.4×10^{-8} (97)
elg1Δ	YKJM1405	1.7×10-8 (49)	YKJM3115	1.3×10-7 (383)
mec1Δ sml1Δ	RDKY3735	4.6×10^{-8} (131)	YKJM3146	1.2×10^{-7} (339)
rad9Δ	RDKY3719	2.0×10^{-9} (6)	YKJM3796	7.5×10^{-8} (215)
rad53Δ sml1Δ	RDKY3749	9.5×10^{-9} (27)	YKJM3816	2.4×10^{-7} (690)
chk1Δ	RDKY3745	1.3×10^{-8} (37)	YKJM3134	2.8×10^{-8} (80)
dun1Δ	RDKY3739	3.4×10^{-8} (97)	YKJM3136	8.4×10^{-8} (239)
*pds1Δ**	RDKY3729	6.7×10^{-8} (190) 25°C	YKJM3117	1.2×10^{-7} (345) 25°C
tel1Δ	RDKY3731	2.0×10^{-10} (0.6)	YKJM3611	2.5×10^{-7} (798)
mre11Δ	RDKY3633	2.2×10^{-7} (629)	YKJM3203	5.5×10^{-7} (1559)
sgs1Δ	RDKY3814	7.7×10-9 (22)	YKJM3612	2.1×10-7 (609)
top3Δ	RDKY3815	9.5×10-9 (27)	YKJM3833	7.9×10-7 (2269)

All strains are isogenic with the wild type strain, RDKY3615 [*ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069::URA3*] with the exception of the indicated mutations. The numbers in parenthesis indicate the fold induction of GCR rate relative to wild type GCR rate.

* GCR rates of strains carrying either *rfc5-1* or *pds1* were determined at 25°C, but the rates determined at 30°C did not show significant difference.

Can^r 5-FOA^r indicates resistant to canavanine and 5-FOA.

Table 6

The *smc6-9* mutation synergistically increased GCRs with mutations affecting telomere maintenance

Relevant genotype	Wild type		*smc6-9*	
	Strain	Mutation rate (Can^r $5\text{-}FOA^r$)	Strain	Mutation rate (Can^r $5\text{-}FOA^r$)
Wild type	RDKY3615	3.5×10^{-10} (1)	YKJM3088	2.7×10^{-8} (76)
pif1-m2	RDKY4343	4.8×10^{-8} (137)	YKJM3091	6.3×10^{-7} (1808)
tlc1Δ	RDKY4224	3.1×10^{-10} (0.9)	YKJM3143	3.2×10^{-7} (914)
est1Δ	RDKY4345	1.5×10^{-10} (0.4)	YKJM3787	6.2×10^{-8} (177)
est2Δ	RDKY4347	1.2×10^{-10} (0.3)	YKJM3137	1.2×10^{-7} (339)
est3Δ	RDKY4349	1.5×10^{-10} (0.4)	YKJM3788	1.1×10^{-7} (300)

All strains are isogenic with the wild type strain, RDKY3615 [*ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069::URA3*] with the exception of the indicated mutations. The *nse3-2* and *nes3-2 pif1-m2* mutations increased GCR rates 1.9×10^{-8} (54) and 3.0×10^{-7} (868), respectively). The numbers in parenthesis indicate the fold induction of GCR rate relative to wild type GCR rate. Can^r $5\text{-}FOA^r$ indicates resistant to canavanine and 5-FOA.