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Def1 and Dst1 play distinct roles in repair of AP lesions in highly transcribed genomic regions

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Abstract

Abasic or AP sites generated by spontaneous DNA damage accumulate at a higher rate in actively transcribed regions of the genome in *S. cerevisiae* and are primarily repaired by base excision repair (BER) pathway. We have demonstrated that transcription-coupled nucleotide excision repair (NER) pathway can functionally replace BER to repair those AP sites located on the transcribed strand much like the strand specific repair of UV-induced pyrimidine dimers. Previous reports indicate that Rad26, a yeast homolog of transcription-repair coupling factor CSB, partly mediates strand-specific repair of UV-dimers as well as AP lesions. Here, we report that Def1, known to promote ubiquitination and degradation of stalled RNA polymerase complex, also directs NER to AP lesions on the transcribed strand of an actively transcribed gene but that its function is dependent on metabolic state of the yeast cells. We additionally show that Dst1, a homolog of mammalian transcription elongation factor TFIIS, interferes with NER-dependent repair of AP lesions while suppressing homologous recombination pathway. Overall, Def1 and Dst1 mediate very different outcomes in response to AP-induced transcription arrest.

INTRODUCTION

Abasic (AP) sites comprise a major portion of endogenously occurring DNA damages and are produced when a variety of lesions including uracil, modified bases and spontaneous or enzymatic deamination products are excised by specific DNA N-glycosylases thereby releasing the damaged bases from DNA (1). In yeast, genetic studies showed that spontaneous AP sites prevalently originate from uracil incorporated during replication by DNA polymerases (2). Following the excision of uracil base by DNA glycosylase, Ung1, AP sites ensue and become a potent block to transcription and replication machineries. Failure

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CONFLICT OF INTEREST STATEMENT

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to repair AP lesions, therefore, can be both mutagenic and cytotoxic. As an alternative to repair, AP lesions can be bypassed by translesion synthesis (TLS) polymerases. In yeast and metazoans, Rev1 and Polζ ensure continued replication albeit at a high mutation cost by inserting mostly C nucleotides opposite the AP lesions (3,4). Base Excision Repair (BER), the major repair pathway for removing AP sites, is initiated when an AP endonuclease, Apn1 in yeast, cleaves the DNA phosphodiester bond at the 5′ side of the lesion (5). Ntg1 and Ntg2 AP lyases can alternatively initiate BER by nicking the sugar phosphate backbone on the 3′ side of the AP site (6). To complete repair, the deoxyribose phosphate residue is removed by a phosphodiesterase and a DNA polymerase fills the gap followed by a DNA ligase that seals the remaining nick.

When BER is disrupted or overwhelmed, Nucleotide Excision Repair (NER) can provide an alternative mechanism for the repair of AP sites (7–10). NER pathway is primarily involved in removing helix-distorting lesions such as UV-induced thymidine dimers (11). The loss of NER has been associated with increased sensitivity to sunlight and predisposition to skin cancer in humans. NER can be divided into two sub-pathways based on the lesion recognition step, global genome repair (GGR) and transcription-coupled repair (TCR)(12). In GGR, repair proteins directly recognize a distortion of the DNA helix and are recruited to the lesion site; Rad7 and Rad16 form a complex required for this step in yeast. GGR is involved in the repair of lesions throughout the genome regardless of the transcriptional status of the genes (13,14). In contrast, TCR is initiated by the stalled RNA polymerase complex at a lesion and thus repair only those lesions located on the transcribed strand of a gene blocking transcription. Rad26, a yeast homolog of human CSB and a DNA dependent ATPase, is uniquely required for the TCR sub-pathway. The abolishment of Rad26 results in significant but partial disruption of TCR (15). Rad14, a homolog of human XPA, has been shown to be essential for the lesion verification step directly following the lesion recognition and is required for both GGR and TCR (16). The subsequent steps are comparable for GGR and TCR pathways; structure-specific endonucleases together with NER-specific helicases create dual incisions around the lesion to release the lesion-containing single strand DNA of 25–30 nucleotides, the resulting gap is filled by DNA polymerases and remaining gap ligated by a DNA ligase.

AP sites are not recognized as helix distorting lesions by Rad7-Rad16 complex but can cause robust transcription block by T7 RNA polymerase or mammalian RNA polymerase II (17). In yeast, our previous findings demonstrated that, while AP sites are predominantly repaired by Apn1-mediated BER pathway, TCR pathway contributes significantly to repair AP lesions and reduce the AP-associated mutations in the context of a highly transcribed gene. When Apn1 is disrupted, the defect in the repair of AP sites result in transcription-associated mutations (TAM), which is further elevated upon the disruption of back-up repair pathway involving Ntg1 and Ntg2, the N-glycosylases/AP lyases. The disruption of NER by the deletion of *RAD14* also led to a dramatic increase in TAM in *apn1* background suggesting the involvement of NER in AP site repair. The deletion of *RAD26*, a TCR specific gene, also led to an increase in TAM although still significantly lower than the increase in mutation due to *RAD14* deletion. In contrast, the deletion of *RAD7*, a GGR specific gene, in *apn1* background did not affect mutagenesis suggesting that TCR, but not GGR, is involved in the repair of AP sites. The increase in TAM observed in *apn1 rad14* or

apn1 rad26 strain was specifically due to unrepaired lesions on the transcribed strand, further confirming TCR repair of AP sites which is likely triggered by stalling of the RNA polymerase II complex (RNAPII) at the AP sites (18). This overlap between TCR and BER was also demonstrated in human cells for the repair of the 8-oxoguanines (GO lesions), which is the most common type of oxidative DNA lesions (19).

The mechanism of what happens to RNAPII stalled at a UV-induced lesion and how the repair machinery accesses the damage obstructed by the stalled RNA polymerase have been extensively studied but still not completely understood (20). The events following RNAPII stalling at an AP lesion are even less understood. Multiple pathways exist that could help RNAPII contend with obstacles. First, the stalled RNAPII can be backtracked thereby allowing access for repair enzymes to the lesion. In *E. coli,* UvrD, a DNA helicase/translocase and NER protein, was shown *in vitro* to facilitate backtracking by binding to RNAPII and forcing it to slide backwards along DNA thereby exposing the DNA lesions (21). Second, alternative to promoting repair, the RNAPII backtracking can promote lesion bypass. *In vitro* studies have shown that transcription elongation past the lesion on the template is promoted by a general transcription elongation factor, TFIIS. Mammalian TFIIS or yeast Dst1 functions to stimulate the intrinsic endonuclease activity of RNAPII, which cleaves the nascent RNA transcript allowing for the transcript re-alignment with the active site (22). Finally, if RNAPII stalls for a prolonged period of time, the RNAPII degradation factor (Def1) promotes the ubiquitination and degradation of Rpb1, the largest subunit of RNAPII (23,24). The degradation of RNAPII could enhance repair by clearing the path for DNA repair enzymes especially since the stalled RNAPII can prevent access to the lesion by repair factors (23,25,26).

