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Replication of O6-methyl-dG in Saccharomyces cerevisiae

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Abstract

This study investigated the mechanisms used to replicate across O^6-methyl-dG (^{Me}G) in *Saccharomyces cerevisiae*. Single stranded oligonucleotide transformation was used to incorporate one ^{Me}G lesion into the yeast genome. By introducing the lesion into several different strain backgrounds and examining the sequences after replication, this study explored the roles of mismatch repair (MMR), translesion synthesis (TLS), and template switching in accurately replicating across the ^{Me}G lesion. In contrast to previous results, this study found that MMR is crucial to the accurate replication of ^{Me}G. TLS also seems to contribute to the replication of the ^{Me}G lesion, particularly when the lesion is in the lagging strand. However, this replication via TLS is typically inaccurate in the absence of MMR. Finally, this study discovered that Rad5, which is typically associated with template switching, plays a significant role in bypassing the lesion and promoting accurate replication across the lesion by influencing other mechanisms, like TLS.

Introduction

Faithful maintenance and replication of the genome is crucial to every organism. Although there are several mechanisms dedicated to promoting accurate replication, DNA damage is still a concern because damaged bases can mispair. If this damage is not prevented or repaired, these mispairs can lead to mutations and disease. Our lab studies the processes of mutagenesis and the mechanisms involved in prevention and repair in *Saccharomyces cerevisiae*. This study focuses on O^6-methyl-dG (^{Me}G) and investigates how this lesion is treated in the cell. Oligonucleotide transformation is used to insert the ^{Me}G lesion into a specific locus in the yeast genome and study its replication.

Alkylation damage

Alkylation lesions are a common type of DNA damage. Alkylating agents are produced endogenously through enzymatic reactions and cellular processes [1, 2]. Environmental compounds, such as those found in tobacco and grilled food, can also cause alkylation damage [3, 4]. Chemotherapy drugs, such as temozolomide and procarbazinem, are often alkylating agents [5].

Regardless of their source, alkylating agents create a variety of DNA lesions, including methylation of the N^7 and O^6 atoms of guanine (G), the N^1 and N^3 atoms of adenine (A), and the N^3 atom of cytosine (C) [6]. Although methylation of the O^6 atom of G, which creates O^6-methyl-dG (^{Me}G), makes up only a small fraction of all the alkylation lesions (~8%), ^{Me}G is the most mutagenic and can cause cell death [6, 7].

O^6-methyl-dG (^{Me}G)

During replication, ^{Me}G can pair with either C or thymine (T). The ^{Me}G:C base pair is more stable than the ^{Me}G:T pair [8] because ^{Me}G forms more hydrogen bonds with C than T. However, the ^{Me}G:T pair has a shape that resembles the geometry of Watson-Crick pairs, while the ^{Me}G:C pair creates a wobble configuration at a neutral pH (fig. 1) [9-11]. As a result, high-fidelity polymerases, which rely on Watson-Crick geometry to correctly replicate, tend to incorporate a T opposite the ^{Me}G. Lower fidelity polymerases, however, do not depend as much on base pair shape and can more often pair the ^{Me}G with a C [12, 13].

The mutagenic properties of ^{Me}G are based on its ability to pair with T. When T is replicated opposite the ^{Me}G, a G:C to A:T transition mutation occurs. In order to prevent this

mutation, mammalian cells have developed enzymes and pathways to block the mutagenic effects of ^{Me}G. For instance, O^6-methylguanine-DNA-methyltransferase (MGMT) repairs the lesion by removing the methyl group from ^{Me}G and restoring it to a normal G [14]. Another method of preventing mutations is mismatch repair (MMR), which corrects replication errors. When MMR encounters high levels of the ^{Me}G lesion, it can signal mammalian cells to undergo apoptosis [15]. Loss of MGMT and MMR have been linked to colon tumorigenesis in mice [16] and colorectal cancers in humans [17]. If MGMT is inactivated through mutation or epigenetic silencing, alkylation damage will increase within the cell. If MMR is then lost as well, the damaged cells will escape apoptosis and show increased mutagenesis.

Methods for coping with ^{Me}G in yeast

Yeast cells have evolved several methods to cope with alkylation damage. One method involves O^6-methylguanine-DNA-methylase (MGT1). MGT1 is the yeast homolog of MGMT [18]. Like MGMT, MGT1 repairs the ^{Me}G lesion by transferring the methyl group from the ^{Me}G onto the sulfur of the cysteine in the enzyme's active site [14, 19]. When the methyl group is removed, the ^{Me}G is reverted to a G, which is replicated normally.

MMR is another system found in yeast that is involved with the ^{Me}G lesion [6]. MMR was initially defined in *E. coli* through the *mutS* and *mutL* genes [20]. Yeast contains six *mutS* homologues (MSH) and four *mutL* homologues (MLH) [20-22]. Of the *MSH* genes in yeast, *MSH2, MSH3,* and *MSH6* are involved in nuclear MMR [21-23]. They code for proteins that form heterodimers and bind to mismatched DNA. The Msh2/Msh6 heterodimer is known as MutSα and recognizes single base pair mismatches and small insertion/deletion loops. The

Msh2/Msh3 heterodimer, on the other hand, is called MutSβ and recognizes larger loops [21-23]. Since the ^{Me}G creates a single base pair mismatch, it is primarily recognized by MutSα.

Another process known as translesion synthesis (TLS) can also be used to replicate across the ^{Me}G damage [12]. In the last 20 years, numerous DNA polymerases that are not essential for replication have been discovered in all organisms, including bacteria and humans [24]. In general, these additional polymerases have low processivity and low fidelity on normal DNA suggesting that they deal with repair, instead of replication. Normal replicative polymerases often stall at lesions like ^{Me}G because the lesion is too big for their tight active sites [24]. Studies focusing on UV damage in cells have shown that Rad6 and Rad18 play a crucial role in replicating across lesions [25, 26]. When polymerase stalling occurs, Rad18 binds to the region of single-stranded DNA and directs Rad6 to the DNA by forming a complex with it [27-29]. This complex then interacts with the proliferating cell nuclear antigen (PCNA), a DNA polymerase sliding clamp present at the replication fork, by monoubiquitinating PCNA at the Lys 164 residue [30] (fig. 2). The monoubiquitination of PCNA inactivates the normal replicative polymerase and recruits translesion synthesis DNA polymerases, primarily polymerase ζ (pol ζ) and polymerase η (pol η) in yeast, to the stalled replication fork [31-33]. Pol ζ is made up of a Rev3-Rev7 complex and tends to be error-prone and mutagenic, while pol η is encoded by *RAD30* and is considered error-free in many contexts [32, 34, 35]. Although pol ζ and pol η are both recruited by the Rad6-Rad18 complex, recent data in the Crouse Lab comparing *rad18* mutants with *rad18 rad30* mutants suggests that pol η may have some function independent of Rad18 (unpublished).

In addition to recruiting pol ζ and pol η, the Rad6-Rad18 complex signals another post-replicative repair pathway called template switching [36]. This pathway involves Rad5 and an

Mms2-Ubc13 complex [30, 33]. These proteins polyubiquitinate PCNA by linking additional ubiquitins, through the Lys 63 residue, to the first ubiquitin added by the Rad6-Rad18 complex [37-39] (fig. 2). This polyubiquitination leads to template switching [33, 36]. In template switching, the lesion on the stalled strand is bypassed using the recently replicated sister strand. There are two types of template switching: fork regression and strand invasion [36] (fig. 3). During fork regression, replication of the two strands becomes uncoupled, and synthesis continues on one strand and stalls on the other. Rad5 then rearranges the strands such that the parent strands re-anneal, the recently replicated strands anneal to each other, and there is regression of the replication fork. At this point, the stalled strand can be replicated past the damaged region using the recently replicated sister strand as a template. After this replication, the fork is reversed and replication can continue normally. The other type, strand invasion, also involves uncoupled replication. However, it includes restarting replication downstream of the damage site and leaving a gap at the lesion. To fill this gap, the 3'-end of the recently replicated strand invades the homologous region of its sister strand, uses it as a template, and then rejoins the replicated portion downstream of the gap [32, 36, 40]. Regardless of specific mechanism, template switching is error-free because the actual lesion is avoided and not replicated, even though it remains in the genome.

Although Rad5 is primarily associated with template switching, a study by Gangavarapu et al. suggests that Rad5 may affect the efficiency of TLS polymerases [41]. In this study, *RAD5*, *MMS2*, or *UBC13* was inactivated and the effects on UV sensitivity were observed. The authors found that the *rad5* mutants had substantially higher UV sensitivity than the *mms2* and *ubc13* mutants. This finding suggests that Rad5 has some effect on lesion bypass independent of its activity with the Mms2-Ubc13 complex. The study concluded that Rad5 could improve

efficiency through two possible mechanisms. One idea involves Rad5 physically interacting with the TLS polymerases and coordinating their activity. The other mechanism suggests that Rad5 improves efficiency by contributing to the ubiquitination of the TLS polymerases.

In addition to TLS and template switching, homologous recombination can play a role in coping with alkylation damage. Pivotal work by Petr Cejka, Josef Jiricny, and others has shown that in *Saccharomyces cerevisiae,* ^{Me}G is highly cytotoxic in large quantities because it can lead to cell cycle arrest and cell death presumably through futile cycles of repair [6, 42, 43]. These studies exposed the yeast cells to severe amounts of alkylation damage and concluded that regardless of which base, C or T, is inserted opposite the ^{Me}G, MMR recognizes the pair as a mistake and attempts to correct it. However, MMR focuses on correcting the newly synthesized strand, and the ^{Me}G lesion remains in the template strand. Since both ^{Me}G:T and ^{Me}G:C appear to be treated as mistakes, every attempt to replicate across the ^{Me}G triggers MMR. Ultimately, this futile cycle of repair may cause the polymerase to stall and leave a gap opposite the ^{Me}G. Homologous recombination (HR) can be used to repair this gap [43]. Since gaps can lead to double-strand breaks and cell death, HR serves an important purpose in cells [6, 42]. In fact, alkylation resistance in yeast, unlike in mammalian cells, is not affected by the status of MMR because HR is more active in yeast than mammalian cells.

