

# The *S. cerevisiae* Mag1 3-methyladenine DNA glycosylase modulates susceptibility to homologous recombination

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## Abstract

DNA glycosylases, such as the Mag1 3-methyladenine (3MeA) DNA glycosylase, initiate the base excision repair (BER) pathway by removing damaged bases to create abasic apurinic/apyrimidinic (AP) sites that are subsequently repaired by downstream BER enzymes. Although unrepaired base damage may be mutagenic or recombinogenic, BER intermediates (e.g. AP sites and strand breaks) may also be problematic. To investigate the molecular basis for methylation-induced homologous recombination events in *Saccharomyces cerevisiae*, spontaneous and methylation-induced recombination were studied in strains with varied *MAG1* expression levels. We show that cells lacking Mag1 have increased susceptibility to methylation-induced recombination, and that disruption of nucleotide excision repair (NER; *rad4*) in *mag1* cells increases cellular susceptibility to these events. Furthermore, expression of *Escherichia coli* Tag 3MeA DNA glycosylase suppresses recombination events, providing strong evidence that unrepaired 3MeA lesions induce recombination. Disruption of *REV3* (required for polymerase  $\zeta$  (Pol  $\zeta$ )) in *mag1 rad4* cells causes increased susceptibility to methylation-induced toxicity and recombination, suggesting that Pol  $\zeta$  can replicate past 3MeAs. However, at subtoxic levels of methylation damage, disruption of *REV3* suppresses methylation-induced recombination, indicating that the effects of Pol  $\zeta$  on recombination are highly dose-dependent. We also show that overproduction of Mag1 can increase the levels of spontaneous recombination, presumably due to increased levels of BER intermediates. However, additional *APNI* endonuclease expression or disruption of *REV3* does not affect *MAG1*-induced recombination, suggesting that downstream BER intermediates (e.g. single strand breaks) are responsible for *MAG1*-induced recombination, rather than uncleaved AP sites. Thus, too little Mag1 sensitizes cells to methylation-induced recombination, while too much Mag1 can put cells at risk of recombination induced by single strand breaks formed during BER.

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## 1. Introduction

Base excision repair (BER) and nucleotide excision repair (NER) preserve sequence integrity by excising damaged nucleotide(s) [1–4]. When excision repair

fails to fully restore the DNA prior to DNA replication, processes involving homologous recombination can help cells tolerate DNA damage [5]. For example, if a DNA polymerase is inhibited by a lesion, homologous recombination may facilitate substitution of an undamaged template for one that contains the lesion (Fig. 1A). In addition, single strand breaks (and possibly unrepaired lesions that inhibit DNA polymerases) in the template DNA may lead to replication

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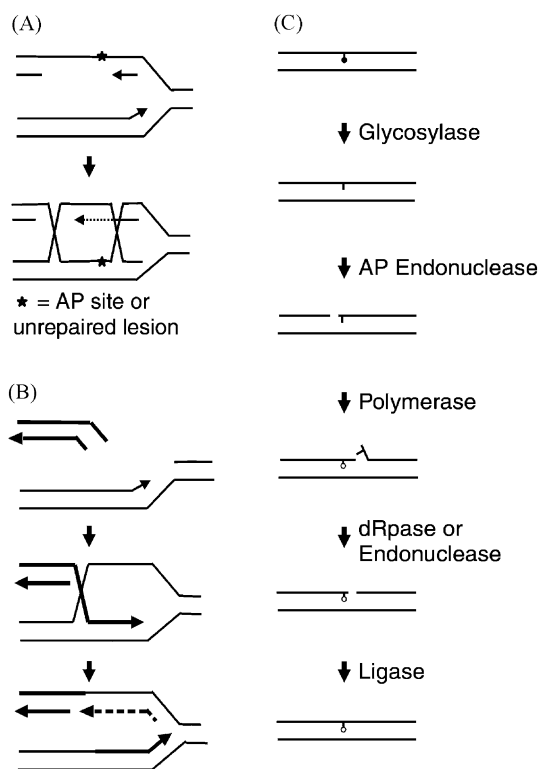


Fig. 1. (A and B) Possible consequences of a replication fork encountering unrepaired lesions or BER intermediates. (A) Repair of a daughter strand gap via sister chromatid exchange (endonucleolytic cleavage of the single stranded DNA gap may initiate this process; not depicted here). (B) A single strand break, or possibly an unrepaired lesion, causes collapse of the replication fork, which can then be reinserted via homologous recombination. Note that endonucleolytic processing to create a single stranded 3' overhang precedes homology searching. Resolution of the resulting Holliday junction restores the replication fork in break-induced replication [5]. (C) Schematic representation of the BER pathway. In this version, only short-patch BER initiated by a monofunctional glycosylase that lacks AP lyase activity (e.g. Mag1) is shown.

collapse, and there is mounting evidence that homologous recombinational repair restores collapsed replication forks (Fig. 1B) [6–9]. Processes that involve homologous recombination generally do not result in sequence changes. However, recombination between misaligned sister chromatids or between homologous chromosomes can lead to deletions and loss of heterozygosity [10]. Although our understanding of the relationship between damaged bases and their resulting point mutational spectrum is rather sophisti-

cated, much less is known about how changes to base structure lead to homologous recombination events.

Alkylating agents comprise one of the broadest classes of DNA damaging agents and these agents are present both in the environment and within normal cells [4,11]. As a model for this class of damage, we have focused on methylation damage, one of the simplest forms of alkylation damage. Methylating agents produce many different types of lesions with varied biological consequences [11,12]. The most abundant methylation lesions are 7-methylguanine (7MeG, 60–80%), *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup> MeG, 0.3–8%) and 3MeA (8–12%) [13]. Although 7MeG is readily removed from the genome of mammalian cells ( $t_{1/2} = \sim 20$  h for mouse embryonic stem cells; [14]), the normal steady state level of 7MeG in mouse cells is >10,000 adducts per cell [15], which suggests that the genome is under constant assault by methylating agents. Among the different types of methylated bases, 3MeA and *O*<sup>6</sup>MeG have some of the most serious biological consequences. While several studies have addressed the effects of *O*<sup>6</sup>MeG on mitotic homologous recombination in eukaryotes (e.g. [16–18]), much less is known about the recombinogenic potential of 3MeA.

3MeA is a toxic lesion that inhibits DNA polymerases in vitro [19]. In *Saccharomyces cerevisiae*, one of the main defenses against 3MeA is the Mag1 3MeA DNA glycosylase [20]. Mag1 initiates BER by cleaving the glycosylic bond between 3MeA and the deoxyribose of the sugar phosphate backbone (Fig. 1C). The resulting apurinic/apyrimidinic (AP) site is then cleaved 5' to the abasic sugar by the major AP endonuclease, Apn1, which is responsible for >95% of the AP endonuclease activity in *S. cerevisiae* [21]. The 3' hydroxyl group created by Apn1 is then extended one or several nucleotides by DNA polymerase  $\delta$  or  $\epsilon$  (Pol  $\delta$  or  $\epsilon$ ) [22,23]. Finally, the abasic deoxyribose is removed by the Rad27 flap-endonuclease [24], or possibly by a deoxyribose phosphatase, to facilitate DNA ligation [1,25]. *S. cerevisiae* deficient in 3MeA DNA glycosylase activity are very sensitive to the toxic effects of agents that create 3MeA [20], and the additional disruption of genes required for homologous recombination makes such cells even more sensitive to methylation damage [26], suggesting that both BER and homologous recombination help prevent methylation-induced toxicity.