While in mammalian cells, CSB, the homolog of Rad26, is sufficient in activating TCR, Rad26 only partially accounts for TCR of AP lesions in *S. cerevisiae* suggesting that additional factors are involved in recruiting NER proteins to the stalled RNAPII (15,18). In the current report, we took advantage of a genetic assay designed to monitor the repair of AP sites within a highly transcribed gene in a strand-specific manner. This assay allowed us to examine the role of multiple factors that were previously implicated in the TCR of UV-induced DNA damage or in the rescue of the arrested RNAPII. Consistent with its function in the repair of UV lesions, the disruption of Def1 resulted in an increase in mutagenesis due to the accumulation of unrepaired AP sites specifically on the transcribed strand. However, we show here that, the disruption of Dst1 resulted in an unexpected decrease in the AP-associated mutagenesis suggesting a complex role for Dst1 when transcription elongation and damage repair are both at stake. These results provide new insights into factors that are involved in the repair or transcriptional bypass of the AP lesions located within transcribed genes.

MATERIALS AND METHODS

Yeast strains and plasmids

Yeast strains used for the mutation and recombination assays were derived from YPH45 (*MATa, ura3-52 ade2-101 trp1Δ1).* Construction of strains containing the *his4Δ::pTET-lys2-TAA* allele was previously described (18). Gene deletions were carried out through one-step

allele replacement by amplification of the loxP-flanked marker cassettes (27). pTDG is a 2-micron plasmid with human TDG expression cassette under *pGAL* control along with TRP1 marker and was a gift from Dr. Bruce Demple (Stony Brooke School of Medicine, Stony Brooke, NY).

Mutation/Recombination Rates and Spectra

Mutation and recombination rates were determined using the method of the median, and 95% confidence intervals were calculated as previously described (28). Each rate was based on data obtained from 12–24 independent cultures and two independently-derived isolates. 1mL yeast extract-peptone (YEP) medium supplemented with 2% glycerol and 2% ethanol (YEPGE) was inoculated with 250,000 cells from an overnight culture grown in the same medium. For the *DEF1*-deleted strains, 500,000 cells from an overnight culture were used to inoculate. Following growth at 30°C in YEP plus 2% dextrose (YEPD) for 3 days or YEPGE for 4 days, cells were washed with water and the appropriate dilutions were plated either on synthetic, lysine-deficient medium containing 2% dextrose (SCD-Lys) to select Lys + revertants or on SCD-Leu medium to determine the total number of cells in each culture. *CAN1* forward mutation rates were determined by plating cells on SCD-Arg medium supplemented with 60μg/mL L-canavanine sulfate (SCD-Arg+Can; Sigma). In TDG expression experiments, indicated strains were transformed with either empty vector or pTDG and selectively plated on SCD-Trp medium. Individual colonies were inoculated into a 1ml SC-Trp cultures supplemented with 2% galactose and 1% raffinose. After 4 days of growth at 30°C, appropriate dilutions were plated on SCD-Trp to determine total cell numbers and on SCD-Trp-Lys to determine the number of Lys+ colonies in each culture.

To determine mutation spectra, individual colonies were used to inoculate 0.3ml YEPGE cultures (or SC-Trp with 2% galactose and 1% raffinose for TDG expression experiments). After 2 or 3 days of growth at 30°C, an appropriate fraction of each culture was plated on SCD-Lys (or SCD-Trp-Lys in TDG expression experiments). A single Lys+ revertant from each culture was purified on YEPD plates, and genomic DNA was prepared using a 96-well format in microtiter plates. The l*ys2-TAA* reversion window was amplified using primers 5′-AGCTCGATGTGCCTCATGATAG-3′ and 5′-CATCACACATACCATCAAATCC-3′ and the PCR product was sent to Eurofins genomics for sequencing using primer 5′-TAGAGTAACCGGTGACGATG-3′. The rates of A>C and T>G were calculated by multiplying the proportion of the events by the total Lys+ mutation rate. The 95% confidence intervals for the rate of A>C or T>G mutation type were calculated first by obtaining the 95% confidence intervals for the proportion of each mutation type (Vassarstats; (29)) and applying the root-sum-squared (RSS) method.

qRT-PCR

Total RNA was extracted using the standard hot acid phenol method and treated with DNase 1 (New England Biolabs). qRT-PCR was performed using SensiFAST SYBR No-ROX one step kit from Bioline and Biorad CFX Connect instrument. The qRT-PCR conditions were as follows: 45°C for 10 mins and 95°C for 2 mins followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec and 72°C for 5 sec. The primers used for amplification were 5′BGLF: 5′-GAGTAACCGGTGACGATGATATT-3′ and 5′BGLR: 5′-

CATTAAATGACCACGTTGGTTGA-3′ for *LYS2*, CAN1F: 5′-GAGTTCTGGGTCGCTTCCAT-3′ and CAN1R: 5′-GGCACCTGGGTTTCTCCAAT-3′ for *CAN1*, UBC6F: 5′-GATACTTGGAATCCTGGCTGGTCTGTCTC-3′ and UBC6R: 5′-AAAGGGTCTTCTGTTTCATCACCTGTATTTGC-3′ for *UBC6* and ACT1F: 5′-ATTCTGAGGTTGCTGCTTTGG-3′ and ACT1R: 5′-TGTCTTGGTCTACCGACGATAG-3′ for *ACT1*. Relative RNA levels were determined by ΔΔCq analysis using *UBC6* or *ACT1* as the reference gene as indicated.

RESULTS

We previously used reversion mutation assays to examine TAM in BER- and/or NER-deficient yeast strains and demonstrated that, under high transcription conditions, AP sites derived from uracil replacing thymine is a major source of TAM (18,30). In this report, the *pTET-lys2-TAA* allele, which has a TAA stop condon inserted in-frame into the *LYS2* ORF, was used in the quantitation of TAM. In this reversion mutation assay, a mutation in the TAA stop codon allows the production of functional Lys2 protein required for lysine synthesis, which can be distinguished by selection on the lysine-lacking media. When the major AP endonuclease Apn1 is disrupted, the A>C and T>G transversion mutations are significantly elevated. These mutations are dependent on the expression of uracil DNA glycosylase Ung1 and significantly reduced when the cellular dUTP level is reduced upon overexpression of the dUTP pyrophosphatase Dut1. Overall, the previous investigation led to the model that A>C and T>G mutations are the net result of Rev1/Polζ-dependent translesion bypass synthesis across from the uracil-derived AP sites during which predominantly C nucleotides are inserted opposite AP (31). The *pTET-lys2-TAA* reversion assay allows us to infer from the mutation spectra whether the relevant AP sites were located on the transcribed or the non-transcribed strand. As diagramed in Fig. 1A, AP sites on the transcribed strand and non-transcribed strand each result in the A>C and T>G mutation signatures, respectively. Of the uracil-associated mutations, A>C but not T>G mutations are greatly elevated when NER factor Rad14 is disrupted (18). A>C mutations were also significantly elevated upon the disruption of TCR-factor Rad26, but not of GGR-factor Rad7 indicating that only TCR subpathway of NER is involved in the repair of AP lesions.

Non uracil-derived AP sites can be repaired by TC-NER

To determine whether those AP lesions originating from sources other than uracil excision could be processed in a TCR-dependent manner, we generated AP sites in the yeast genome by the expression of modified human glycosylase TDG, which was shown to excise unmodified thymine residues from oligonucleotide substrates *in vitro* and to induce the accumulation of AT>CG transversions *in vivo* (32,33). Expected mutations from TDG-induced AP lesions at the *pTET-lys2-TAA* reversion allele are shown in Fig. 1A. Compared to the vector control, TDG expression in yeast repair-deficient strains, *apn1 ung1* (BER^-), *apn1 rad26 ung1* (BER^- TCR^-), *apn1 rad14 ung1* (NER^- (TCR^- GGR^-)) and *apn1 ntg1 ntg2 ung1 (*BER^-), resulted in elevation of the overall mutation rate by 5- to 11-fold (Fig. 1B). These elevated mutations are specifically due to TDG glycosylase activity and not from the uracil excision by yeast Ung1 as the experiments were carried out in *ung1* backgrounds. TDG-expression in *apn1 ntg1 ntg2 ung1* strains led to an accumulation of T>G mutations.