Oligonucleotide transformation in yeast

Single-stranded oligonucleotides (oligos) were first used to transform the yeast genome by the Sherman Lab in 1988 [44]. Since then, the Crouse, Kow, and Jinks-Robertson Labs have developed a general model for this type of transformation in yeast [45]. An electric shock is used to disrupt the cell wall, which allows the oligos to enter the yeast cell. Once in the cell, the

oligos travel to the nucleus and anneal to single-stranded portions of DNA at replication forks. The oligos act as a primer for the replicative polymerase [45]. If the oligos contain different bases than the template sequence and successfully anneal, the new bases will be incorporated into the genome. However, MMR can recognize the mismatches formed when the oligo anneals to the genome and cause excision of the mismatched portion of the oligo.

Experiments in this study

This study focused on the effects of ^{Me}G on DNA replication in *S. cerevisiae*. A collection of strains that lack certain repair pathways, such as MMR and template switching, were used to analyze the roles of these pathways in the replication of ^{Me}G. Single-stranded oligonucleotide transformation was used to incorporate the ^{Me}G lesion into a specific locus in the strains' genomes. Since the ^{Me}G lesion was selectively inserted into one location in the genome, this approach differed substantially from previous *in vivo* studies of ^{Me}G that created a relatively large number of ^{Me}G lesions throughout the genome [6, 42, 43]. In comparison to the effects observed in these previous studies, the low amount of damage used in this study led to different results. For instance, the data in this study demonstrated that MMR not only distinguishes between ^{Me}G:C and ^{Me}G:T, but also plays a crucial role in promoting accurate replication of ^{Me}G by ensuring that it is paired with C. The results suggest that TLS, in addition to MMR, is one of the main mechanisms used to replicate across the ^{Me}G lesion. However, this study did not examine the role of HR. Thus, HR could also be responsible for replicating across ^{Me}G, but further experiments are necessary to examine this role.

Methods

Yeast strains

All yeast strains were derived from GCY2196 and GCY2297 [46], which originated from strains containing the G148C mutation in the *TRP5* gene in the forward (F) and reverse (R) orientation [47]. The common background for all strains was *MATα his3-Δ200 ura3-52 leu2-Δ1 lys2-CT$_{1265}$GA*. The mutants in this study contained CGATGTTATCCAACTGGGA starting at position 138 of *TRP5*, where the underlined bases indicate differences from the original G148C sequence. The strains have been further modified by others in the Crouse Lab to disrupt various genes, such as *MSH6*, *RAD18*, and *RAD5* (Table 1).

Since this study focused on the ^{Me}G lesion, the *MGT1* gene was knocked out in all the relevant strains to prevent removal of the alkylation damage. A strain containing the *MGT1* knockout (KO) and a G418^R marker was obtained from the yeast gene deletion consortium [48]. The G418^R marker was then replaced with a *his5+* marker from *S. pombe* using the plasmid pUG27 [49]. This *his5+* marker complements the *his3* mutation found in GCY2196 and GCY2297. After the marker was replaced, the *MGT1* KO with the *his5+* marker was amplified using polymerase chain reaction (PCR) and the other strains in this study were transformed with the PCR product.

Lithium acetate (LiOAc) transformation [50] was used to insert the *his5+* marker in order to disrupt the *MGT1* gene. The cells were grown on SD-His plates [51] to screen for positive transformants. Because the strains were originally *his3* and the *his5+* marker complements this *his3* mutation, only the cells that were successfully transformed grew on SD-His media. The transformed cells were also confirmed by PCR using two pairs of primers. For the first pair of primers, one primer anneals upstream of the *MGT1* gene, while the other anneals within the

marker. If these primers yielded a PCR product of the correct size, the marker was correctly inserted and the *mgt1* mutant was confirmed. The second pair of primers included a primer that also anneals upstream and a primer that anneals within the *MGT1* gene. Thus, these primers were used to indicate the presence of the wildtype *MGT1* gene. When these primers were used, a lack of PCR product suggested that the cells contained only an *mgt1* mutation. This technique was used to turn the strains in Table 1 into *mgt1* mutants (Table 2).

Oligonucleotides used

Three oligos were used in this study (fig. 4). Two of the oligos, Primer ^{Me}G and Primer G, create three mismatches when they anneal to the yeast DNA. Primer ^{Me}G creates a mismatch between a ^{Me}G and a G. If this mismatch is not removed, the ^{Me}G becomes incorporated into the strain's genome. The orientation of the strain, forward or reverse, determines whether the ^{Me}G lesion will ultimately be in the lagging or leading strand. When the oligos are transformed into a strain that is in the forward orientation (F), they anneal to the leading strand during the first round of replication. When the second round of replication begins, the ^{Me}G lesion is in the lagging strand. For strains that have the reverse orientation (R), the opposite is true (fig. 5). Primer G behaves similarly and acts as a control by creating G:G mismatch, leading to the incorporation of a G. In addition, both oligos create a C:C mismatch downstream of the ^{Me}G:G mismatch. If the C is incorporated, the cell reverts to Trp+. They also create a G:A mismatch upstream of the ^{Me}G, which leads to the formation of an *SphI* restriction site if the G is incorporated. The locations of these three mismatches provide an opportunity to screen for cells that have successfully incorporated the ^{Me}G. Since all three mismatches are within nine base

pairs, any cells that are Trp+ and contain the *SphI* restriction site would most likely also have incorporated the ^{Me}G.

The third oligo, known as Primer Lys2, reverts the *lys2* mutation to wild type by introducing a G:C→C:G transversion. Initially, this oligo was supposed to act as a control. However, recent data in the Crouse Lab has shown that the number of *LYS2* revertants that arise from this oligo and the number of *TRP5* revertants that result from oligos like Primer ^{Me}G and Primer G are not always correlated (unpublished). As a result, Primer Lys2 cannot be used as an internal control. However, it was still used in this study because it is serves as a way of assessing the efficiency of each electroporation. If a particular strain yields few *TRP5* revertants, it is difficult determine whether the low number is due to the strain's background or electroporation failure. Primer Lys2 can help distinguish between these two scenarios. If the number of *TRP5* revertants is low but the number of *LYS2* revertants is normal, we know the low numbers are due to the strain's genotype, instead of electroporation failure.

Single-stranded oligonucleotide transformation

All twelve strains (Table 2) were transformed using the following electroporation protocol, modified from [45]. Each strain was cultured and grown overnight. The following day, 1 mL of the overnight culture was inoculated into 50 mL of YPAD [51] solution and grown while shaking at 30°C. When the cell cultures reached an OD_{600} of 1.3, the cells were centrifuged at 3000 RPM and 4°C for 4 minutes, washed twice with chilled ddH_2O, and washed once with chilled 1.0M sorbitol. After the last washing, the samples were centrifuged, the supernatant was removed, and the cells were resuspended in 1 mL of 1.0 M sorbitol per 1 g of cells. The suspension was separated into two 200 µL aliquots. One aliquot was mixed with 200

pmol each of Primer Lys2 and Primer ^{Me}G, while the other aliquot was mixed with 200 pmol each of Primer Lys2 and Primer G. Both mixtures were transferred to 2-mm gap electroporation cuvettes and electroporated at 1.5 kV, 200 Ω, and 25 μF (BTX Harvard Apparatus ECM 630). After electroporation, the cells were immediately mixed with 5 mL of YPAD and allowed to recover at 30°C with shaking for 2 hours. After the 2 hour recovery time, the cells were centrifuged, washed with 20 mL of ddH_2O, and resuspended in 1 mL of ddH_2O. Of this 1 mL suspension, 50 μL were plated onto one SD-Lys plate and 200 μL were plated onto four SD-Trp plates [51].

SphI restriction site test

Only electroporated colonies that grew on the SD-Trp plates were tested for the *SphI* restriction site, following a protocol developed by Gina Rodriguez. Of the Trp+ colonies, 48 per strain per oligo (either Primer ^{Me}G or Primer G) were tested. If the electroporation yielded fewer than 48 Trp+ colonies, all the colonies were tested.

The Trp+ colonies were resuspended in 96 round deep well microplates containing 150 μL of SD-Trp liquid media per well and grown overnight at 30°C with shaking. The following day, the samples were replicated from the microplates with SD-Trp media to microplates with 300 μL of YPAD per well using a 96-well replicator (Fisher, 05-450-9, Boekel No. 140500). These microplates were incubated overnight at 30°C with shaking. After the overnight growth, the cells were replicated from the microplates with YPAD to PCR microplates containing 15 μL of 2 mg/mL Zymolyase 20T per well. These PCR microplates were incubated at 37°C for 30 minutes and 95°C for 10 minutes to allow the Zymolyase to lyse the cells.

After incubation, 85 μL of ddH_2O were added to each well of the PCR microplates to dilute the lysed cells. These lysed cells were then used to amplify the *TRP5* region of the yeast genome. The amplification was done using 5 μL of the lysed cell solution, 0.5 μL of Takara DNA Polymerase (TAKRF001A, Takara Bio Inc.), 10 μL of Takara 5X buffer, 4 μL of Takara dNTP mixture, 25 pmol each of primer trpseq2 and primer trpseq8 (Table 3), and 29.3 μL of ddH_2O. The PCR involved 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 20 sec, and extension at 72°C for 15 sec. This PCR yielded a 246-bp product that contained the region modified by Primer ^{Me}G and Primer G.

The PCR products were then exposed to the *SphI* restriction enzyme. A master mixture of 20 μL of the *SphI* restriction enzyme (New England BioLabs, #R0182L, 10,000 U/mL), 96 μL of the 10X concentrate buffer provided by New England BioLabs, and 892 μL of ddH_2O was created and 10 μL of this mixture were placed in each well of a 96 well PCR microplate. Then 4.8 μL of PCR product were added to each well of the microplate and incubated overnight at 37°C. After the incubation, gel electrophoresis was used to determine whether the samples contained the *SphI* restriction site. If a sample did not contain the restriction site, it would appear on the gel as a single band that is 246 bp long. However, if a sample was positive for the restriction site, it would appear as two shorter bands.

Sequencing

The colonies that were electroporated with Primer ^{Me}G, were Trp+, and contained the *SphI* restriction site were eligible for sequencing because they should have incorporated the O^6-methyl-dG lesion. To obtain the sequences, 45 μL of the colonies' PCR products were sent to

Agencourt® Bioscience for Quicklane sequencing. Only 24 samples, or however many were eligible if there were fewer than 24, were sequenced per strain.

Results

Oligo transformation

As described earlier, the Crouse Lab has found that transformation with Primer Lys2 cannot serve as an internal control for Primers ^{Me}G and G. However, it can act as an indicator of transformation efficiency and help determine whether a low number of Trp revertants is due to electroporation failure or the strain background. Also, transformation with Primer Lys2 is somewhat independent of MMR because it creates only a C:C mismatch, as demonstrated by the fact that the average number of Lys revertants for strains with MMR is relatively similar to the average number of revertants for strains lacking MMR.