NER deficient *S. cerevisiae* show very little sensitivity to methylation damage [27]. However, *S. cerevisiae* lacking both Mag1 and NER are far more sensitive to the toxic effects of the methylating agent methyl methanesulfonate (MMS) than are cells lacking only Mag1, suggesting that NER can effectively remove potentially toxic lesions normally repaired by Mag1 [27,28]. NER involves concomitant dual incisions on either side of the original damage, generating an oligonucleotide 25–30 bases long for removal [2]. In *S. cerevisiae*, NER involves over a dozen proteins. Unlike BER, wherein components of the pathway are able to act independently of one another, most of the NER components are required to act in concert to cleave the DNA. As an example, although Rad4 and Rad23 interact to form a damage recognition complex [29,30], Rad4 is also absolutely required to be present in the NER complex in order for incision to take place [29,31].

Mag1 deficient cells are highly sensitive to the toxic effects of methylation damage [20]. Paradoxically, expression of *MAG1* from the *GAL1* promoter also makes *S. cerevisiae* sensitive to methylating agents [32]. Since 7MeG comprises the vast majority of methylation-induced lesions, high levels of Mag1 activity may overburden downstream BER enzymes by converting otherwise benign 7MeG lesions into cytotoxic abasic sites or other downstream BER intermediates [32,33].

Another mechanism for tolerating DNA lesions is translesion replication. Pol  $\zeta$ , formed from a complex of the Rev3 and Rev7 proteins, is a non-essential polymerase that facilitates DNA replication past AP sites and other DNA lesions known to inhibit DNA replication [34–38]. Cells deficient in Pol  $\zeta$  have an increased susceptibility to recombination caused by AP sites, presumably because inhibition of DNA replication induces homologous recombination [39]. It is not yet known if Pol  $\zeta$  plays an analogous role in the case of 3MeA lesions.

In this work, we have explored the ability of 3MeA and its downstream BER intermediates to induce homologous recombination in *S. cerevisiae*. In the studies presented here, we show that cells deficient in the ability to repair 3MeA lesions have an increased susceptibility to methylation-induced mitotic homologous recombination, which is consistent with a model wherein unrepaired 3MeA induces recombination. On

the other hand, overexpression of *MAG1* increases cellular susceptibility both to methylation-induced recombination events and to spontaneous recombination events, suggesting that BER intermediates are potentially recombinogenic. The effects of Pol  $\zeta$  on spontaneous and damage-induced homologous recombination were also investigated.

## 2. Materials and methods

### 2.1. Media and growth conditions

*S. cerevisiae* strains were grown non-selectively in yeast extract–peptone supplemented with adenine (1% yeast extract–2% Bacto-peptone–2% dextrose–1.5% agar for plates–30 mg/ml adenine). Cells were grown in synthetic complete (SC) medium [40] lacking leucine during liquid culture (to prevent expansion of recombinant cells) and in SC medium lacking histidine to select for recombinants (controls were grown in SC medium). For gene induction experiments, medium was supplemented with 2% glucose, 2% raffinose, or 2% galactose. When galactose-inducible protein expression was required, cultures were expanded in media supplemented with raffinose, incubated in liquid media supplemented with galactose for 2.5 h, and plated on solid media supplemented with galactose. MMS was purchased from Sigma (St. Louis, MO) and was added to molten media at 55 °C at the indicated concentrations; cells were plated within 2.5 h after the media solidified. 5-Fluoroorotic acid (5-FOA) was purchased from Sigma (St. Louis, MO) and plates were prepared according to standard protocols [41].

### 2.2. Strains

Table 1 lists yeast strains used in this study. Strains CHY7 and CHY34 were created from RSY6 and Y433, respectively [42], by one-step gene replacement of *APN1* with an *EcoRI/BamHI* deletion/disruption cassette released from pSCP108 (a kind gift of B. Demple) [21]. Transformants that were *apn1::URA3* were identified by MMS sensitivity (data not shown). Strains CHY8 and CHY35 were constructed from RSY6 and Y433, respectively, using a *mag1*-deletion/disruption cassette generated by PCR amplification of the 1.6 kb gene blaster

Table 1  
Strains used in this study

Strain	Genotype	Reference
<b>Haploids</b>		
RSY6	<i>MAT<math>\alpha</math> HIS3::pRS6 leu2-3,112 ura3-52 trp5-27 ade2-40 ilv1-92 arg4-3</i>	Schiestl et al. [42]
CHY7	Same as RSY6 except <i>apn1<math>\Delta</math>::hisG</i>	This study
CHY8	Same as RSY6 except <i>mag1<math>\Delta</math>::kan<sup>r</sup></i>	This study
CHY9	Same as RSY6 except <i>rad4<math>\Delta</math>::hisG</i>	This study
MRY11	Same as RSY6 except <i>rev3<math>\Delta</math>::hisG</i>	This study
CHY10	Same as RSY6 except <i>mag1<math>\Delta</math>::kan<sup>r</sup> rad4<math>\Delta</math>::hisG</i>	This study
MRY12	Same as RSY6 except <i>mag1<math>\Delta</math>::kan<sup>r</sup> rad4<math>\Delta</math>::hisG rev3<math>\Delta</math>::hisG</i>	This study
Y433	<i>MAT<math>\alpha</math> his3-<math>\Delta</math>200 leu2-<math>\Delta</math>98 ura3-52 ade2-101 lys2-801</i>	Schiestl et al. [42]
CHY34	Same as Y433 except <i>apn1<math>\Delta</math>::hisG</i>	This study
CHY35	Same as Y433 except <i>mag1<math>\Delta</math>::kan<sup>r</sup></i>	This study
CHY36	Same as Y433 except <i>rad4<math>\Delta</math>::hisG</i>	This study
MRY38	Same as Y433 except <i>rev3<math>\Delta</math>::hisG</i>	This study
CHY37	Same as Y433 except <i>mag1<math>\Delta</math>::kan<sup>r</sup> rad4<math>\Delta</math>::hisG</i>	This study
MRY39	Same as Y433 except <i>mag1<math>\Delta</math>::kan<sup>r</sup> rad4<math>\Delta</math>::hisG rev3<math>\Delta</math>::hisG</i>	This study
<b>Diploids</b>		
RS112	RSY6 X Y433	Schiestl et al. [42]
CHY113	CHY7 X CHY34	This study
CHY114	CHY8 X CHY35	This study
CHY116	CHY9 X CHY36	This study
MRY113	MRY11 X MRY38	This study
CHY115	CHY10 X CHY37	This study
MRY114	MRY12 X MRY39	This study