Compared to the vector-control in the same strain background, the rates of T>G and A>C mutations were elevated by 150- and 13-folds, respectively. These results indicate that, in the absence of Apn1 activity, the Ntg1/Ntg2-mediated subpathway of BER largely repairs those AP sites generated by excision of T's in the non-transcribed strand as previously observed with uracil-derived mutations (Fig. 1C and 1D) (18). In *apn1 rad14 ung1* strains, the rates of A>C and T>G mutations were elevated by 25.3-and 2.5-fold, respectively, indicating that a defect in NER led to accumulation of unrepaired AP sites mostly on the transcribed strand. In *apn1 rad26 ung1* strains, the rate of TDG-induced A>C mutations (42×10^{-8}) was higher compared to that in *apn1 ung1* (32×10^{-8}) albeit considerably lower compared to that in *apn1 rad14 ung1* (121×10^{-8}). Overall, TCR subpathway of NER is a major back-up mechanism to repair TDG-induced AP lesions on transcribed strand within a highly transcribed gene and Rad26 is partially involved in mediating TCR.

Srs2 or Sub1 is not involved in TCR-dependent repair of AP sites in yeast

When RNAPII encounters DNA damage, it stalls at the damage site and the lesion must be repaired to ensure continued elongation. TCR of AP lesions is only partially affected by the disruption of Rad26 and additional factors must be involved in mediating efficient repair of AP sites by TCR (18). *E. coli* protein UvrD is a DNA helicase/translocase with known roles in NER and replication-associated processes (34,35). UvrD was recently shown *in vitro* to bind stalled RNA polymerase and to force it to slide backwards along DNA allowing greater access for the DNA repair factors to the lesion for more efficient repair (21). Srs2, a yeast paralog of UvrD, is a helicase and a DNA dependent ATPase that is involved in multiple facets of genome maintenance (i.e. homologous recombination, checkpoint recovery, and replication fork progress) (36–38). In order to determine whether Srs2, like *E. coli* UvrD, promotes TCR repair of AP lesions, we deleted *SRS2* gene in *WT*, *apn1* and *apn1 rad26* strains and determined the rates of spontaneous reversion mutations at the *pTET-lys2-TAA* allele. Overall mutation rate was not affected by Srs2 disruption in *WT* or *apn1* backgrounds and reduced by ~1.7-fold in *apn1 rad26* background (Fig. 2A). In *WT* or *apn1* backgrounds, mutation spectra also showed no significant change due to Srs2 disruption. However, in *apn1 rad26* background, there was a 14-fold decrease in the rate of T>G mutations (from AP sites on the non-transcribed strand) with no significant change in the rate of A>C mutations (from AP sites on the transcribed strand) (Figs. 2B and 2C). This strand specific effect of Srs2-disruption indicates that this factor is likely involved in promoting repair of AP lesions on the non-transcribed strand.

The transcriptional co-activator Sub1 was shown to be involved in the tolerance to the hydrogen peroxide-induced DNA damage in a TCR-dependent manner (39). The mutation rates and spectra at the *pTET-lys2-TAA* allele were determined in WT, *apn1* and *apn1 rad26* backgrounds with additional deletion of the *SUB1* gene. The rates of overall Lys+ mutations or A>C transversions were not affected by Sub1-disruption in all three strain backgrounds (Figs. 2D and 2E and Table S1). The rate of T>G mutation rates in *apn1 rad26 sub1* strains was ~4-fold reduced compared to *apn1 rad26* strains but this difference was not statistically significant (Fig. 2F).

DST1 deletion reduces mutations at pTET-lys2-TAA

During the DNA damage-induced transcription arrest, TFIIS can promote backtracking of the stalled RNAPII by stimulating the cleavage of nascent RNA but can also promote the resumption of transcription elongation and lesion bypass by RNAPII by realigning mRNA termini and DNA template (20,40). To determine whether TFIIS is involved in the TCR repair of AP sites, we deleted *DST1*, the gene encoding TFIIS in yeast and determined the mutation rates. We will from hereon refer to TFIIS as Dst1. Dst1-disruption resulted in a 3.1-, 3.4- and 6-fold reduction in the overall mutation rates in WT, *apn1,* and *apn1 rad26* backgrounds, respectively (Fig. 3A). The rates of both A>C and T>G mutations were reduced suggesting that this reduction is not specific to AP lesions on the transcribed strand (Figs. 3B and 3C). We also disrupted Dst1 in the BER and NER deficient backgrounds (*apn1 ntg1 ntg2* and *apn1 rad14,* respectively) and determined the mutation rates as well as the mutation spectra. We observed a ~ 6-fold reduction in the mutation rates in both *apn1 ntg1 ntg2 dst1* and *apn1 rad14 dst1* strains (Fig. 3A). There was a ~6-fold reduction in the rates of A>C in *apn1 rad14* background (Fig. 3B). For the T>G mutation rates, Dst1-disruption resulted in a 10-fold reduction in *apn1 ntg1 ntg2* background, further indicating that the function of Dst1 is not limited to the AP sites on the transcribed strand or to the TCR pathway (Fig. 3C).

The effect of Dst1 disruption is not due to change in transcription activity

In order to determine whether the decrease in mutagenesis upon Dst1-disruption is dependent on the level of transcription, we looked at the mutation rates at *CAN1,* a moderately transcribed locus. *CAN1* encodes an arginine transporter and deleterious mutations at this locus confer resistance to the toxic arginine analog canavanine. Dst1-disruption led to a ~2-fold reduction in the canavanine-resistant (Can^R) mutation rates in *apn1 rad26* and a ~4.5 fold reductions in both *apn1 rad14* and *apn1 ntg1 ntg2* backgrounds (Fig. 4A). The Can^R mutation rate in *apn1* background was not affected.

We previously demonstrated that mutation rate at the *pTET*-regulated *lys2* reporter linearly correlates with the rate of transcription; repressing transcription by addition of doxycycline resulted in the proportional decrease in the mutation rate (41). In order to determine whether the decrease in mutation rate upon *DST1* deletion was due to a decrease in the transcription rate, we determined the *LYS2* expression levels in *WT, dst1* and *apn1 dst1* strains using the qRT-PCR method. The *LYS2* expression level was not significantly affected upon *DST1*-deletion indicating that the effect of *DST1* deletion on mutation rate is not due to changes in the transcription levels of *LYS2 (*Fig. 4B). The expression level of *CAN1* was also not affected by *DST1*-deletion (Fig. 4C).

Dst1 disruption increases recombination

A reduction in mutation rate upon *DST1*-deletion could be due to the repair of AP lesions by pathways other than BER and NER. Thus, we disrupted Dst1 in yeast strains containing three different homologous recombination reporter constructs that vary in their sequence context (G-rich, C-rich or G/C-rich). Detailed description of these reporter constructs as well as the recombination rates in *WT* strain backgrounds were previously reported (42). *DST1*-

deletion resulted in 2- to 3-fold increases in recombination rates for all three constructs (Fig. 4D).