As demonstrated by the number of Lys revertants (Table 4), the transformation efficiency varied between different experiments. Some experiments, such as the second set of data for 2584, were much more efficient than average leading to a large number of Lys revertants and presumably a correspondingly large number of Trp revertants. On the other hand, other experiments had below average efficiency, as seen in the first set of data for 2585, and yielded an aberrantly low number of Trp revertants.

The number of Trp revertants also varied greatly depending on the primer and the strain background. When Primer ^{Me}G was used, the number of revertants was generally lower than the number of revertants obtained with Primer G, suggesting that the ^{Me}G:G mismatch is recognized more than the G:G mismatch during the first round of replication. In terms of strain background, the *msh6* mutants yielded more Trp revertants than the wild type strains (fig. 6). This result

suggests that MMR may play a role in recognizing and removing annealed oligos during transformation.

In addition, after the oligos are incorporated, ^{Me}G and G are treated differently during the second round of replication. Unlike G, ^{Me}G is recognized as abnormal and can trigger template switching. In many cases, template switching could bypass the C needed for Trp reversion as well as the ^{Me}G, leading to Trp- cells. Since G is a normal base, cells transformed with Primer G should not experience template switching. The data support this idea because in the absence of template switching, through the inactivation of Rad18, the ratio of the number of Trp revertants created by Primer ^{Me}G to Primer G increased for the leading strand (Table 5). This result suggests that the lower number of Trp revertants obtained with Primer ^{Me}G, in comparison to Primer G, is partially due to template switching during the second round of replication, at least on the leading strand.

Unlike the leading strand, the lagging strand did not exhibit a change in the ratio of the number of Trp revertants due to Primer ^{Me}G and Primer G (Table 5). This phenomenon was most likely the result of homologous recombination. In addition to MMR and template switching, homologous recombination could also lead to fewer Trp revertants. The ^{Me}G lesion is known to stimulate recombination [43]. If recombination is activated during the second round of replication, the C needed for Trp reversion will probably be bypassed along with the ^{Me}G because they are located close to each other. This bypass will result in loss of Trp revertants because it returns the cells to Trp-. Since it is triggered by ^{Me}G, but not the G incorporated by Primer G, recombination probably contributes to the difference in the number of Trp revertants generated by Primer ^{Me}G and G.

Recombination is also known to affect the lagging strand more than leading strand [52]. This strand bias arises because right when stalling occurs on the leading strand there is probably no completely replicated lagging strand. However, when stalling takes place on the lagging strand, there will mostly likely be a completed leading strand to serve as a template. As a result, the lagging strand probably experienced more recombination, which led to loss of Trp revertants and maintained the ratio of ^{Me}G to G Trp revertants even in the absence of template switching (Table 5). The leading stand was not as affected by recombination, which led to a noticeable change in the ratio when Rad18 was inactivated (Table 5). Although homologous recombination is an important pathway in yeast, it was not examined in detail in this study because all the strains were capable of recombination.

SphI **restriction site test**

Like the number of Trp revertants, the percentage of colonies with *SphI* restriction sites differed from strain to strain (Table 4 and fig. 7). The *mgt1* strains generally had the lowest percentage of *SphI*+ colonies. When MMR was inactivated, like in the *msh6 mgt1* mutants, the percentage increased noticeably. For Primer G, the percentage of *SphI*+ colonies increased to almost 100% in the absence of MMR. This result suggests that MMR recognizes the ^{Me}G:G and G:G mismatch that occurs when the oligos anneal, and this recognition leads to removal of a fraction of the oligo, not including the C:C mismatch because it is poorly recognized. After this removal, the resulting cells are Trp+ and *SphI*- (fig. 8). This hypothesis is further supported by recent data from the Crouse Lab which demonstrates that an oligo creating an additional mismatch on the other side of the C:C mismatch yields only Trp+ and *SphI*+ cells even in the presence of MMR (unpublished). In this case, MMR most likely recognized the new mismatch

along with the ^{Me}G:G and G:G mismatches, resulting in removal of that entire region of the oligo, including the C necessary for Trp reversion. Thus, only oligos that completely escaped MMR detection were incorporated, which led to only Trp+ and *SphI*+ cells.

Although cells transformed with Primer G are affected primarily by MMR, cells transformed with Primer ^{Me}G are also influenced by template switching. Loss of the *SphI* restriction site could occur during the second round of replication through template switching. As explained earlier, template switching is triggered by the ^{Me}G incorporated by Primer ^{Me}G, but not the G incorporated by Primer G. In most cases, when template switching is activated, one would expect not only the ^{Me}G, but also the G necessary to create the *SphI* site to be bypassed. If this occurs, the resulting cells would be Trp+ but *SphI*- (fig. 9). This phenomenon is supported by the fact that in the absence of MMR, nearly 100% of the cells transformed with Primer G contained the *SphI* site, but only 83% to 89% of the cells transformed with Primer ^{Me}G had the restriction site. However, when template switching was disabled through inactivation of Rad18 or Rad5, the cells transformed by Primer ^{Me}G were almost 100% *SphI*+ (Table 4 and fig. 7).

Sequencing data

As expected, only C was replicated opposite the ^{Me}G lesion in the wildtype strains. In these strains, MGT1 is fully functional and can fix the lesion allowing for normal replication. When the *MGT1* gene was inactivated, ^{Me}G was still primarily paired with C (Table 4 and fig. 10). This result is surprising because ^{Me}G is known to be mutagenic [13]. Thus, the accurate replication of the ^{Me}G in the absence of MGT1 suggests that there are other pathways or enzymes, besides MGT1, that lead to correct replication. One such pathway is MMR because, when MMR was inactivated, ^{Me}G was mostly mispaired with T.

The sequencing data also revealed replication of the ^{Me}G lesion is affected by the replication strand of the lesion. The location of the ^{Me}G lesion becomes particularly relevant for the *rad18 mgt1* mutants (fig. 11). On the lagging strand, C was incorporated opposite the ^{Me}G. On the leading strand, however, ^{Me}G was paired almost evenly with C and T. When MMR, TLS, and MGT1 were inactivated, the accuracy of replication became even worse with almost even incorporation of C and T on the lagging strand and mainly incorporation of T opposite the ^{Me}G on the leading strand (Table 6).

The removal of MMR and Rad5 in the *msh6 rad5* mutants led to primarily inaccurate replication of the ^{Me}G lesion (fig. 12). In both orientations, the ^{Me}G was mostly mispaired with T. In fact, the frequency of T incorporation in the absence of MMR and Rad5 was greater than when only MMR was inactivated. This result suggests that Rad5, in addition to MMR, may play an important role in accurately replicating across the ^{Me}G lesion. A description of the mechanism will be given in the Discussion.

Discussion

Oligo incorporation

As mentioned earlier, any oligo that anneals during electroporation can be removed if it creates mismatches that are recognized as incorrect during replication. In the cases of Primer ^{Me}G and Primer G, complete removal of the oligos results in cells that are Trp- because the C:C mismatch, which causes *trp5* reversion, is removed. The data suggest that MMR is responsible for removing the oligos after they have annealed. When MMR is inactivated, the number of Trp revertants increased (fig. 6) suggesting that more of the oligos that anneal are incorporated into

the genome and cause *trp5* reversion. Thus, MMR can recognize ^{Me}G:G and G:G pairs as mismatches and remove the entire oligo.

In addition to complete removal, the annealed oligos can be partially removed. This situation occurs if the C:C mismatch created by the oligos is poorly recognized but the ^{Me}G:G mismatch and/or the G:A mismatch are recognized as mistakes. In these cases, the oligo is cleaved off up to the C:C mismatch (fig. 8). Since the C:C mismatch is maintained but the G:A mismatch is removed, the resulting cell will be Trp+ but *SphI*-. As demonstrated in Table 4, the lowest percentage of *SphI*+ sites occurred in the wildtype strains and the *mgt1* mutants. When MMR was disabled, the *SphI*+ percentage increased to 100% in cells transformed with Primer G (fig. 7). This suggests that the partial removal of annealed oligos is the primary cause of Trp+ and *SphI*- cells when Primer G was used. For the cells transformed with Primer ^{Me}G, disabling MMR led to only a slight increase in the *SphI*+ percentage. To obtain *SphI*+ percentages near 100%, both MMR and Rad18 had to be inactivated (fig. 7). This finding suggests that the partial removal of oligos by MMR and template switching during the second round of replication play a role in creating Trp+ and *SphI*- cells when Primer ^{Me}G is used.

Replication of the ^{Me}G lesion

If an annealed oligo escapes complete and partial removal and contains different nucleotides than the template DNA, these new nucleotides are incorporated into the genome. When Primer ^{Me}G is incorporated, the resulting cells are Trp+, *SphI*+, and contain the ^{Me}G lesion. By incorporating the lesion into a variety of genetic backgrounds and observing the resulting number of Trp revertants and sequences, this study analyzed which mechanisms are used to replicate ^{Me}G.

Past research has suggested that MMR cannot discriminate between ^{Me}G:C and ^{Me}G:T, which leads to a futile cycle of repair [6, 42, 43]. The sequencing data in this study, however, demonstrate that yeast MMR can distinguish between the two different pairs. Surprisingly, even in the absence of MGT1, ^{Me}G was paired with C almost every time. However, when MMR was inactivated, T was primarily incorporated opposite the lesion (fig. 10). The increase in ^{Me}G:T mispairing that was allowed in the absence of MMR suggests that MMR plays a major role in ensuring accurate replication of the ^{Me}G lesion.

By comparing the number of ^{Me}G:C pairs to ^{Me}G:T pairs in the absence of MMR, this study found that replication of ^{Me}G is only accurately performed about once in every five attempts (fig. 10). MMR would then recognize the ^{Me}G:T mispairs, which would lead to removal of the recently replicated region near the mismatch and another attempt at replication. If several ^{Me}G lesions occurred in the genome, it would take numerous rounds of repair to accurately replicate every lesion. Since there is a finite quantity of MutSα in every cell, many lesions could even overwhelm MMR. In these cases, the repair driven by MMR would resemble a futile cycle of repair.

This phenomenon can be used to explain the difference between previously published results and the findings in this study. In previous studies, numerous ^{Me}G lesions were created within the cell [42, 43]. The amount of lesions was probably high enough to overwhelm MMR and require countless rounds of repair to yield completely accurate replication, leading to the resemblance of a futile cycle of repair. In this study, however, only one ^{Me}G was created within the genome. Since MMR could effectively cope with one lesion, it ensured accurate replication of the ^{Me}G.