fragment of pUG6 [43]. The primers contained 58–60 bp overhangs with homology to sequences flanking the *MAG1* coding sequence. Properly targeted G418<sup>r</sup> *mag1* clones were identified by sensitivity to MMS-induced toxicity (data not shown). To create CHY9 and CHY10, *RAD4* was disrupted using a *rad4*-deletion/disruption cassette generated by PCR amplification of the 2.0 kb gene blaster fragment of pMPY-ZAP [44]. The primers contained 55–57 bp overhangs with a homology to the regions directly 5' and 3' to the *RAD4* sequence. Strains CHY36 and CHY37 were constructed using a 5.3 kb *KpnI* *rad4*-deletion/disruption cassette released from pNKY-rad4 (for construction of pNKY-rad4, see later). All *rad4 $\Delta$ ::URA3* clones were confirmed both by PCR amplification of a fragment bridging the inserted and flanking sequences and by increased sensitivity to UV-induced toxicity (data not shown). The *REV3* gene was disrupted in MRY11, MRY12, MRY38, and MRY39 by one-step gene replacement with an 8 kb *REV3*-deletion/disruption cassette released from pYPG101 by *KpnI* (pYPG101 was a

gift of C. Lawrence). The *rev3 $\Delta$ ::URA3* clones were confirmed both by PCR amplification of a fragment bridging inserted and flanking sequences (the appropriate sequence for the flanking primer was obtained by determining the sequence of 200 bases on the 5' end of the targeting cassette and obtaining sequences outside the targeting vector from The *Saccharomyces* Genome Database<sup>TM</sup>). Proper disruption of *REV3* was also confirmed by increased sensitivity to the toxic effects of both UV (data not shown) and MMS. All *URA3* markers were recycled [45] by selection on 5-FOA [46] immediately following strain verification. Diploid strains were constructed by mating the *MAT $\alpha$*  and *MAT $\alpha$*  haploid strains and selecting on plates lacking lysine and tryptophan.

### 2.3. Plasmids

Vectors pYES-Tag, pYES-MAG, and pYES-MGT (gifts of B. Glassner and L. Samson) have the sequences of 3MeA glycosylases (*Escherichia coli* Tag and *S. cerevisiae* *MAG1*), and the *S. cerevisiae*

repair methyltransferase (*MGT1*) cloned into pYES2.0 (Invitrogen) between *Bam*HI and *Xho*I. For AP endonuclease overexpression, pYES-APN1 was constructed by subcloning the *APN1* coding sequence (PCR amplified from YEpAPN1, a gift of B. Demple [21]) into pYES2.0 between *Bam*HI and *Xho*I. Effective expression was confirmed by reversion of the MMS sensitivity of CHY7 (data not shown). Plasmid pYES-MAG-APN was constructed to co-express *MAG1* under the galactose-inducible promoter and *APN1* under its own promoter. The *Bgl*III fragment of the YEpAPN1 plasmid, containing the *APN1* gene under its own promoter, was PCR-amplified and subcloned into the pYES-MAG vector at *Spe*I. pNKY-rad4 is a derivative of pNKY51 (gifts of R. Bennett and B. Demple) which contains a *URA3* marker flanked by *Salmonella typhimurium hisG* sequences [45]. To create pNKY-rad4, 0.5–0.7 kb PCR products corresponding to the regions flanking *RAD4* were directionally cloned 5' and 3' to the gene blaster fragment of pNKY51.

#### 2.4. Cell survival and recombination assays

Non-quantitative determination of MMS sensitivity of strains was evaluated by gradient plate analysis of stationary phase cells as described previously [47]. Non-quantitative determination of UV sensitivity was similarly determined by gradient plate analysis of log phase cells.

Cells were grown to log phase (UV studies) or stationary phase (MMS studies and spontaneous recombination studies) in minimal media lacking leucine, serially diluted, and 20 ml aliquots were placed on solid minimal complete media and solid media lacking histidine. For studies of UV effects, cells were exposed to UV in a Stratalinker (Stratagene Inc., La Jolla, CA) immediately after plating. For studies of MMS effects, strains were plated onto control and MMS containing plates in 20 ml aliquots (up to 36 aliquots per 9 cm<sup>2</sup> square dish). Plates were incubated at 30 °C for 1–4 days. Colonies were counted under a 10× dissecting microscope once a standard average colony size was reached. A minimum of 10 colonies total were counted among at least two independent aliquots for each measurement. The frequency of *HIS3* recombinants was calculated as colonies/ml on media lacking histidine divided by colonies/ml on minimal complete media.

#### 2.5. Nucleic acid techniques

Restriction endonucleases were from New England Biolabs (Beverly, MA). Taq polymerase was from Gibco BRL, while Advantage 2 polymerase was from Clontech (Palo Alto, CA). Taq polymerase was used for analytical PCR and Advantage 2 polymerase for PCR subcloning. Plasmid DNA and yeast chromosomal DNA were isolated and manipulated according to standard procedures [41]. Gene disruption cassettes and plasmid DNA were transformed into yeast by electroporation, or by lithium acetate transformation [41]. Cell lysates for PCR from single colonies were prepared by lyticase disruption (2.3 mg/ml lyticase, 15 min, 30 °C) followed by one freeze thaw cycle at –78 °C. More than 25 sets of oligonucleotides (provided by Amitof Co., Allston, MA) were used to create vectors and confirm accurate gene targeting. Sequences are available upon request.

### 3. Results

#### 3.1. Overexpression of *S. cerevisiae* *MAG1* sensitizes cells to both MMS-induced cell death and mitotic recombination

MMS creates 7MeG (80–85%), 3MeA (9–12%), 3MeG (0.3–0.7%), O<sup>6</sup>MeG (0.3%), 7-methyladenine (1.8%), phosphotriesters (0.8%) as well as more minor lesions [13]. Although MMS has been shown to induce homologous recombination in *S. cerevisiae* [48], the lesions responsible for this effect were not known. To explore the basis for methylation-induced mitotic homologous recombination, we created diploid strains of *S. cerevisiae* with varied repair and damage tolerance capacities in which recombination can be observed by reconstitution of two mutant copies of the *HIS3* gene. One mutant allele containing a deletion in the 3' end (*his3-Δ3'*) precedes another mutant allele containing a 5' deletion (*his3-Δ5'*), while the entire open reading frame of the *HIS3* allele on the other homologue has been deleted [42] (Fig. 2A). The two non-functional *his3* alleles flank a *LEU2* cassette and pBR322 sequences. Note that *LEU2* is lost in ~99% of homologous recombination events [42]. Consequently, by growing cells in the absence of leucine, *HIS3* recombinants can be prevented from expanding in culture,