We additionally determined the effect on AP-associated mutagenesis of disrupting Rpb9, which was previously reported to increase the UV sensitivity of *rad26* and *rad7* strains indicating its involvement in TCR (43,44). Rpb9 is also a non-essential subunit of RNAPII that shares homology with Dst1 and was reported to be required for Dst1 to stimulate the transcript cleavage and the read-through by RNAPII (45). Similar to *DST1*-deletion, the deletion of *RPB9* in WT, *apn1,* and *apn1 rad26* backgrounds significantly reduced the rates of A>C and T>G mutations but did not affect the transcription rate of *pTET-lys2-TAA* reporter (Fig. 5A – D). Rpb9-disruption, however, did not result in a significant elevation in recombination (Fig. 5E). This could be due to the technical difficulty associated with the severely affected growth rate in this strain.

Def1-disruption elevates mutations from AP lesions on the transcribed strand

Def1 is recruited to the stalled and/or arrested RNAPII and promote the ubiquitination and degradation of RNAPII thereby clearing the way for the subsequent RNA polymerases and ensure the continued transcription as well as possibly allowing the access of repair proteins to DNA damage (23,26). Although previous studies have suggested that Def1 is not involved in TCR *per se* but functions as a last resort to allow for the resumption of transcription (23), other studies identified Def1 as a potential transcription-repair coupling factor by showing that Def1-disruption elevated the UV-sensitivity in the absence of GGR (44). We deleted *DEF1* in *apn1* and *apn1 rad26* backgrounds to determine whether it leads to a specific elevation in the A>C mutations indicative of further interruption in TCR. The overall mutation rate in *def1* and *apn1 def1* mutants showed no significant difference compared to WT and *apn1,* respectively (Fig. 6A and Table S2). However, the deletion of *DEF1* in *apn1 rad26* background led to a 1.7-fold increase in the overall mutation rate and a ~2-fold increase in the rate of A>C mutations (Fig. 6A and 6B). The rate of T>G mutations were generally lower in comparison to the A>C mutations and were slightly reduced upon the disruption of Def1 (Fig. 6C). This suggests that, in the absence of Rad26, the Def1-mediated degradation of the stalled RNAPII can alternatively allow the recruitment of repair enzymes to the AP lesions on the transcribed strand. However, when both Def1 and Rad26 are disrupted, the repair of the AP sites is partially compromised and mutations accumulate. The disruption of Def1 did not affect the T>G mutation rates in *apn1 rad14* background. The rate of A>C mutations in *apn1 rad26 def1* strains is significantly less than that in *apn1 rad14* strains (Fig. 6B; Table S2) where TCR as well as GGR are completely abolished suggesting that, even though both Def1 and Rad26 contribute to repairing the AP lesions on the transcribed strand, additional factor must be functioning to mediate TCR. The deletion of *RAD7,* which specifically disables GGR, did not affect the overall rate of mutagenesis in *apn1* or *apn1 rad26* strains indicating that GGR is not significantly contributing to the repair of AP lesions. When we deleted *DEF1* in *apn1 rad7* background, we observed a 2.2-fold increase in the overall mutation rate further supporting our findings that Def1 contributes in repairing AP sites. Surprisingly, the overall mutation rate of *apn1 rad7 def1* strains was 2.1-fold higher compared to *apn1 def1* strains suggesting that Def1 and Rad7 might be operating in the same pathway to promote the repair of AP sites. Def1-disruption in *apn1 rad14*

background led to a slight decrease in the mutation rate indicating that the degradation and removal of the stalled RNAPII might interfere with the efficient repair of AP sites by pathways other than NER. Our attempt to generate *apn1 rad26 rad7 def1* strain by a consecutive one-step allele replacement approach was not successful. It is possible that this combination of deletion is lethal, although we lack the definitive proof since the construction of mutant diploids followed by the tetrad analysis was not carried out.

The effect of Def1-disruption on mutations from AP lesions is dependent on the growth conditions

For all of the data described above, we determined the reversion mutation rates of the *pTET-lys2-TAA* allele by growing the relevant yeast strains in the rich media supplemented with 2% glycerol and 2% ethanol as the primary carbon source (YEPGE). Because the *DEF1*-deletion strains showed a slow growth phenotype in YEPGE, we repeated the experiments in the rich medium supplemented with the fermentable sugar dextrose (YEPD). Although the growth conditions did not significantly affect the mutation rates for WT or *def1* strain (Table S2 and S6), in *apn1* background, the rate of overall Lys+ reversion mutation was >20-fold lower when the cells are grown in YEPD than in YEPGE (Figs. 6A and 7A). In YEPD, in *apn1 def1* strain compared to *apn1* strain, there were ~15-fold and ~19-fold increases in the overall and A>C mutation rates, respectively. Compared to the modest 1.7- and 2-fold changes in the overall and A>C mutation rates observed in YEPGE, when analyzed in YEPD, *DEF1*-deletion in *apn1 rad26* background led to an overwhelming ~53-fold increase in the overall mutation rate and a ~60-fold increase in the A>C mutations. The effect of Def1-disruption on the rate of T>G mutations was nominal in all backgrounds. In YEPD, the rates of overall and A>C mutations in a*pn1 rad26 def1* strain were comparable to those in *apn1 rad14* strain where TCR is completely abolished (Fig. 7). We also determined the mutation rates in *apn1 rad7 def1* strains in YEPD. The deletion of *DEF1* in YEPD led to a 4.8-fold increase in mutagenesis in *apn1 rad7* strain background indicating the involvement of *DEF1* in the TCR repair of AP sites. However, unlike in YEPGE where we observed a 2.1-fold increase in the mutation rate in *apn1 rad7 def1* strain compared to *apn1 def1* strain, we observed a 2.3-fold reduction in the rate of mutagenesis. And, in *apn1 rad14* background, no further elevation in the overall mutation rate resulted from Def1-disruption. However, the rates of A>C mutations were slightly increased and the rates of T>G mutations were reduced by two-fold further confirming that the effect of Def1 is specific to the AP lesions located on the transcribed strand.

DISCUSSION

AP lesions, arguably the most prevalent type of spontaneously occurring DNA damage, are produced in cells through multiple pathways. Notably, AP lesions are obligatory intermediates in the repair of many different types of DNA damage including those produced by endogenously generated reactive oxygen species and by chemotherapeutic agents targeting thymidine synthesis pathway or directly damaging DNA by alkylation. AP sites present on the transcribed strand of a gene are particularly problematic since they not only endanger the genome integrity but also compromise efficient transcription by blocking RNA polymerases. We previously reported that the TCR subpathway of NER can be

recruited to AP lesions on the transcribed strand and removes this type of damage to a significant extent (18). A key aspect of the reporter assay used to demonstrate the involvement of Rad26 in TCR repair of AP lesions is that we can infer from the mutation spectra whether the relevant AP lesion was present on transcribed or non-transcribed strand. Exploiting this assay, we previously showed that, when TCR is compromised in *apn1 rad26* background, AP sites derived from uracil excision from DNA accumulate specifically on the transcribed strand. By expression of the engineered human glycosylase TDG that can excise undamaged thymine to generate AP sites, we demonstrate here that non-uracil derived AP sites are also processed and repaired *via* TCR.