In addition to MMR, this study examined the effects of TLS and template switching using *rad18 mgt1* and *msh6 rad18 mgt1* mutants. When these pathways were inactivated, the number of Trp revertants decreased substantially (Table 4). However, the ratio of Primer ^{Me}G to Primer G Trp revertants increased for the leading strand and remained the same for the lagging strand (Table 5). In this case, recombination was most likely the primary pathway involved in bypassing the ^{Me}G lesion, particularly on the lagging strand. In the process of dealing with the lesion, recombination would also bypass the C necessary for Trp reversion, leading to fewer Trp+ cells. Thus, in strains that had functional TLS and template switching, the Trp+ cells would be able to replicate across the ^{Me}G lesion using some mechanism other than recombination. One possible candidate for replicating across the ^{Me}G in Trp+ cells is pol ζ. Data obtained from the *msh6 mgt1* mutants showed that replication of the ^{Me}G lesion was primarily inaccurate (fig. 10). As the traditionally error-prone polymerase involved in TLS, pol ζ could be replicating across the ^{Me}G, but only inserting the correct base approximately once every five times.

When *RAD18* was inactivated, the number of Trp revertants became extremely low. However, some revertants did form. Their presence suggests that other pathways, besides recombination and Rad18 dependent enzymes, can be used to replicate across the ^{Me}G. One likely possibility is that pol η is acting independently of Rad18 and yielding a small amount of Trp+ cells that can replicate across the ^{Me}G lesion. Although most models claim that pol η cannot function without Rad18, recent data in the Crouse Lab has shown that *rad18* and *rad18 rad30* mutants do not behave exactly alike (data not shown). If pol η was completely dependent on Rad18, the inactivation of *RAD30*, which encodes pol η, would have no effect on *rad18* mutants. Since inactivation of *RAD30* has an effect, pol η probably has some limited function

independent of Rad18. This pol η function could be used to replicate across the ^{Me}G in the absence of Rad18.

Unlike pol ζ, pol η is typically considered an accurate polymerase. However, little is known about its accuracy while replicating across ^{Me}G. If pol η was in fact responsible for the replication that occurred in the absence of Rad18, its accuracy varied depending on whether the ^{Me}G was in the leading or lagging strand during the second round of replication (fig. 11 and Table 6). In the absence of MMR, the accuracy of replication was approximately the same for both strands. When Rad18 was inactivated in the *msh6 mgt1* mutants, the accuracy remained the same on the leading strand, but increased on the lagging strand (Table 6). This result demonstrates that there may be a difference in the polymerases replicating the lagging and the leading strands. Since a loss of Rad18 function only altered the accuracy of replication on the lagging strand, the data suggest that the polymerases that are replicating in the absence of Rad18 are more accurate only on the lagging strand, in comparison to the polymerases that replicate in the presence of Rad18.

Similar to the polymerases affected by Rad18, MMR seems to have some strand bias. Without MMR, replication is inaccurate (fig. 11 and Table 6). When MMR was fully functional, the accuracy of replication increased to 100% on the lagging strand. On the leading strand, however, accuracy increased to 91% in the *mgt1* mutants and only 53% in the *rad18 mgt1* mutants (Table 6). Therefore, MMR recognized and repaired all of the errors made during replication of the lagging strand but not the leading strand. Like these results, previous studies have also found more MutSα activity of the lagging strand compared to the leading strand [45, 53]. These results suggest that the accuracy of not only Rad18 independent replication, but also MMR could be strand dependent.

In addition to analyzing the effects of removing both TLS and template switching through Rad18 inactivation, this study used *msh6 rad5 mgt1* mutants to gain insight into the role of template switching. Although Rad5 is typically associated with template switching, there is evidence suggesting that Rad5 could also affect TLS [41]. When Rad5, MMR, and MGT1 were all inactivated, replication of the ^{Me}G became almost completely inaccurate and T was inserted opposite the lesion. Comparing these results to the sequencing data of the *msh6 mgt1* mutants (fig. 12) revealed that the small amount of accuracy observed in the absence of MMR is due to Rad5 activity. Since Rad5 is associated with template switching, that pathway is a possible mechanism for the accurate replication. However, in order for it to bypass the ^{Me}G lesion without also compromising the Trp reversion and the formation of the *SphI* restriction site, template switching must occur between the G:A and C:C mismatches created by the oligo. This window consists of 7 bps, which means that template switching would have to create a small, precise loop to accurately bypass the ^{Me}G lesion (fig. 13).

Because template switching involving such a short region of DNA seems unlikely, the influence of Rad5 could be due to another mechanism besides template switching. Like the study by Gangavarapu [41], this study suggests that Rad5 may affect the accuracy of TLS. When Rad5 is inactivated, its effects are lost and the accuracy decreases. Further research is needed to explore the effects of Rad5 on TLS and other lesion bypass mechanisms.

Conclusions and future experiments

This study has demonstrated several significant findings. First, it confirmed that MMR can affect oligo transformation. MMR can recognize ^{Me}G:G and G:G mismatches and cause partial removal of the oligo, which yields Trp+ and *SphI*- cells, or presumed complete removal,

which would lead to Trp- and *SphI*- cells. Second, results with *rad5* mutants demonstrated that Trp reversion and the formation of the *SphI* restriction site are affected by not only oligo removal, but also template switching. Unlike a normal G, ^{Me}G can trigger template switching if it is recognized as an abnormal base. While bypassing the lesion, template switching can also bypass the C required for Trp reversion or the G necessary to create the *SphI* site. If this occurs, the resulting cells will be Trp- or *SphI*-, respectively.

In addition to examining oligo incorporation, sequencing was used to explore the accuracy of replication of the ^{Me}G lesion. This study discovered that when a manageable amount of damage occurs within the genome, yeast MMR can distinguish between ^{Me}G:C and ^{Me}G:T and ensure accurate replication of the lesion. In the absence of MMR, replication was inaccurate. This finding suggests that pol ζ, which is considered a low fidelity polymerase, may be responsible for replicating across the ^{Me}G lesion. Future experiments can be done to determine whether pol ζ is in fact responsible. Pol ζ is encoded by *REV3* and *REV7*. Thus, experiments with *rev3 mgt1* or *rev7 mgt1* mutants can be used to reveal pol ζ's specific role in ^{Me}G replication.

Although the findings suggest that pol ζ plays a role in replication of ^{Me}G, the results also show that other polymerases and mechanisms may be involved. In the absence of Rad18, pol ζ should not be functional. Even without pol ζ activity, however, replication still occurred across the lesion. This replication could be performed by pol η as suggested by recent experiments indicating that pol η may have Rad18 independent function. To confirm this pol η activity, future experiments with *rad30* mutants should be performed. Since *RAD30* codes for pol η, these mutants should provide insight into whether this Rad18 independent replication is due to pol η.

In addition to demonstrating the possible involvement of pol ζ and pol η, this study showed that replication of the ^{Me}G lesion in the absence of Rad18 is affected by the replication strand of the lesion. Replication was generally more accurate when the lesion was in the lagging strand than in the leading strand. Even with full MMR activity, replication of the ^{Me}G lesion in the leading strand without Rad18 was partially inaccurate. Further experiments are required to determine the cause of this strand bias.

Finally, this study showed that Rad5 plays a role in replicating across the ^{Me}G lesion. Comparing *rad5 msh6 mgt1* mutants to *msh6 mgt1* mutants revealed that Rad5 is somewhat involved in accurate replication. Although Rad5 is traditionally associated with template switching, this mechanism is probably not responsible for the accurate replication. In order for template switching to bypass the ^{Me}G lesion and yield Trp+ and *SphI+* cells, it must create a small, precise loop, which is difficult and unlikely. As a result, the most probable explanation is that Rad5 can affect another pathway, like TLS. Future research will be necessary to determine which pathways are affected by Rad5 and how Rad5 promotes accurate replication of the ^{Me}G lesion.

Although this study explored several mechanisms for replicating across ^{Me}G, it did not investigate the effects of HR. Since ^{Me}G is known to trigger recombination, future experiments should be performed to investigate the effects of recombination on oligo incorporation and replication of the lesion. In yeast, HR can be disrupted by inactivating *RAD52*. Thus, *rad52* mutants can be used to explore the role of recombination in the cell's response to the ^{Me}G lesion.

In summary, this work has contributed the groundwork for the study of ^{Me}G replication. By examining cells that contain only one ^{Me}G lesion, this study explored the effects of low levels of alkylation damage. Since low levels of ^{Me}G damage likely represents the typical situation in

cells, these experiments provide insight into how cells cope with alkylation damage on a daily basis. Because alkylation damage is detrimental to the organism as a whole and can lead to diseases like cancer, understanding how cells replicate over alkylation lesions is a significant area of research that should be pursued further.

List of Figures

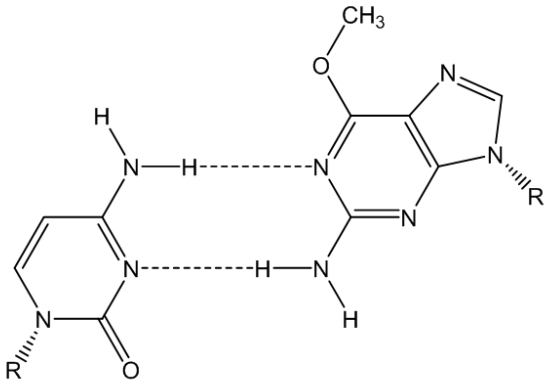

Figure 1: The wobble configuration created when ^{Me}G (right) pairs with C (left) at a neutral pH. The dashed lines represent hydrogen bonds [9].