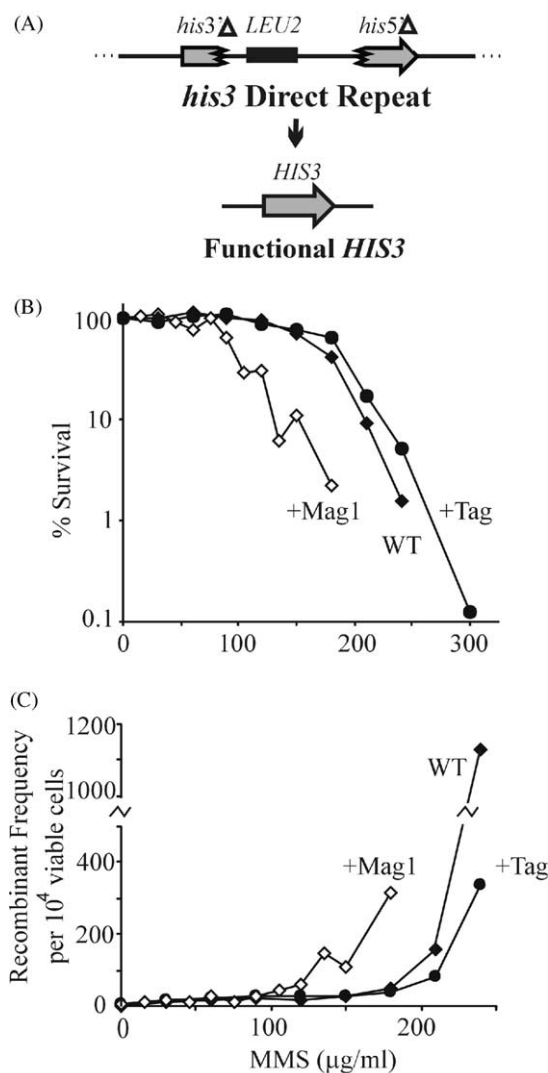


Fig. 2. (A) Schematic representation of the *his3* direct repeat used to detect recombination. (B and C) MMS-induced toxicity and recombination in RS112 diploid yeast induced to express *S. cerevisiae* *MAG1* 3MeA DNA glycosylase (open diamonds), *E. coli* Tag 3MeA DNA glycosylase (filled circles), or carrying the pYES control vector (wild type, WT; filled diamonds). Stationary phase cells were cultured in galactose containing media for 2.5 h to induce *MAG1* expression prior to exposure to MMS on solid media containing galactose. Percent survival was calculated from a colony forming assay and recombination frequency is plotted as the number of *HIS3* revertant colonies per viable cell. Representative data from two independent experiments are shown.

thus minimizing variation in recombination frequency. It is worth noting that diploid strains were used throughout these studies because the effect of Mag1 on MMS-induced recombination was not apparent in haploid cells. The underlying cause(s) for the observed differences between haploid and diploid *mag1* mutant cells are not yet known.

Uncleaved abasic sites inhibit DNA replication [49], and AP endonuclease deficient cells show increased levels of spontaneous homologous recombination, presumably due to replication inhibition caused by increased levels of uncleaved abasic sites [39]. Glassner et al. have shown that expression of *MAG1* from the galactose-inducible *GAL1* promoter leads to increased abasic site-induced point mutations [32]. Thus, we first set out to explore the possibility that overexpression of *MAG1* might induce mitotic homologous recombination by creating replication-blocking uncleaved abasic sites. Wild type diploid cells harboring the *his3* direct repeat and carrying the galactose-inducible pYES-MAG vector [32] were expanded in medium lacking leucine, incubated in the presence of galactose to induce *MAG1* expression, and subsequently plated on solid media containing varying concentrations of MMS. Consistent with previous studies [32], cells overexpressing *MAG1* have increased susceptibility to MMS-induced toxicity compared with their wild type counterparts expressing normal levels of Mag1 (Fig. 2B). This effect is thought to be due to increased levels of potentially toxic BER intermediates [32]. Here, we find that cells overexpressing Mag1 also have an increased susceptibility to MMS-induced homologous recombination (Fig. 2C). These data suggest that Mag1 creates BER intermediates that are more recombinogenic than the lesions being removed by Mag1.

Mag1 not only removes 3MeA, but it also takes out 7MeG, 7-methyladenine and even normal bases [20,50–52]. We were, therefore, interested in the nature of the lesions that were being converted into recombinogenic intermediates by Mag1. To explore this question, we asked if overexpression of a glycosylase that has a more narrow substrate range than Mag1 has the same effect. *E. coli* Tag almost exclusively repairs 3MeA and does not act on 7MeG or normal bases [52,53]. Unlike Mag1, overexpression of *tag* does not affect either MMS-induced toxicity or homologous recombination (Fig. 2B and C) (*tag* expression, however,

does rescue Mag1 deficient cells from MMS toxicity, see later). Thus, the increased levels of recombination observed in cells overexpressing *MAG1* does not appear to be caused by removal of 3MeA. Furthermore, the observation that expression of *tag* does not induce recombination suggests that there are not enough 3MeA lesions in the DNA to cause levels of BER intermediates to rise to toxic or recombinogenic levels. Alternatively, 3MeA itself may be recombinogenic, so that *tag* expression facilitates conversion of a recombinogenic lesion into a recombinogenic repair intermediate.

### 3.2. *MAG1* overexpression induces spontaneous homologous recombination in *S. cerevisiae*

Even in the absence of exogenous methylating agent, high levels of *MAG1* expression leads to a significant increase in mitotic homologous recombination (Fig. 3A). Although the identity of the Mag1 substrates is not known, the ability of Mag1 to take out normal bases, albeit inefficiently, may contribute to this effect [52]. Induced expression of *tag*, on the other hand, does not affect spontaneous homologous recombination (Fig. 3A). Therefore, removal of 3MeA by Mag1 does not appear to be responsible for the observed increase in spontaneous recombination in cells overexpressing *MAG1*.