AP lesions are poorly recognized by NER proteins but can be repaired by TCR by stalling RNAPII (17), which subsequently interferes with transcription elongation and might be detrimental to the cell. In order to determine how multiple mechanisms involved in overcoming the obstacle to transcription might interact with the TCR repair of AP sites, we examined four potential regulators of stalled RNAPII. Sub1, Srs2, Dst1 and Def1 have been variously implicated in TCR repair of UV induced damages and/or transcription elongation (21–23,39,40,45). In the current report, we demonstrate that Srs2 and Sub1 are not involved in TCR repair of AP lesions since disruption of either proteins did not affect the spontaneous AP-associated A:T to C:G mutations at the *pTET-lys2-TAA* reporter in *apn1* or *apn1 rad26* background (Fig. 2). *DST1* deletion, on the other hand, resulted in up to a 10-fold reduction in mutation rates, which we showed was not due to the reduced transcription of the reporter gene. The reduction in mutation rate could be explained by more efficient repair in the absence of Dst1. We speculate that the action of Dst1 might be interfering with repair by encouraging transcriptional bypass of the AP lesion. In fact, Dst1 has been shown to induce RNAPII to bypass oxidative DNA damage (e.g. 8-oxoG) but not UV-induced damage (46) leading us to suggest that Dst1 might also promote transcriptional bypass of the AP lesions, which, like 8-xoG, is not helix-distorting. Interestingly, the effect of Dst1 disruption is not limited to the transcribed strand or TCR since mutations arising from AP lesions on both the transcribed (A>C) and the non-transcribed (T>G) DNA strands are significantly reduced and AP-associated mutagenesis reduced in both NER^{-} (*apn1 rad14)* and BER^{-}(*apn1 ntg1 ntg2*) strains. The elevation in homologous recombination upon the deletion of *DST1* suggests that, when present, Dst1 prevents AP lesion-repair by recombination through a mechanism yet to be defined. Disruption of *E. coli* GreA or GreB, which are the functional homologs of yeast Dst1, was recently shown to decrease mutations by increasing homologous recombination (C. Herman, Baylor College of Medicine, Houston, TX; Personal communications). In fact, in *E. coli*, *GreA* and *GreB,* which carry out similar functions as Dst1 during RNA polymerase stalling, promote transcription elongation at the expense of repair and repress recombination. This result is fully consistent with our data that recombination is enhanced when *DST1* is deleted (Fig. 4D). To further understand the roles of Dst1, we disrupted homologous recombination by the deletion of *RAD51* (Fig. S1). In *apn1* or *apn1 dst1* background, *RAD51*-deletion did not affect the mutation rates indicating that recombination might be occurring in *RAD51*-independent manner (47). It is also possible that the effect of Dst1-disruption on the AP-dervied mutations is independent of its role in repressing homologous recombination. Further studies need to be performed to explore how yeast Dst1 or *E. Coli* GreA/GreB represses homologous recombination.

The degradation and clearance of RNAPII arrested at lesions require Def1, which mediates ubiquitination of the catalytic subunit of RNAPII (23,24). Our effort to determine whether Def1 allows for the more efficient repair of AP lesions on the transcribed strand yielded a surprising result that the effect of Def1 disruption was highly dependent on the growth conditions (Fig. 6 and 7). When analyzed in media containing glycerol and ethanol as the major carbon source, we observed a modest (1.7-fold) increase in the mutation rate in *apn1 rad26* background as expected from previous findings that Rad26 normally inhibits the activity of Def1 and the degradation of RNAPII is used as a mechanism of last resort (23). However, in contrast to experiments in YEPGE, in dextrose-containing rich media (YEPD), Def1-disruption resulted in much greater increase in the mutation rate in both *apn1* (~15-fold) and *apn1 rad26* (~53-fold) backgrounds (Figs. 6A and 7A). During the fermentative growth in YEPD, the mutation rate of *apn1 rad26 def1* was similar to that of *apn1 rad14* suggesting that Def1 is involved in TCR repair of AP sites and that Rad26 and Def1 are sufficient in repairing all the AP sites in the transcribed strand without the need for additional factors when the cells are grown in dextrose. The effect of Def1 disruption in *apn1 rad14* background was also dependent on the growth conditions.

Yeast cells preferably grow *via* fermentation using dextrose as the carbon source. Using the non-fermentable carbon source such as ethanol and glycerol, yeast cells respire or grow oxidatively relying on the mitochondria. According to our data, the effect of cell metabolic state extends beyond the role of Def1; the overall mutation rates in *apn1* and *apn1 rad14* backgrounds showed significant differences depending on the growth condition (Fig. 6A and 7A). We also observed that, although the effect of Srs2- or Sub1-disruption on the repair of AP lesion remained constant in YEPGE or YEPD, the effect of *DST1* deletion in *apn1 rad26* background on the AP-associated mutation rate was significantly dependent on the carbon source (Fig. S2). This difference is not due to different number of cell divisions during the culturing process since the starting and ending cell density during the experiments were not significantly different in these two growth conditions (Table S7). It has been previously shown in yeast that the metabolic state can be a determining factor in the types and numbers of endogenously-occurring DNA damage (48). There is a major difference in the accumulation of reactive oxygen species (ROS) in yeast cells growing by respiration (glycerol/ethanol) and by fermentation (dextrose) (49). The difference in the extent of the ROS-induced DNA damage in these two growth conditions is one potential explanation for the growth-condition-dependent effect of DEF1 deletion. However, the mutations in the *pTET-lys2-TAA* assay are originating not from oxidative damage but from uracil incorporated during DNA synthesis, leading us to suggest that the cell metabolic state and growth condition can also affect the various pathways involved in AP repair and/or transcriptional bypass. In *E. coli,* ppGpp, a small molecule mediator of the stringent response, was recently shown to play an important role in promoting TCR of helix-distorting DNA lesions (50). Stress response could similarly play an important role in shifting the balance among multiple pathways involved in the repair of AP lesions in yeast.

In summary, we report that Def1 and Dst1 play distinct yet significant function in how AP lesions in the transcribed genes are repaired (Fig. 8). We show that Def1, in addition to Rad26, can facilitate repair of AP lesions located on the transcribed strand by TCR as well as other repair pathways. We also describe a novel function of Dst1 in interfering with AP

repair possibly by suppressing homologous recombination. Dst1-disruption reduces mutations resulting from the AP lesions located on both the transcribed and the non-transcribed strands leading us to suggest that further study is needed to understand how the role of Dst1 in AP lesion repair is mechanistically connected to its previously characterized role in rescuing stalled transcription complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Def1 directs NER to AP lesions on the transcribed strand of an actively transcribed genes.
- The function of Def1 is dependent on the growth conditions.
- Dst1, a homolog of mammalian transcription elongation factor TFIIS, interferes with NER-dependent repair of AP lesions.