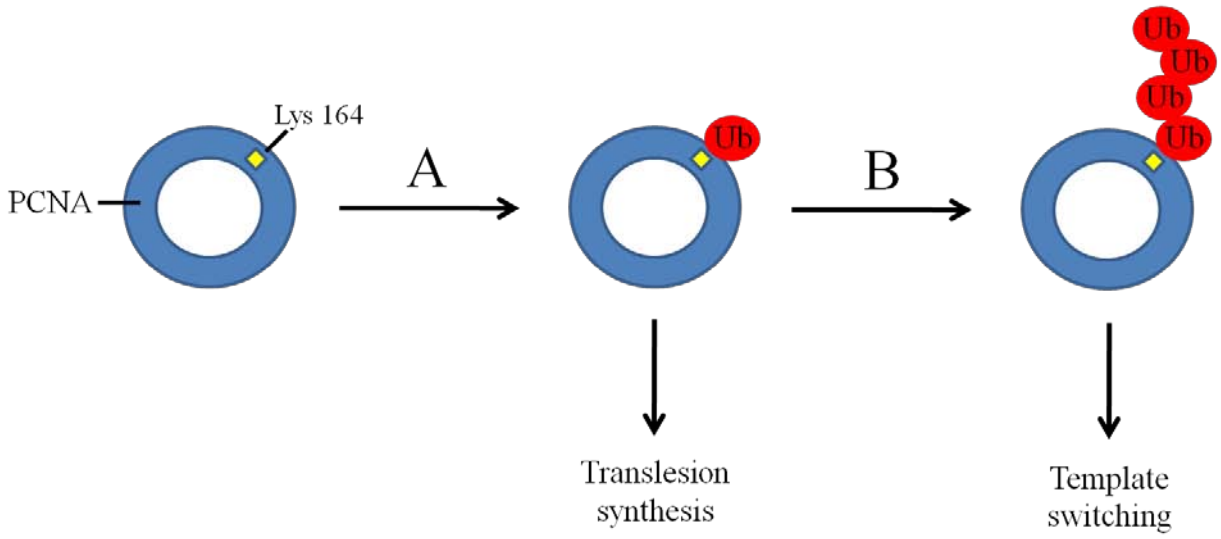

Figure 2: A. The Rad6-Rad18 complex monoubiquitinates PCNA on the Lys 164 residue, leading to translesion synthesis. B. Rad5 and the Mms2-Ubc13 complex create a polyubiquitin chain by linking additional ubiquitins, through the Lys 63 residue, to the first ubiquitin added by the Rad6-Rad18 complex. Polyubiquitination then leads to template switching [33, 36].

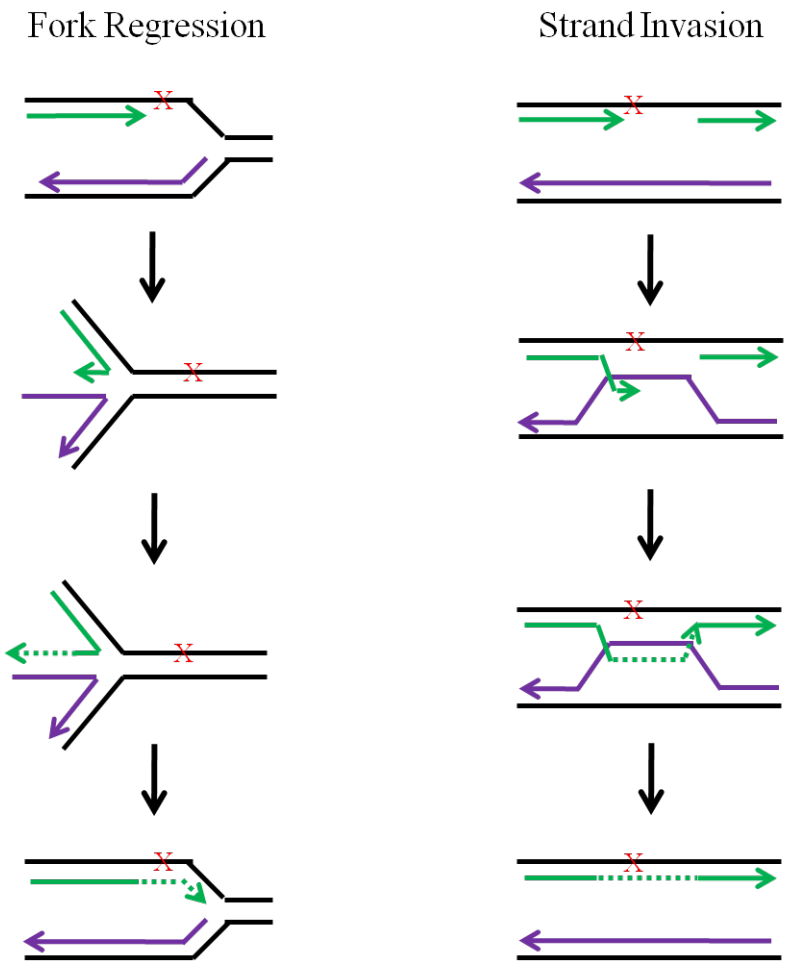

Figure 3: The two models of template switching. Fork regression (left) involves re-annealing of the parent strands (black) and annealing of the recently replicated strands (green and purple). The stalled strand (green) replicates past the lesion (red) using the recently replicated sister strand (purple) as a template. Strand invasion (right) can be used to fill in a gap left at the lesion (red). The stalled strand (green) invades the homologous region of the recently replicated sister strand (purple) and uses it as a template [36].

Primer ^{Me}G:

5’-GAGAAGGGCATGCC^{Me}GAGTTCGATAACATCGACACCACCAT-3’

Primer G:

5’-GAGAAGGGCATGCCGAGTTCGATAACATCGACACCACCAT-3’

Primer Lys2:

5’-CCAACCCTATCTTTCACATCAGGTTCCGAAGGTATTCCTA-3’

C = Introduces a C:C mismatch, reverts the *trp5* allele to wildtype

G = Introduces a G:A mismatch, forms a *SphI* restriction site

^{Me}**G** = Introduces a ^{Me}G:G mismatch, incorporates the O^6-methyl-dG lesion

G = Introduces a G:G mismatch, acts as a control for the ^{Me}G

C = Introduces a C:C mismatch, reverts the *lys2* allele to wildtype

Figure 4: The oligonucleotides used in this study

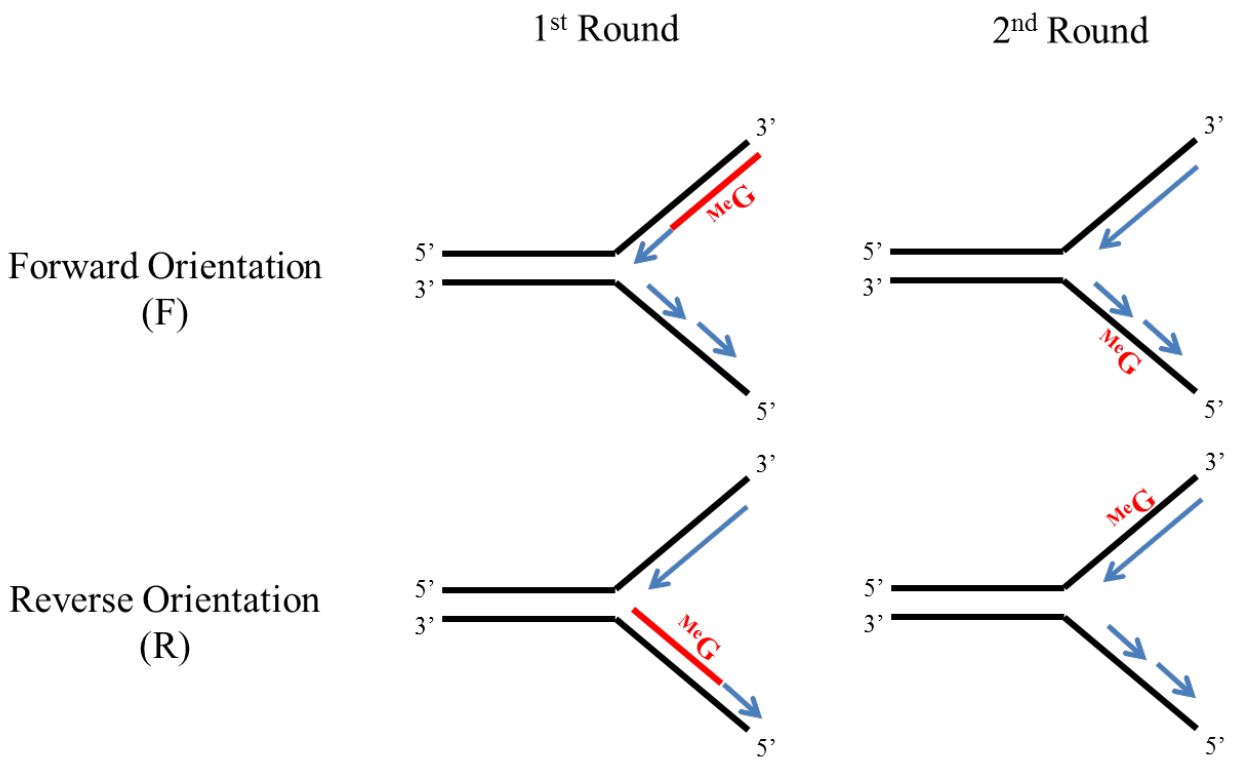

Figure 5: When the strain is in the forward orientation, the oligo with the ^{Me}G (shown in red) anneals to the leading strand. During the second round of replication the ^{Me}G lesion is on the lagging strand. The opposite occurs in the reverse orientation.