*MAG1* overexpression may increase the steady state levels of any of several different BER intermediates, including uncleaved abasic sites, single strand breaks, and possibly flaps created if repair synthesis displaces one or more nucleotides (Fig. 1C) [1]. To explore the possibility that overexpression of *MAG1* induces homologous recombination by increasing the levels of uncleaved abasic sites, we varied the levels of AP endonuclease activity in cells overexpressing *MAG1*. Although wild type and *apn1* mutant cells show similar levels of spontaneous recombination (Fig. 3B), overexpression of *MAG1* in either wild type cells or *apn1* mutant cells has very different effects. Overexpression of *MAG1* in a wild type background causes an ~7-fold increase in the levels of spontaneous recombination, while overexpression of *MAG1* in an *apn1* mutant strain causes a dramatic ~20-fold increase in spontaneous recombination (Fig. 3B). When *APN1* expression is restored, this increase is suppressed, suggesting that uncleaved abasic sites generated by *MAG1* can induce recombination. Nevertheless, it remains possible that single strand breaks, rather than uncleaved abasic sites, underlie *MAG1*-induced recombination in wild type cells that harbor normal levels of *APN1*. Thus, *APN1* was coexpressed in cells that overexpress *MAG1*. Expression of additional *APN1* activity did not suppress *MAG1*-induced recombination in wild type cells, suggesting that uncleaved AP sites were not

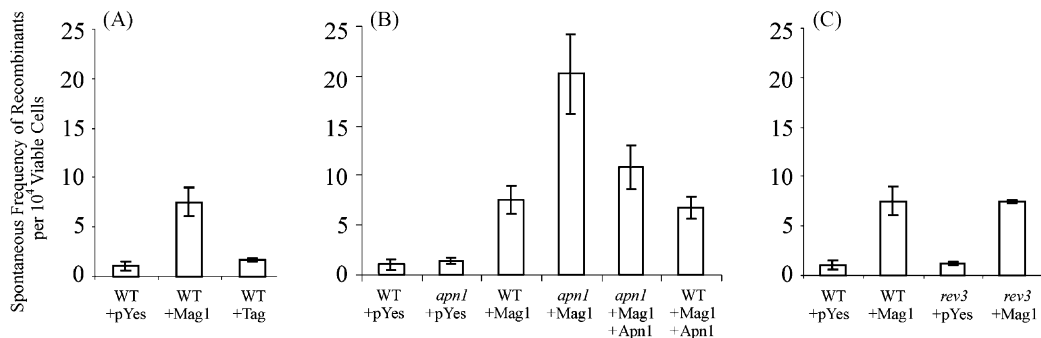


Fig. 3. Spontaneous recombination frequency for diploid strains carrying the indicated mutations and expressing *S. cerevisiae* *MAG1* or *E. coli* Tag from a galactose inducible promoter. When coexpressed with *MAG1*, *S. cerevisiae* *APN1* is under its own promoter. Approximately 1000 cells were expanded to stationary phase in 3–5 independent cultures (media lacking leucine was used to suppress expansion of recombinant cells). Stationary phase cells were cultured under inducing conditions for 2.5 h and subsequently diluted onto galactose plates to determine viable cell number and spontaneous recombination frequency. Consistent results were observed in at least three independent experiments. The error bars indicate 1 S.D. where each independent culture is weighted equally. (A) Comparison of the effects of overexpression of *tag* and *MAG1*. (B) The effects of varied expression of *MAG1* and *APN1*. (C) The effects of *REV3* status on spontaneous recombination and *MAG1*-induced recombination. The same data for wild type cells (WT and WT +pYes) and wild type cells induced to express *MAG1* (WT +Mag1) are shown in (A), (B) and (C) to facilitate comparisons.

responsible for Mag1-induced recombination in cells harboring normal levels of *APN1* (Fig. 3B). We conclude that in the absence of *apn1*, *MAG1* overexpression creates enough uncleaved AP sites to induce recombination, but that wild type levels of AP endonuclease activity may be sufficient to prevent uncleaved AP sites from inducing recombination. Alternatively, *APN1* expression may be converting recombinogenic uncleaved AP sites into downstream BER intermediates that are comparably recombinogenic. To differentiate among these two possibilities, the effects of *MAG1* overexpression were determined under conditions where cells have increased susceptibility to recombination induced by uncleaved AP sites, as described below.

Disruption of Pol  $\zeta$  in AP endonuclease deficient cells causes a dramatic increase in spontaneous homologous recombination events [39], which is consistent with a model wherein the ability of Pol  $\zeta$  to bypass AP sites prevents such uncleaved AP sites from inducing mitotic homologous recombination. To address the question of whether or not uncleaved abasic sites are responsible for *MAG1*-induced recombination, we disrupted *REV3*, which is essential for Pol  $\zeta$  activity [34]. Pol  $\zeta$  does not affect *MAG1*-induced recombination (Fig. 3C). Taken together, the results presented in Fig. 3B and C are consistent with a model wherein uncleaved AP sites are not responsible for *MAG1*-induced recombination in cells that express normal levels of AP endonuclease activity. It is likely that the nicks and gaps that are the inevitable consequence of processing such AP sites are responsible for *MAG1*-induced recombination in cells expressing normal levels of AP endonuclease.

### 3.3. Unrepaired methylated bases cause intrachromosomal homologous recombination

Although it is known that unrepaired UV-induced lesions induce recombination in yeast [54], little is known about potential of unrepaired methylated bases to induce recombination. Previous work shows that mouse cells deficient in 3MeA DNA glycosylase activity have increased susceptibility to 3MeA-induced sister chromatid exchanges (SCEs) [15,55], which is consistent with the possibility that unrepaired 3MeA induces recombination. However, NER is still active in these mouse cells and it may be the case that NER

intermediates, rather than unrepaired 3MeA, are the cause of the observed increase in SCEs. To explore the possibility that unrepaired 3MeA can induce homologous recombination in eukaryotic cells, we created cells deficient in both Mag1 and NER in which recombination can be assayed at the *his3* direct repeat (Table 1).

Before determining the frequency of methylation-induced recombination in such strains, it was first necessary to optimize the experimental approach. A standard approach for measuring MMS-induced homologous recombination in *S. cerevisiae* involves culturing cells for 17 h in liquid media that contains MMS and that lacks leucine (to prevent *HIS3* revertants that have lost the *LEU2* allele from expanding in culture) [48]. After 17 h, the frequency of *HIS3* revertants/viable cell is determined by a colony forming assay. However, because MMS breaks down in water, the effective concentration of MMS is expected to decrease during the 17 h exposure time. Given that eukaryotic cells lacking 3MeA DNA glycosylase activity are more prone to MMS-induced cell cycle arrest than are wild type cells [15], it is possible that certain concentrations of MMS may be high enough to induce cell cycle arrest in repair deficient cells, while having no effect on the doubling time of wild type cells. Since the recombination frequency is dependent on the total number of viable cells, a delayed cell division would reduce the culture density so that it would appear that when compared to wild type cells, the repair deficient strain has an increased number of recombinants/survivors. Cells were, therefore, exposed to MMS or UV only after being plated, so that the number of recombination events is evaluated by colony number (rather than by the total number of *HIS3* cells), which is independent of doubling time.