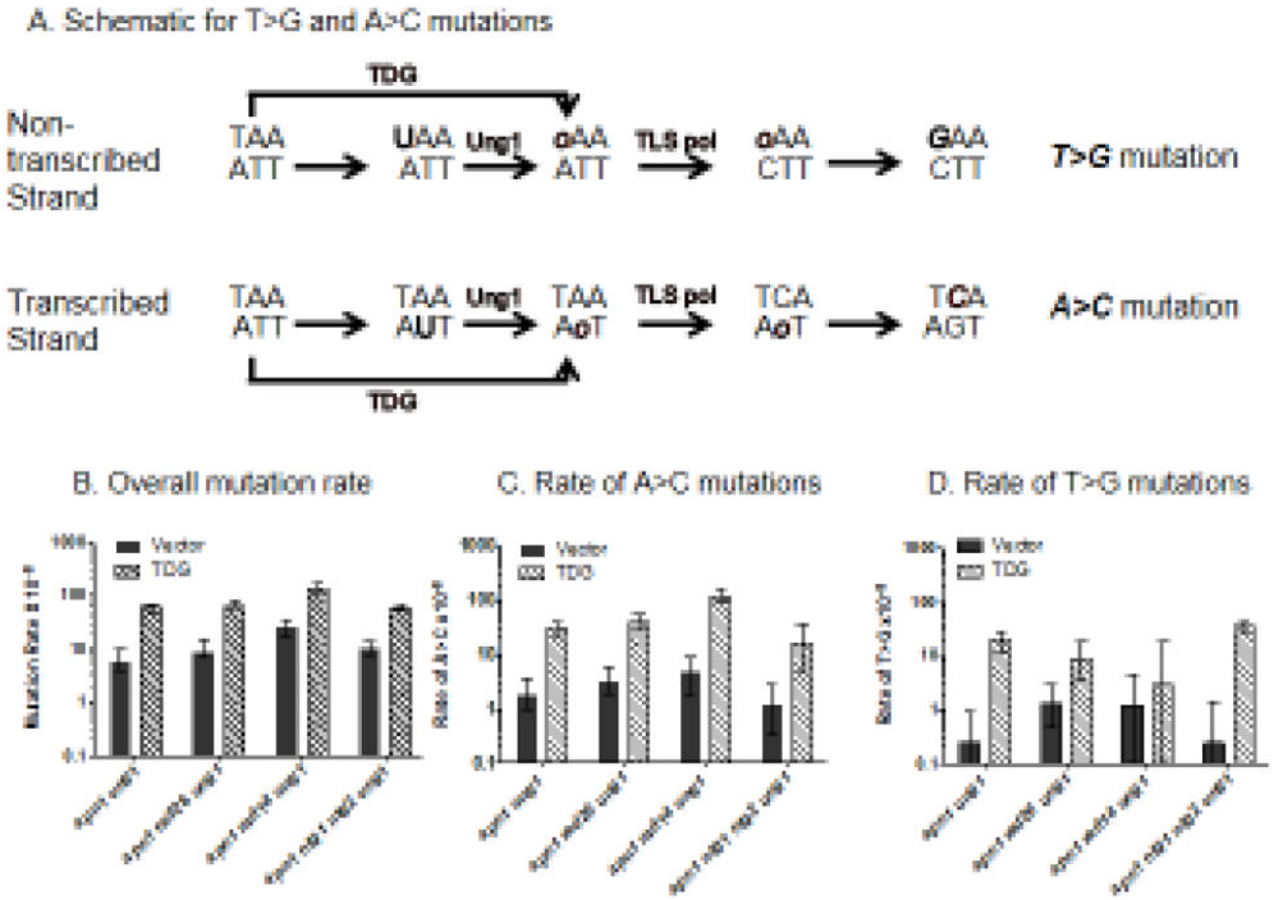

Figure 1.

The effect of TDG expression on the rates of mutation at the *pTET-lys2-TAA* reporter. **A)** Schematic representation of the substitution mutations arising from base excision by Ung1 or TDG. ***o*** Indicates AP sites. B) Overall mutation rates in yeast strains with empty vector or TDG glycosylase-expressing plasmid. Error bars represents 95% confidence intervals. **C)** and **D)** Rates of A>C and T>G transversions, respectively. For the actual numbers see Table S1. See **MATERIALS AND METHODS** regarding calculation of these rates and confidence intervals.

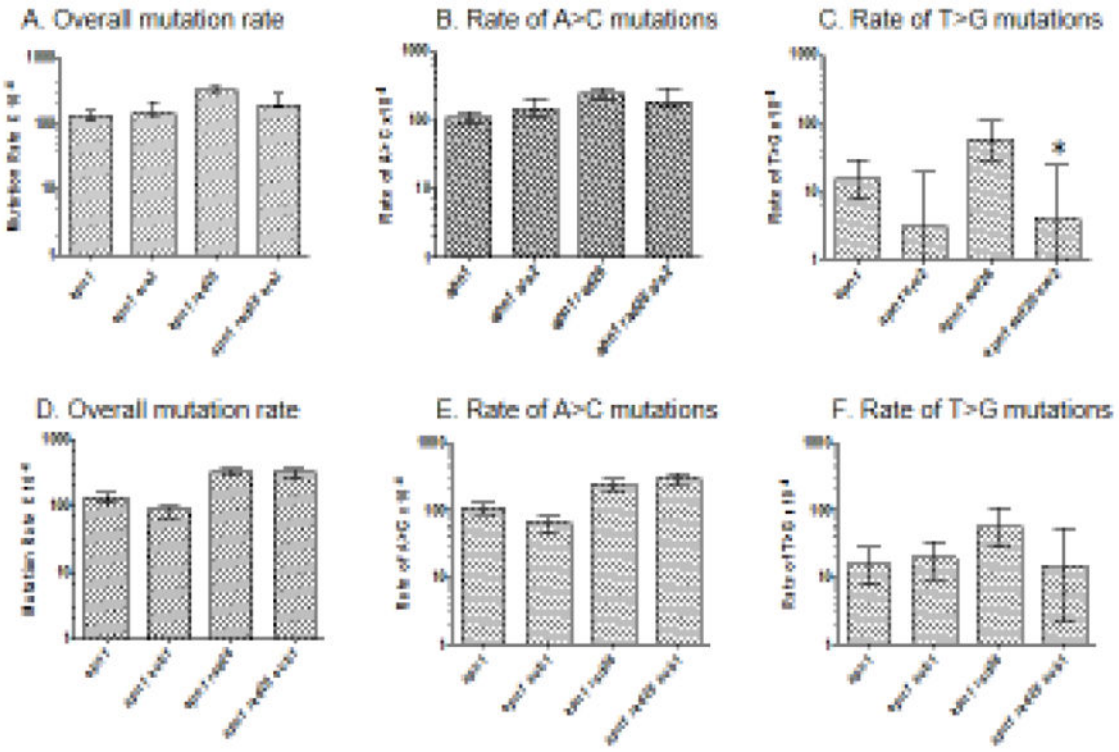

Figure 2.

The effect of Srs2 or Sub1 disruption on the rates of mutations.

A) and **D)** Overall rates of mutations at *pTET-lys2-TAA.* Error bars represent 95% confidence intervals. **B)** and **E)** Rates of A>C mutations. **C)** and **F)** Rates of T>G mutations. *Zero mutation event of this class was detected and the mutation rate was calculated assuming 1 event. For the actual numbers see Table S2. Total, A>C, and T>G mutation rates in *apn1* and *apn1 rad26* backgrounds in **D) – F)** is identical to the rates shown in **A – C**.