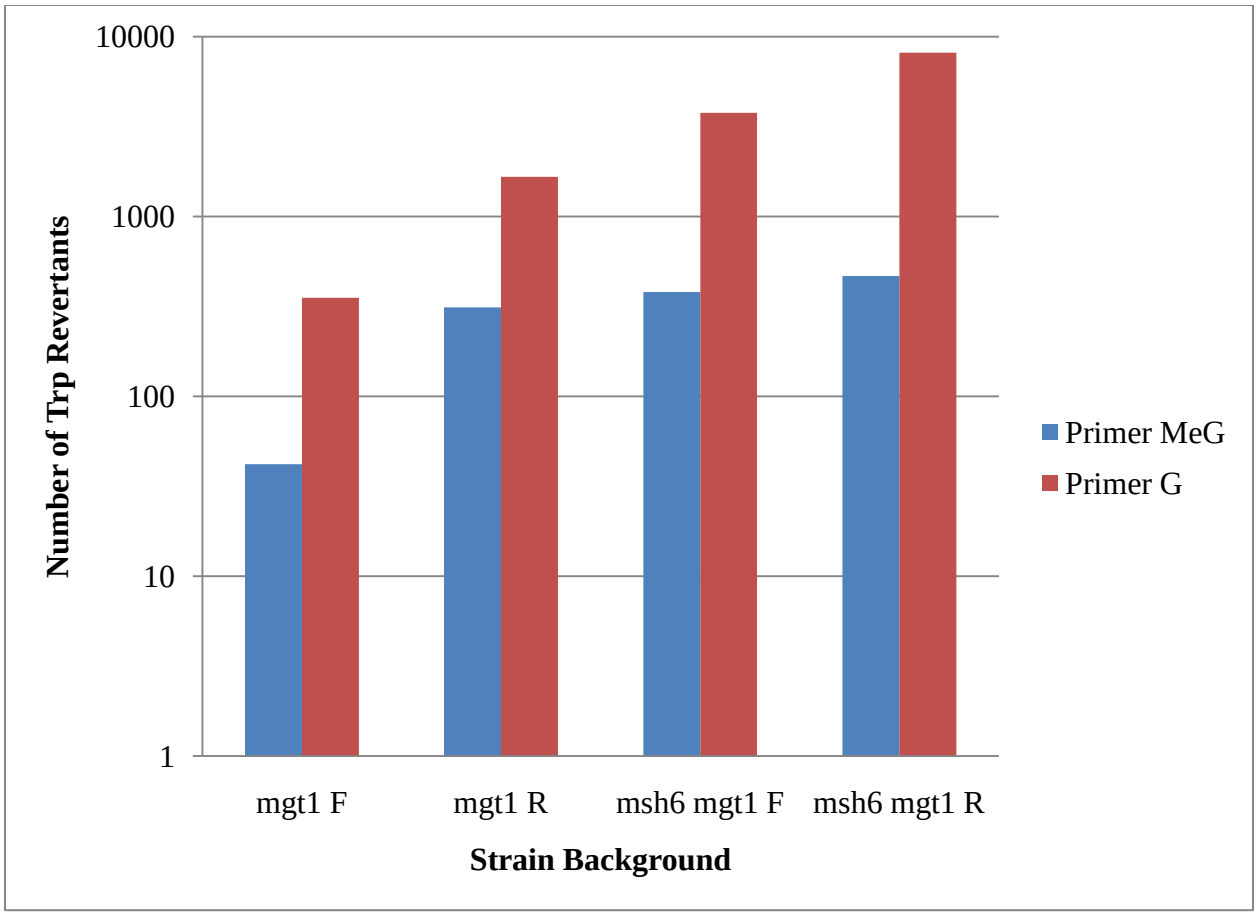

Figure 6: The increase in Trp revertants caused by inactivation of MMR. The increase was less prominent for the strains in the R orientation transformed with Primer ^{Me}G, but this was probably due to electroporation efficiency.

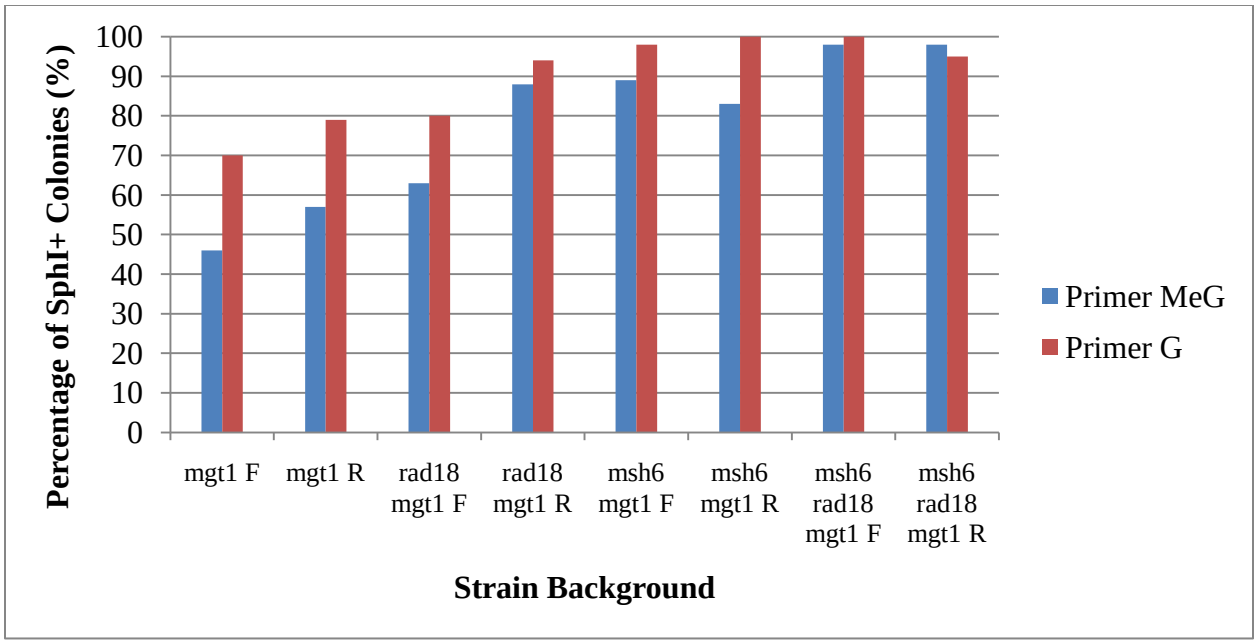

Figure 7: The increase in the percentage *SphI+* colonies caused by the inactivation of template switching, MMR, and the combination of template switching and MMR

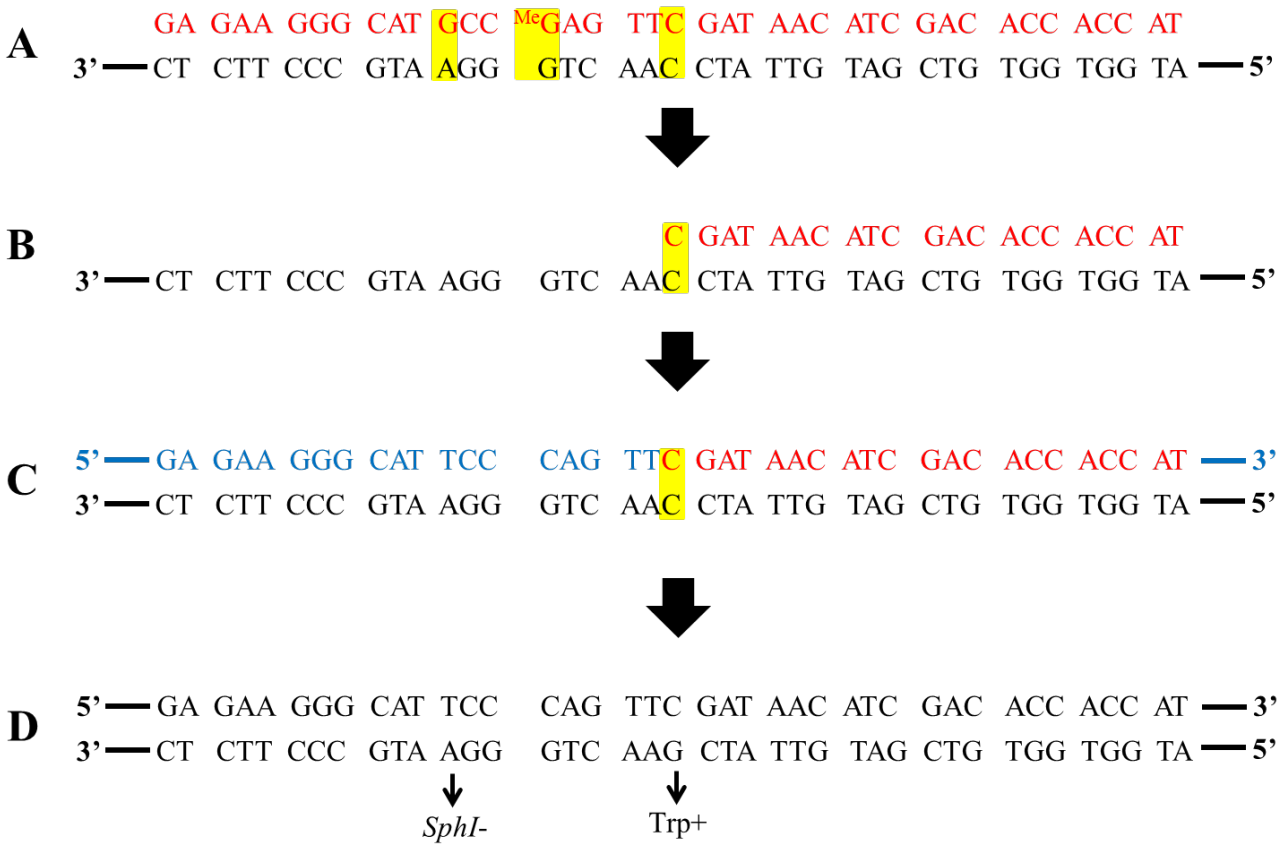

Figure 8: The partial removal of Primer ^{Me}G due to recognition by MMR. The same phenomenon can occur with Primer G. (A) The oligo (red) anneals to the yeast DNA (black). (B) The ^{Me}G:G mismatch is recognized and the oligo is removed up to the C:C mismatch. (C) Replication occurs around the oligo (blue). (D) The resulting DNA after replication.

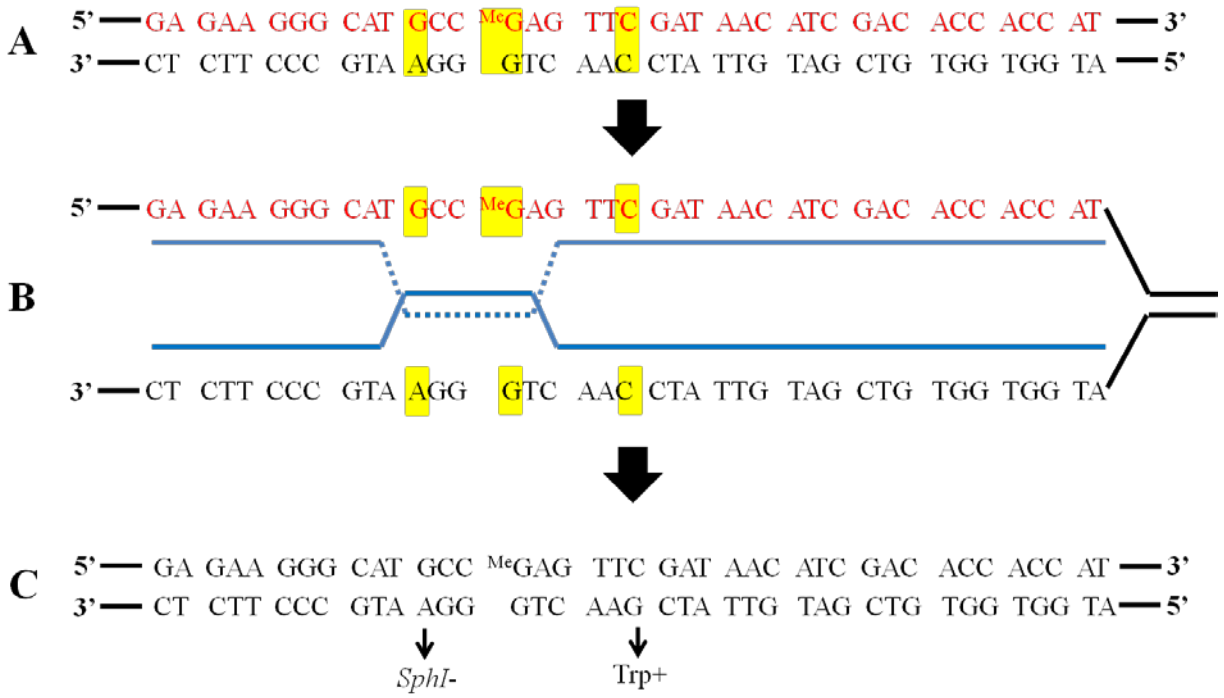

Figure 9: Template switching during the second round of replication. (A) Primer ^{Me}G (red) anneals to the yeast DNA and is completely incorporated. (B) During the second round of replication (blue), template switching (dashed line) is activated and used to bypass the ^{Me}G lesion. In the process, the G necessary for the formation of the *SphI* site is also bypassed. (C) The resulting DNA is Trp+ and *SphI*-.