Doubly BER and NER deficient diploid cells were created by gene disruption, plated at various dilutions on control and selective media, and exposed to UV light or MMS. As expected from previous studies of NER mutant cells [54], the *rad4* mutant cells have increased sensitivity to UV-induced killing and increased susceptibility to UV induced-homologous recombination (Fig. 4A and B). The additional disruption of *MAG1* expression in the *rad4* null cells has no effect, which is consistent with the inability of Mag1 to act on UV damage. When exposed to methylation damage, cells lacking *MAG1* expression have

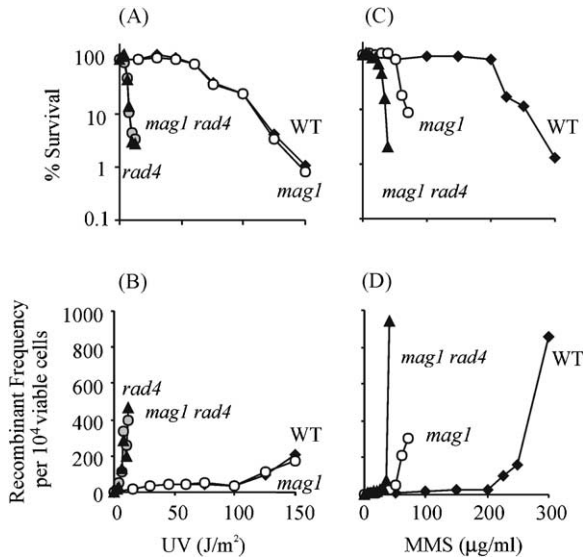


Fig. 4. The effects of deficiencies in 3MeA DNA glycosylase activity (*mag1*) and/or NER (*rad4*) on UV and MMS-induced recombination and toxicity. Diploid cells deficient in the yeast 3MeA DNA glycosylase (*mag1*) and/or nucleotide excision repair (*rad4*) were challenged with exposure to either UV (A and B) or MMS (C and D) and their sensitivity to DNA damage-induced killing and recombination were compared to wild type cells. The strains are wild type (WT; filled diamonds), *mag1* (open circles), *rad4* (shaded circles), *mag1 rad4* (filled triangles). For UV experiments, log phase cells were plated at various dilutions and exposed to the indicated doses of UV. For MMS experiments, cells were grown to stationary phase and plated on solid media containing the indicated concentrations of MMS. Experiments were repeated a minimum of three times and the data presented here show a representative curve.

increased susceptibility to both methylation-induced toxicity and recombination (Fig. 4C and D). To determine if NER intermediates are causing recombination in the *mag1* mutant cells, we compared methylation-induced recombination in *mag1* and *mag1 rad4* double mutant cells. Disruption of NER (*rad4* mutants) did not suppress methylation-induced recombination in *mag1* cells, which suggests that NER intermediates are not causing recombination in cells lacking Mag1. On the contrary, *mag1 rad4* cells have increased susceptibility to methylation-induced recombination compared to *mag1* mutant cells (Fig. 4D), which is consistent with a model wherein both Mag1 and NER prevent unrepaired methylated bases from inducing recombination.

It is worth noting that recombination tends to rise at the same doses of MMS that cause significant toxicity (Fig. 4C and D). While it is often observed that the frequency of homologous recombination increases at lethal levels of DNA damaging agents, in nature, cells are often exposed to sublethal levels of damaging agents. We were, therefore, interested in the possibility that Mag1 might prevent methylation-induced recombination even at sublethal levels of exposure. Fig. 5A and B show that cells lacking Mag1 have an increased susceptibility to methylation-induced recombination, even at doses of methylating agent that do not affect survival. This effect is even more apparent in cells lacking both Mag1 and NER. We, therefore, conclude that Mag1 excises potentially recombinogenic lesions from the genome of eukaryotic cells.

Since methylated bases can be produced endogenously in eukaryotic cells at measurable levels (e.g. [15]), we also examined whether or not Mag1 prevents spontaneous homologous recombination. Although cells lacking NER show a significant increase in susceptibility to spontaneous mitotic recombination (as has been shown previously [56]), disruption of *MAG1* expression has no effect on spontaneous mitotic recombination, either on its own or in combination with an NER deficiency (data not shown). These results indicate that the levels of spontaneous damage repaired by Mag1 are not sufficient to induce detectable changes in the frequency of homologous recombination. The basis for the increased spontaneous recombination in NER deficient cells is not yet known.

### 3.4. Unrepaired 3MeA is a primary cause of methylation-induced homologous recombination in excision repair-deficient cells

From the studies of MMS-induced recombination presented earlier, we cannot conclude that a particular lesion is the primary cause of homologous recombination in Mag1 deficient cells, because MMS creates many different types of methylated bases, several of which can be repaired by Mag1.

*E. coli* Tag 3MeA DNA glycosylase is highly specific for 3MeA [53]. To determine if unrepaired 3MeA can cause homologous recombination, cells were induced to express *tag* using the pYes-Tag vector [32]. Expression of *tag* virtually eliminates MMS-induced recombination in *mag1 rad4* cells at both non-toxic