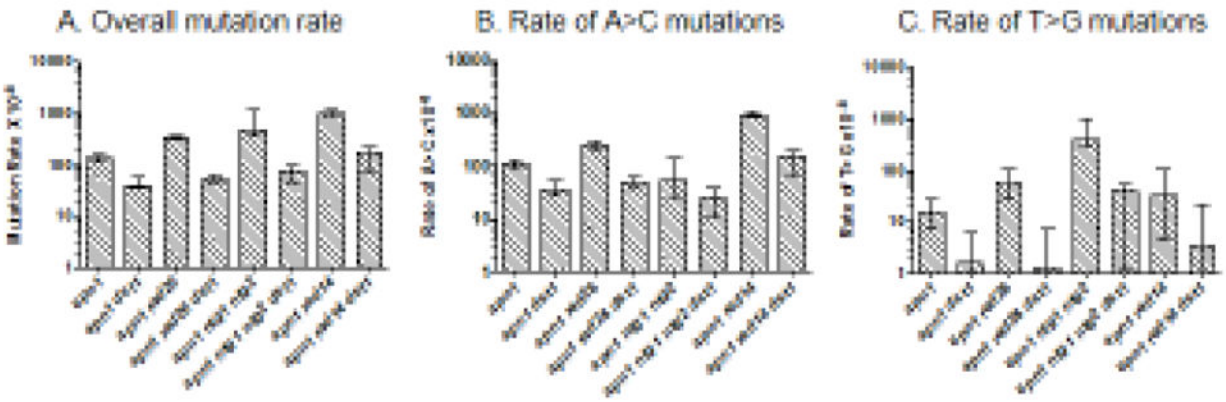

Figure 3.

The effect of Dst1 disruption on the rates of mutations.

A) Overall rates of mutations at *pTET-lys2-TAA*. Error bars represents 95% confidence intervals. **B)** Rates of A>C mutations. **C)** Rates of T>G mutations. For the actual numbers see Table S2. Total, A>C, and T>G mutation rates in *apn1* and *apn1 rad26* backgrounds in this figure is identical to the rates shown in Figure 2.

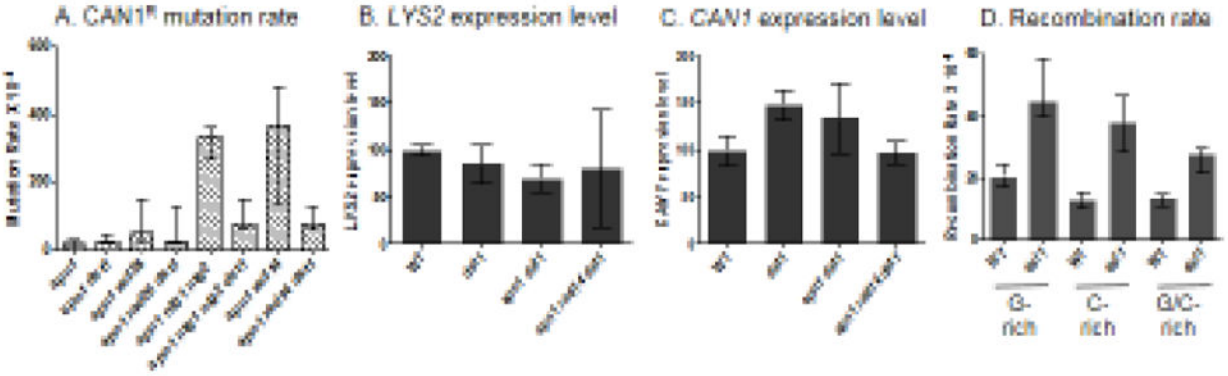

Figure 4.

The effect of Dst1 disruption on transcription and recombination.

A) Overall rate of *CAN1* forward mutations in the indicated genetic backgrounds. Error bars represent 95% confidence intervals. For the actual numbers see Table S3. **B)** *LYS2* and **C)** *CAN1* expression levels calculated by RT-QPCR using *UBC6* as the reference gene. RNA was extracted from yeast cells with the indicated genetic strains. Error bars indicate the standard deviation calculated from 3 or 4 independent experiments. For the actual numbers see Table S4. **D)** The rates of recombination at the *G-rich*, *C-rich* or *G/C-rich* cassettes were determined using the method of the median. Error bars represent 95% confidence intervals. For the actual numbers see Table S5.

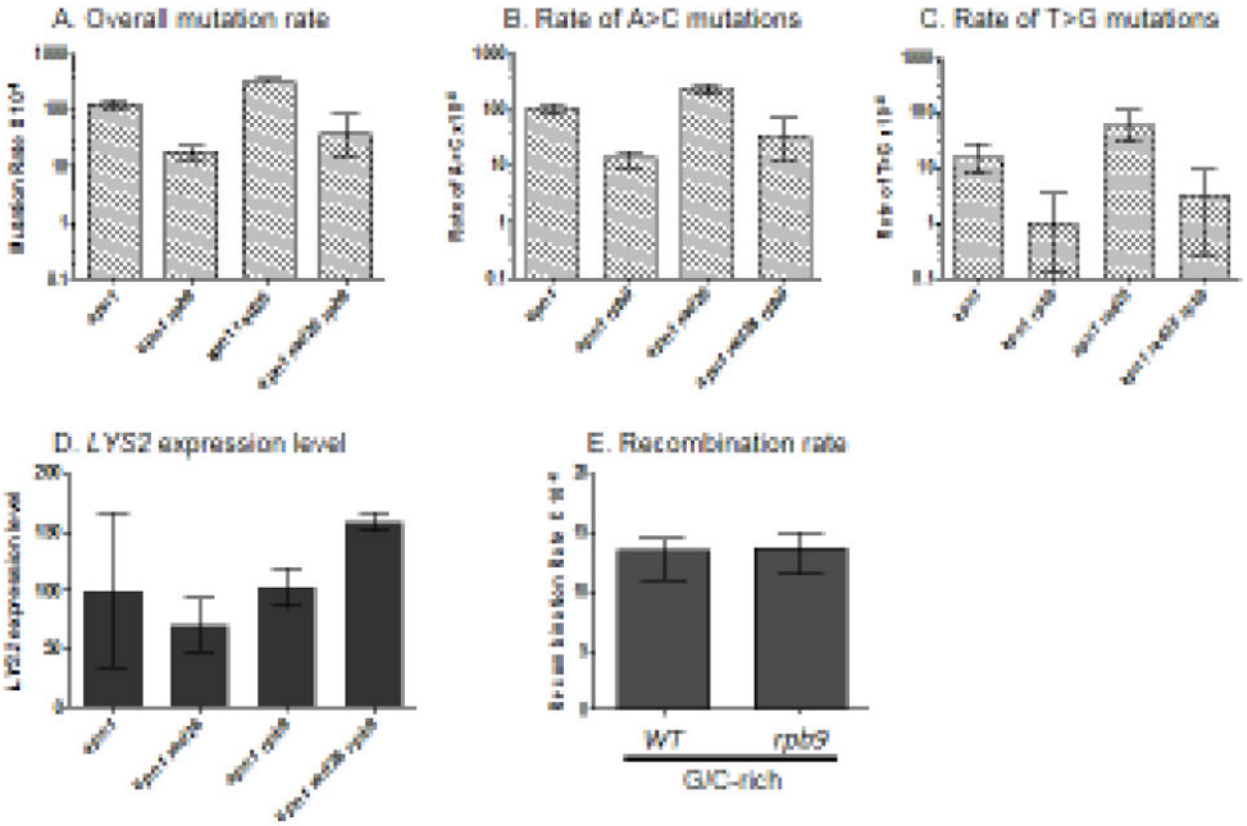

Figure 5.

The effect of Rpb9 disruption on the rates of mutations and recombination.