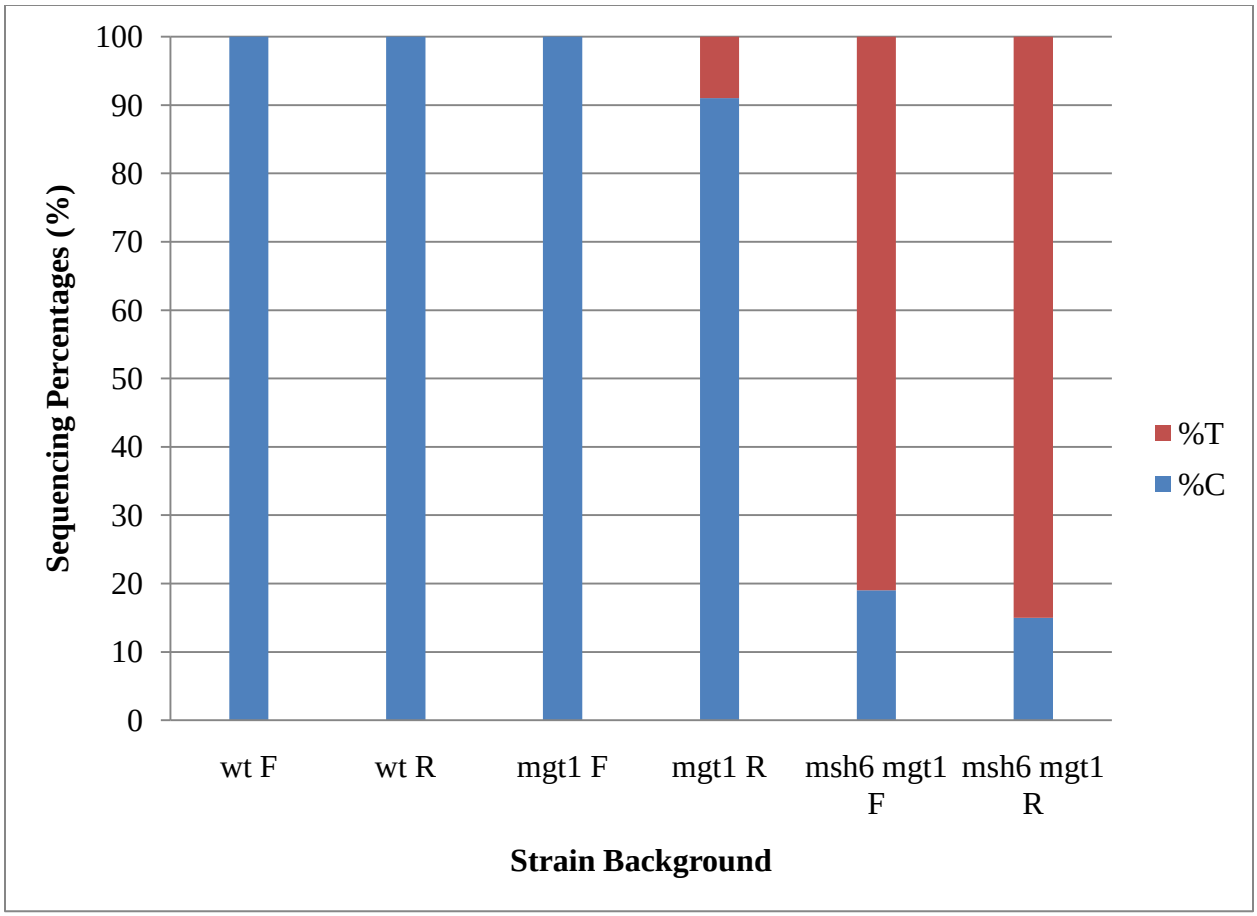

Figure 10: The effect of Mgt1 and MMR inactivation on replication accuracy

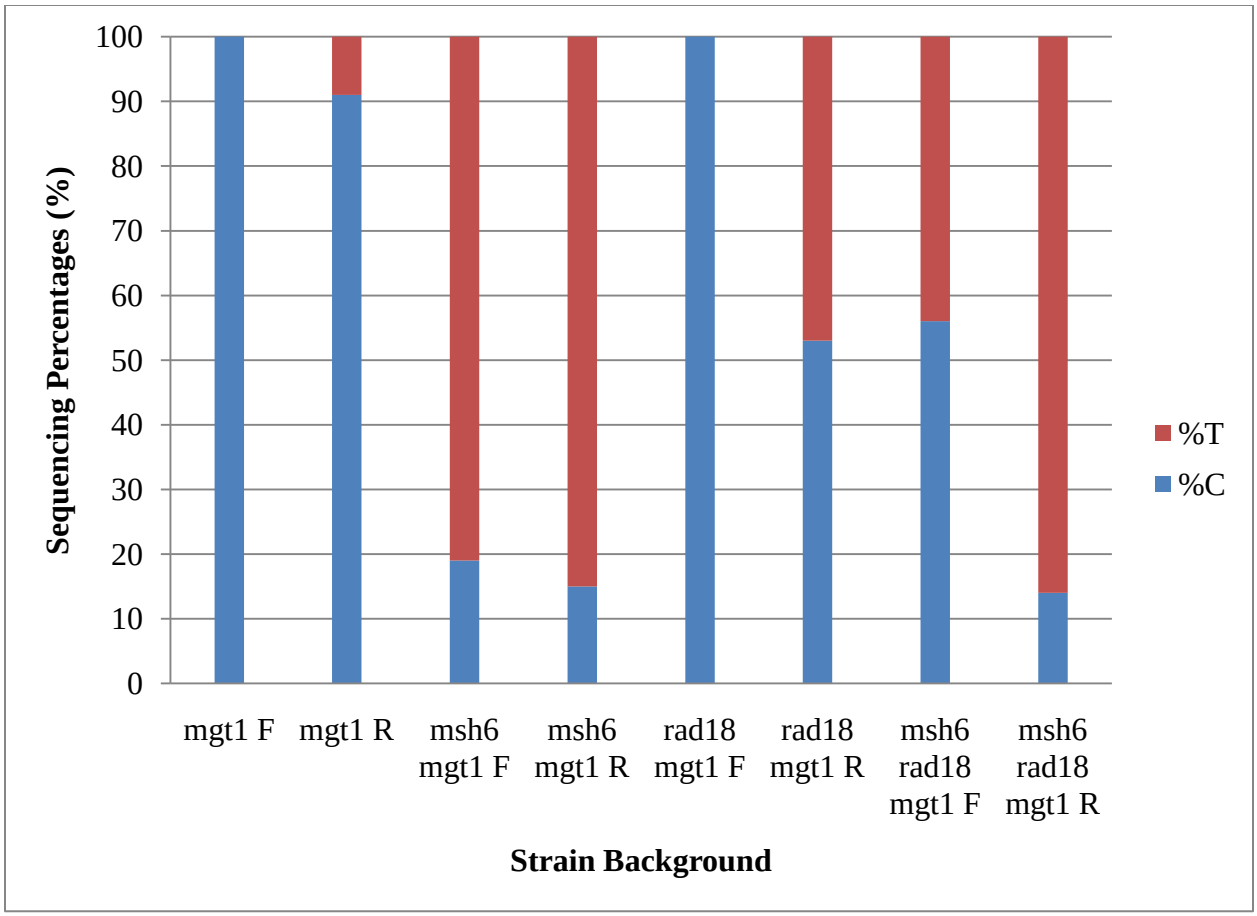

Figure 11: The effect of lesion location and MMR on the accuracy of Rad18 independent replication. In strains with the F orientation, the ^{Me}G lesion is in the lagging strand. In strains with the R orientation, the lesion is in the leading strand.