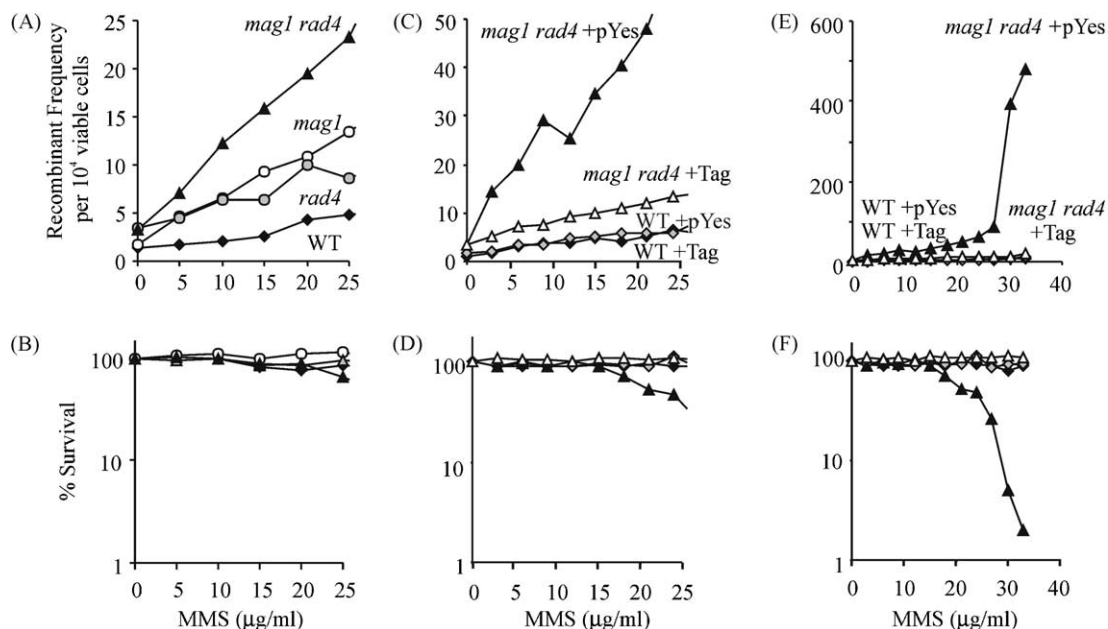


Fig. 5. The effects of deficiencies in 3MeA DNA glycosylase activity (*mag1*) and/or NER (*rad4*) and expression of *E. coli* Tag 3MeA DNA glycosylase on MMS-induced recombination and toxicity. (A) The frequency of *HIS3* recombinants is shown relative to the number of viable cells for wild type cells (WT; filled diamonds) and the following repair deficient strains: *rad4* (shaded circles), *mag1* (open circles), and *mag1 rad4* (filled triangles). (B) At the indicated doses of MMS, there is essentially 100% survival for all genotypes. (C–F) Tag suppresses both MMS-induced toxicity and recombination in *mag1 rad4* cells exposed to sublethal (C and D) and lethal (E and F) levels of MMS. The strains are: wild type *S. cerevisiae* carrying the pYes control vector (solid diamonds); wild type cells expressing *tag* (shaded diamonds); *mag1 rad4* carrying pYes (solid triangles); *mag1 rad4* expressing *tag* (open triangles). Stationary phase diploid cells were plated on solid media containing the indicated concentrations of MMS. For *Tag* induction conditions, see Section 2. Experiments were repeated a minimum of three times and the data presented are a representative curve.

(Fig. 5C and D) and toxic levels of exposure (Fig. 5E and F), providing very strong evidence that unrepaired 3MeA lesions are indeed recombinogenic. Consistent with these results, induced expression of the yeast O<sup>6</sup>MeG repair methyltransferase had no effect on MMS-induced recombination in the *mag1 rad4* cells (data not shown), showing that the NER deficiency does not make cells vulnerable to recombination induced by O<sup>6</sup>MeG lesions.

### 3.5. *Pol ζ* modulates methylation-induced recombination in excision repair deficient cells

*Pol ζ* suppresses AP site-induced mitotic homologous recombination, presumably by facilitating bypass of replication-blocking AP sites [39]. To explore the possibility that *Pol ζ* plays an analogous role in modulating the effects of 3MeA lesions, *rev3*

mutations were introduced into wild type and *mag1 rad4* strains (Table 1). Fig. 6A clearly shows that *Rev3* helps prevent MMS-induced toxicity in wild type cells, which is consistent with previous studies [26]. Here we show that at toxic levels of exposure, *rev3* mutant cells also have increased susceptibility to methylation damage-induced homologous recombination (Fig. 6B).

In cells lacking both *Mag1* and NER, disruption of *REV3* causes a concomitant decrease in survival and increase in methylation-induced recombination (Fig. 6A and B). The deficiency in *Pol ζ* in the *mag1 rad4 rev3* cells causes significant toxicity at about half the concentration of MMS required to elicit a similar response in the *mag1 rad4* cells. Since AP sites are not being generated by *Mag1* in *mag1* mutant strains, these results are consistent with a model wherein *Pol ζ* helps prevent the toxic and recombinogenic effects of

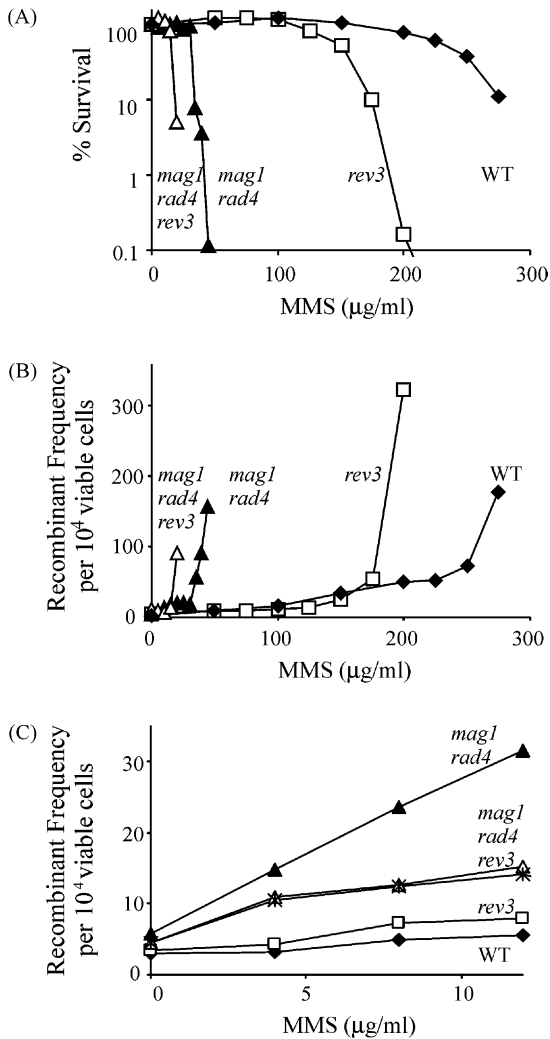


Fig. 6. Modulation of methylation-induced toxicity and homologous recombination by Pol  $\zeta$ . Diploid *S. cerevisiae* carrying the indicated mutations were grown to stationary phase and various dilutions were plated onto either SC media or media lacking histidine. The strains are wild type (WT; filled diamonds), *rev3* (open squares), *mag1 rad4* (filled triangles), *mag1 rad4 rev3* (open triangles and asterisks). (A and B) The effects of Mag1, Rad4, and Rev3 on MMS-induced toxicity and *HIS3* reversion. This experiment was repeated two times, and a representative curve is shown. (C) MMS-induced *HIS3* reversion frequencies at non-toxic doses. This experiment was repeated three times and a representative curve is shown.

3MeA by facilitating translesion replication. Interestingly, disruption of *REV3* does not make *mag1 rad4* cells more sensitive to methylation-induced recombination at lower levels of exposure (Fig. 6C). Indeed, the *mag1 rad4 rev3* cells show decreased susceptibility to methylation-induced recombination compared with their *mag1 rad4* counterparts when exposed to non-toxic doses of MMS. Thus, the effects of Pol  $\zeta$  status are highly dose-dependent. Possible explanations for these observations are discussed later.

#### 4. Discussion

In this study, we have explored the genetic basis for methylation-induced mitotic homologous recombination and the interplay between BER, NER, replicative bypass, and homologous recombination. Although elegant studies exploiting meganucleases that introduce site-specific double strand breaks have made it abundantly clear that double strand breaks created in such a direct repeat are highly recombinogenic (e.g. [57,58]), it is not yet clear how base damage causes homologous recombination. Here, we have explored how the BER pathway affects susceptibility of cells to homologous recombination. We have found that cells lacking Mag1 are highly sensitive to methylation-induced recombination and that disruption of NER sensitizes *mag1* mutant cells to methylation-induced recombination, which clearly indicates that NER intermediates are not causing recombination in *mag1* cells. Furthermore, expression of *E. coli* Tag prevents methylation-induced recombination in the *mag1 rad4* cells, providing very strong evidence that unrepaired 3MeA lesions are responsible for the observed increase in recombination. Given that Mag1 is able to remove a broad spectrum of lesions, there has been a great deal of interest in identifying those Mag1 substrates that are the basis for the evolutionary conservation of Mag1 activity in nearly every species. The results presented here make a case for Mag1 serving to remove potentially recombinogenic 3MeA lesions. Although a deficiency in Mag1 has no detectable effect on spontaneous recombination, it is possible that exposure to conditions that cause methylation damage may play a role in the evolutionary pressure to retain Mag1.