A) Overall rates of mutations at *pTET-lys2-TAA*. Error bars represents 95% confidence intervals. **B)** Rates of A>C mutations. **C)** Rates of T>G mutations. For the actual numbers see Table S2. **D)** *LYS2* expression levels calculated by RT-QPCR using *ACT1* as the reference gene. RNA was extracted from yeast cells with the indicated genetic strains. QRT-PCR was performed using primers targeting *LYS2*. For the actual numbers see Table S4. Error bars indicate the standard deviation calculated from three independent experiments. **E)** The rates of recombination at the *G/C-rich* cassette were determined using the method of the median. Error bars represent 95% confidence intervals. For the actual numbers see Table S5. Total, A>C, and T>G mutation rates in *apn1* and *apn1 rad26* backgrounds in this figure is identical to the rates shown in Figure 2.

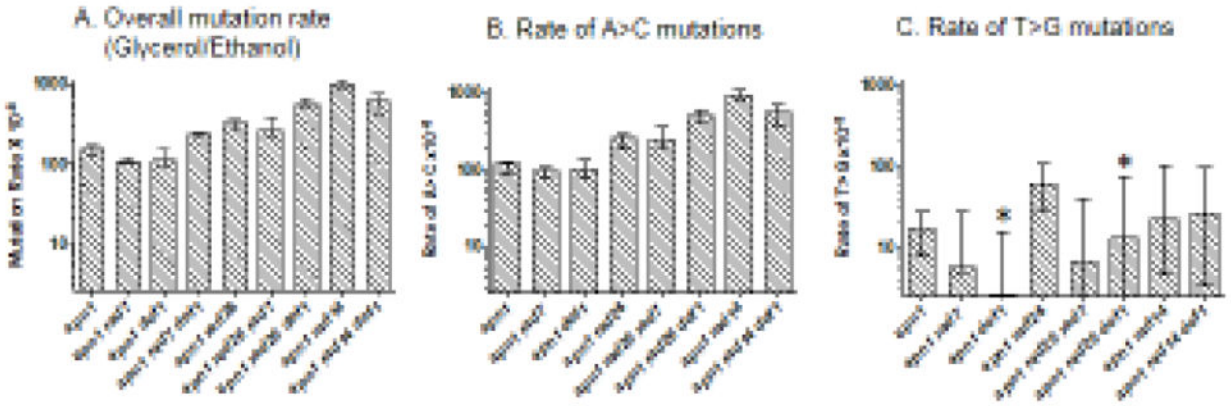

Figure 6.

Effects of Def1 disruption on the rates of mutations in YEPGE.

A) Overall rates of mutations at *pTET-lys2-TAA*. Error bars represent 95% confidence intervals. **B)** Rates of A>C mutations. **C)** Rates of T>G mutations. *Zero mutation event of this class was detected and the mutation rate was calculated assuming 1 event. For the actual numbers see Table S2. Total, A>C, and T>G mutation rates in *apn1, apn1 rad14,* and *apn1 rad26* backgrounds in this figure is identical to the rates shown in Figure 2 and Figure 3.

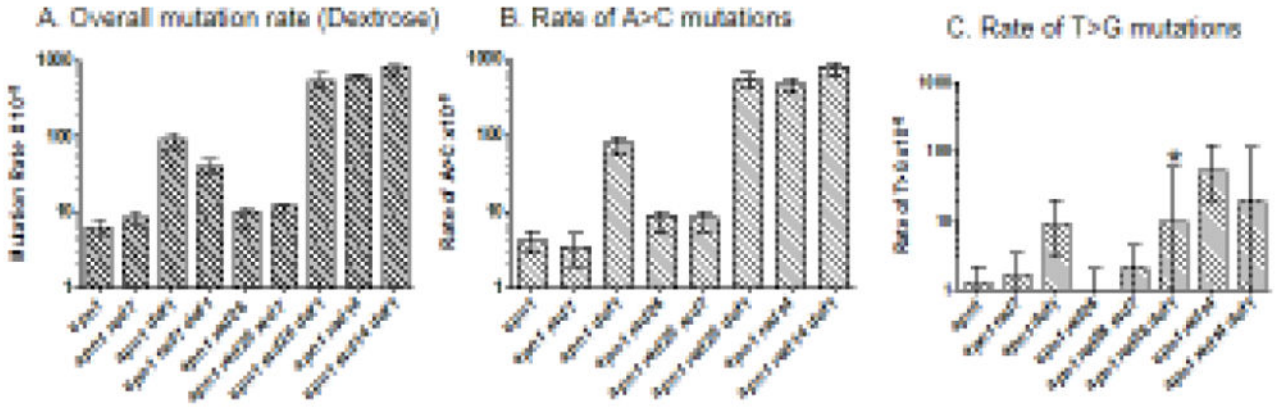

Figure 7.

Effects of Def1 disruption on the rates of mutations in YEPD.

A) Overall rates of mutations at *pTET-lys2-TAA*. Error bars represent 95% confidence intervals. **B)** Rates of A>C mutations. **C)** Rates of T>G mutations. *Zero mutation event of this class was detected and the mutation rate was calculated assuming 1 event. For the actual numbers see Table S6.

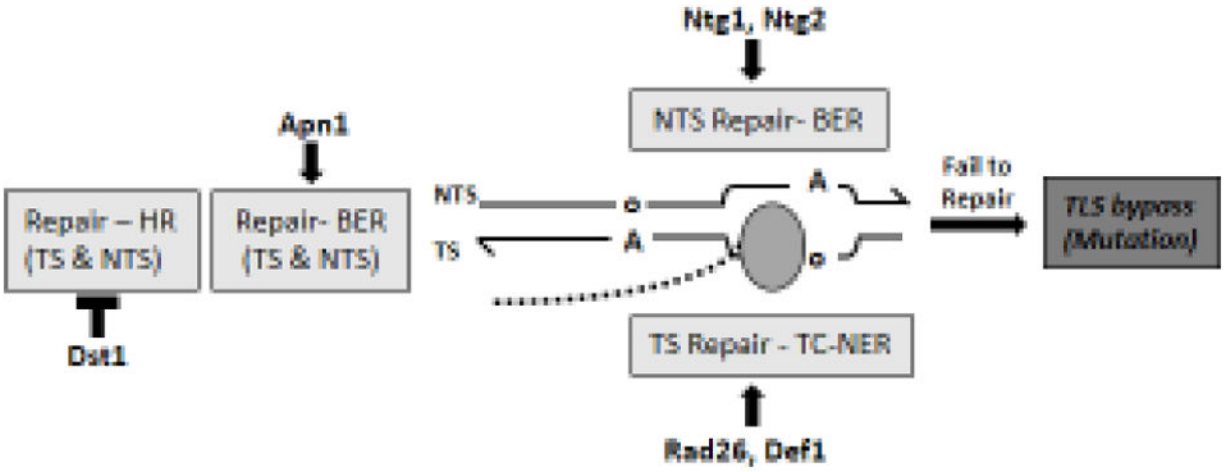

Figure 8.

Proposed model of AP repair at transcribed regions.

AP sites on both transcribed strand (TS) and non-transcribed strand (NTS) are repaired by either Apn1-mediated BER or homologous recombination pathway (HR). Repair by HR is suppressed by Dst1. While Ntg1/Ntg2-mediated BER repairs mostly the AP sites present on TS, Rad26- or Def1-mediated TC-NER repairs the AP sites on NTS. Those AP sites left unrepaired will invoke mutagenic translesion synthesis (TLS). Grey oval and dotted line represent RNA polymerase complex and nascent RNA chain, respectively. AP lesion is denoted by o.