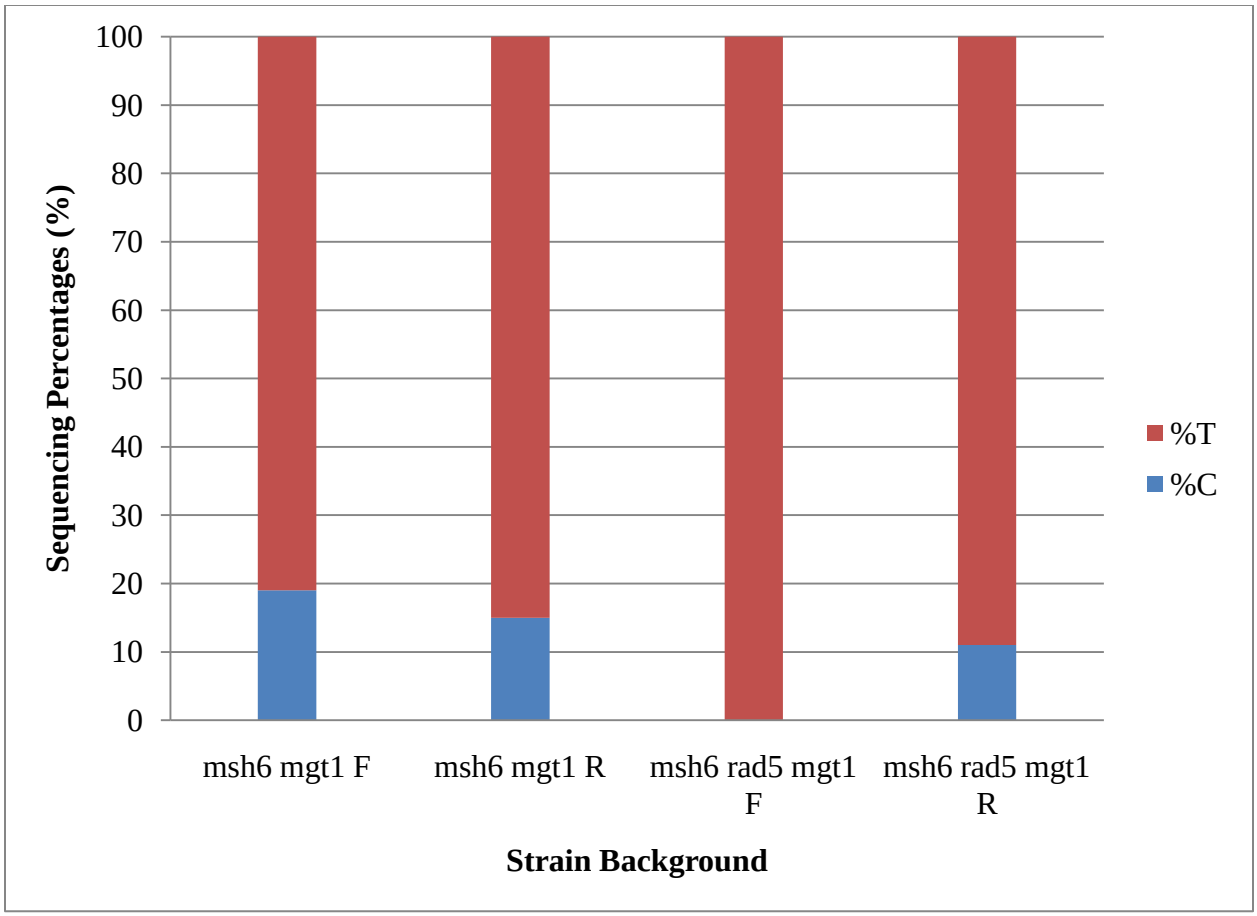

Figure 12: Inactivating Rad5 in *msh6 mgt1* mutants further decreases replication accuracy

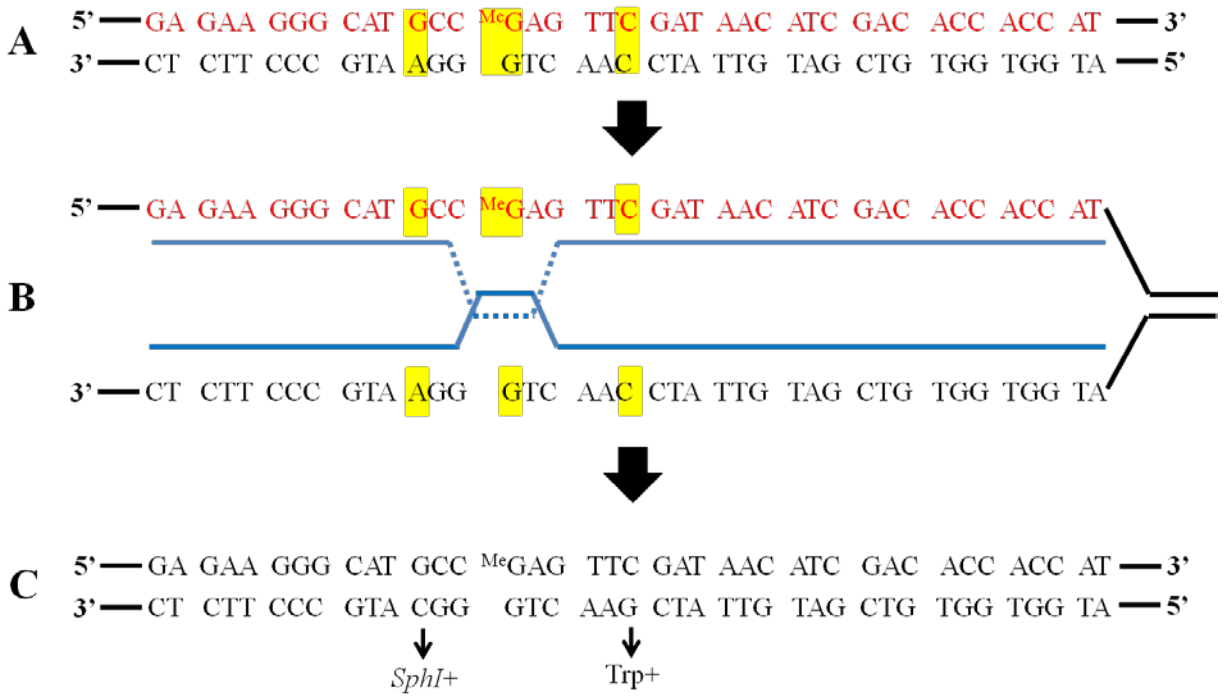

Figure 13: In order to create cells that are Trp+ and *SphI+* and accurately bypass the ^{Me}G lesion, template switching must create a small, precise loop. (A) Primer ^{Me}G (red) anneals to the yeast DNA and is completely incorporated. (B) During the second round of replication (blue), template switching (dashed line) is activated and used to bypass the ^{Me}G lesion. (C) The resulting DNA is Trp+ and *SphI+*.

List of Tables

Table 1: A list of the strains used in this study and their genotypes (all from the Crouse Lab)

Strain	Orientation of *trp5* mutation	Relevant Genotype
GCY2196	Forward (F)	wt
GCY2297	Reverse (R)	wt
GCY2335	F	*msh6Δ::hyg*
GCY2336	R	*msh6Δ::hyg*
GCY2477	F	*rad18Δ::kan*
GCY2478	R	*rad18Δ::kan*
GCY2492	F	*msh6Δ::hyg; rad18Δ::kan*
GCY2493	R	*msh6Δ::hyg; rad18Δ::kan*
GCY2525	F	*msh6Δ::hyg; rad5Δ::kan*
GCY2537	R	*msh6Δ::hyg; rad5Δ::nat*

Table 2: The strains used in this study and their genotypes

Strain	Orientation	Relevant Genotype
GCY2196	F	wt
GCY2297	R	wt
GCY2543	F	*mgt1Δ::his3*
GCY2579	R	*mgt1Δ::his3*
GCY2580	F	*msh6Δ::hyg; mgt1Δ::his5+*
GCY2581	R	*msh6Δ::hyg; mgt1Δ::his5+*
GCY2582	F	*rad18Δ::kan; mgt1Δ::his5+*
GCY2583	R	*rad18Δ::kan; mgt1Δ::his5+*
GCY2584	F	*msh6Δ::hyg; rad18Δ::kan; mgt1Δ::his5+*
GCY2585	R	*msh6Δ::hyg; rad18Δ::kan; mgt1Δ::his5+*
GCY2595	F	*msh6Δ::hyg; rad5Δ::kan; mgt1Δ::his5+*
GCY2596	R	*msh6Δ::hyg; rad5Δ::nat; mgt1Δ::his5+*

Table 3: The sequences of the primers used to amplify the relevant region of *TRP5*

Primer Name	Sequence
trpseq2	5'-CAGGAACGCCTTGGTCACAT-3'
trpseq8	5’-ATGGGTACGGTAACACCTTC-3’

Table 4: Single-stranded oligo transformation, *SphI* restriction site, and sequencing results based on strain background

Strain	Primer	Lys	Trp	SphI+	%C	%T
2196 (wt F)						
	^{Me}G	11300	168	70%	100%	0%
	G	4380	126	72%		
2297 (wt R)						
	^{Me}G	1000	65	86%	100%	0%
	G	1300	228	100%		
2543 (*mgt1* F)						
	^{Me}G	9260	42	46%	100%	0%
	G	7060	353	70%		
2579 (*mgt1* R)						
	^{Me}G	2860	313	57%	91%	9%
	G	4340	1661	79%		
2580 (*msh6 mgt1* F)						
	^{Me}G	7450	380	89%	19%	81%
	G	8460	3773	98%		
2581 (*msh6 mgt1* R)						
	^{Me}G	2400	467	83%	15%	85%
	G	4200	8145	100%		
2582 (*rad18 mgt1* F)						
	^{Me}G	6050	7	63% *	100% *	0% *
	G	5150	84	80% *		
	^{Me}G	4170	7			
	G	3700	33			
2583 (*rad18 mgt1* R)						
	^{Me}G	1900	12	88% *	53% *	47% *
	G	4260	75	94% *		
	^{Me}G	6640	32			
	G	9740	315			
2584 (*msh6 rad18 mgt1* F)						
	^{Me}G	3160	35	98% *	56% *	44% *
	G	3500	305	100% *		
	^{Me}G	25900	329			
	G	21500	2468			
2585 (*msh6 rad18 mgt1* R)						
	^{Me}G	310	32	98% *	14% *	86% *
	G	340	179	95% *		
	^{Me}G	5040	137			
	G	3360	338			
2595 (*msh6 rad5 mgt1* F)						
	^{Me}G	1800	12	98% *	0% *	100% *
	G	2100	278	98% *		
	^{Me}G	8790	180			
	G	9910	606			
2596 (*msh6 rad5 mgt1* R)						
	^{Me}G	7280	298	96%	11%	89%
	G	14600	1425	98%		

* When two transformations were performed per primer per strain, the *SphI+* percentage and the sequencing percentages were based on a combination of both transformation

Table 5: The ratio of the number of Trp revertants generated by Primer ^{Me}G to the number created by Primer G

	Ratio of Primer ^{Me}G to Primer G Trp revertants	
Strain Background	**Lagging Strand (F)**	**Leading Strand (R)**
mgt1	0.12	0.19
msh6 mgt1	0.10	0.06
msh6 rad18 mgt1	0.11	0.41

Table 6: The accuracy of replication depending on the replication strand of the ^{Me}G lesion

	Accuracy of replication based on the location of the ^{Me}G lesion	
Strain Background	**Lagging Strand (F)**	**Leading Strand (R)**
mgt1	100%	91%
msh6 mgt1	19%	15%
rad18 mgt1	100%	53%
msh6 rad18 mgt1	56%	14%
msh6 rad5 mgt1	0%	11%

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