Although too little Mag1 is problematic, overproduction of Mag1 results in increased susceptibility to

spontaneous homologous recombination. This may be due to the ability of uncleaved abasic sites to inhibit DNA replication [49], or it may be that other downstream BER intermediates, such as single strand breaks, are recombinogenic. Studies by Swanson et al. clearly show that Pol  $\zeta$  plays an important role in suppressing recombination induced by uncleaved abasic sites in AP endonuclease deficient cells [39]. Here, we show that while overexpression of *MAG1* induces spontaneous homologous recombination, neither disruption of *REV3* (which is required for Pol  $\zeta$ -mediated translesion replication), nor increased levels of AP endonuclease activity have any effect on Mag1-induced recombination. Thus, although uncleaved abasic sites can induce recombination, this induction is dependent upon the combined conditions of increased expression of a DNA glycosylase that is able to take out normal bases and simultaneous disruption of expression of the major AP endonuclease (shown here) or by the combined disruption of multiple enzymes able to cleave such AP sites (shown by Swanson et al. [39]). We propose that even under conditions of imbalanced BER initiated by overexpression of *MAG1*, normal levels of AP endonucleases may be sufficient to prevent uncleaved abasic sites from playing a major role in inducing recombination in *S. cerevisiae*. Instead, base damage may become recombinogenic by its conversion into single strand breaks, which cannot be bypassed by translesion polymerases. Indeed, Galli and Schiestl have shown that single strand breaks, specifically introduced into a direct repeat induce recombination in a replication-dependent fashion [60]. In addition, it has been proposed that persistent single strand breaks, which are thought to be converted into recombinogenic double strand ends during DNA replication (Fig. 1), are responsible for the high levels of recombination observed in ligase mutants [59]. Thus, we propose that downstream BER intermediates, such as single strand breaks, are most likely responsible for the observed effects of Mag1 overexpression on recombination.

In this study, we also explored the role of Pol  $\zeta$  in modulating the effects of unrepaired methylation damage by disrupting *REV3* in cells lacking excision repair. When cells lacking Mag1 and NER are exposed to toxic levels of methylation damage, *REV3* prevents methylation-induced recombination. These results are consistent with a model wherein Pol  $\zeta$  is

able to replicate past 3MeA lesions. However, at lower levels of methylation damage, *REV3* expression does not offer any protection against recombination, and if anything, *mag1 rad4 rev3* cells are more resistant to methylation-induced recombination than are *mag1 rad4* cells. This apparent paradox can be explained by a model that takes into consideration the presence of multiple polymerases with varied ability to replicate past damaged bases [61–63]. It is not yet clear how polymerases gain access to a damaged template, but it seems likely that eliminating one polymerase may make it possible for other polymerases to gain access. In the absence of Pol  $\zeta$ , unrepaired 3MeA lesions may become accessible to other DNA polymerases, such as Pol  $\eta$  [64]. At low levels of exposure, another polymerase may be more effective than Pol  $\zeta$  at replicating past 3MeA, and thus, be able to suppress homologous recombination. As the dose increases, this polymerase may become saturated, making the cells extremely sensitive to both 3MeA-induced toxicity and recombination. The results presented in this work clearly show that the effects of Rev3 are highly dose-dependent; further studies are necessary to elucidate the relationships among those polymerases that are able to replicate past damage.

An elegant study by Kadyk and Hartwell provides strong evidence that replication is required for unrepaired UV lesions to induce recombination [54]. Furthermore, studies by Galli and Schiestl show that MMS induced recombination is suppressed in cells arrested in G1, and that the frequency of UV-induced recombination increases with time in S phase [65]. Several models have been proposed for mechanisms by which an encounter between a replication fork and a replication-blocking lesion causes recombination at a direct repeat, and these are briefly summarized here. If the lesion is in the lagging strand, a daughter strand gap would be created that is either itself recombinogenic (as shown in Fig. 1A) or is converted into a recombinogenic double strand break (not shown). If the lesion is in the leading strand, and lagging strand synthesis continues, single stranded DNA in the leading strand may promote recombination with the sister chromatid. Alternatively, an encounter with a lesion in the leading strand template may lead to replication fork breakdown, creating a recombinogenic double strand end [66–68] (Fig. 1B). Finally, studies by Lovett et al., suggest that replication slippage can

convert direct repeats into single copies in *E. coli* [69,70]. However, there is no direct evidence that slippage can happen between repeats as long as those used in this study, particularly when there is an intervening ~2 kb cassette between the repeats. Furthermore, UV-induced recombination at this *his3* direct repeat is *RAD52* dependent (Schiestl, personal communication), which is consistent with models that involve homologous recombination rather than replication slippage. Taken together, the results of this work and previous studies are consistent with a model wherein unrepaired 3MeA lesions inhibit DNA replication, which leads to single stranded regions and/or double strand ends that are subject to recombinational repair.

Previous studies suggest that single strand annealing (SSA) is the predominant mechanism underlying recombination at the *his3* repeat [42]. SSA involves a double strand break positioned between the repeats, followed by reannealing of the flanking sequences. Since MMS does not directly create two-ended double strand breaks, we do not think it is likely that SSA is the predominant mechanism of recombination in our studies. Instead, we think it more likely that the repeats are recombined during replication fork reconstitution, as described earlier. It is worth noting that SSA and replication fork reconstitution would be indistinguishable at a direct repeat such as the one used in these studies.

In this work, we have explored the role of Mag1 in modulating mitotic homologous recombination. Together, the Mag1 initiated BER pathway and the NER pathway normally help prevent 3MeA-induced recombination events, but a very high level of expression of Mag1 (which is able to remove normal bases) causes the accumulation of recombinogenic BER intermediates. Thus, in wild type cells exposed to high concentrations of methylation damage, both unrepaired 3MeA and BER intermediates are potentially recombinogenic. Indeed, neither expression of Tag nor expression of Apn1 (data not shown) significantly suppressed MMS-induced recombination in wild type cells, which indicates that ridding the cell of any residual unrepaired 3MeA or uncleaved AP sites only causes an increase in other recombinogenic downstream intermediate(s). Thus, it seems that the BER capacity of *S. cerevisiae* is optimal for tolerance of low levels of damage, and when the damage levels become too high, both unrepaired lesions and BER

intermediates can become problematic to genomic stability. In conclusion, the benefit of removing a particular lesion must be weighed against the cost of creating BER intermediates. The optimal balance of these forces depends upon both the quality and the quantity of the lesions and the relative proportions of the enzymes in the BER pathway.

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