Published in final edited form as:

DNA Repair (Amst). 2008 May 3; 7(5): 801–810.

Identification of Pathways Controlling DNA Damage Induced Mutation In *Saccharomyces cerevisiae*

Ewa T. Lis, Bryan M. O'Neill, Cristina Gil-Lamaignere, Jodie K. Chin, and Floyd E. Romesberg¹

Department of Chemistry, The Scripps Research Institute, La Jolla, CA, 92037

Abstract

Mutation in response to most types of DNA damage is thought to be mediated by the error-prone sub-branch of post-replication repair and the associated translesion synthesis polymerases. To further understand the mutagenic response to DNA damage, we screened a collection of 4848 haploid gene deletion strains of *Saccharomyces cerevisiae* for decreased damage-induced mutation of the *CAN1* gene. Through extensive quantitative validation of the strains identified by the screen, we identified ten genes, which included error-prone post-replication repair genes known to be involved in induced mutation, as well as two additional genes, *FYV6* and *RNR4*. We demonstrate that *FYV6* and *RNR4* are epistatic with respect to induced mutation, and that they function, at least partially, independently of post-replication repair. This pathway of induced mutation appears to be mediated by an increase in dNTP levels that facilitates lesion bypass by the replicative polymerase Pol δ , and it is as important as error-prone post-replication repair in the case of UV- and MMS-induced mutation, but solely responsible for EMS-induced mutation. We show that Rnr4/Pol δ -induced mutation is efficiently inhibited by hydroxyurea, a small molecule inhibitor of ribonucleotide reductase, suggesting that if similar pathways exist in human cells, intervention in some forms of mutation may be possible.

Keywords

yeast; post-replication repair; induced mutagenesis

1.Introduction

The stability of the genome is critical for life, but mutation occurs as a result of both exogenous and endogenous mutagens [1]. Mutations contribute to ageing [2], oncogenesis, tumor progression [3–5], and resistance to chemotherapy [6–8]. Ironically, because many chemotherapeutic drugs are mutagens, they may themselves play a role in inducing the mutations that give rise to resistance and therapy failure [9–11].

For many years, it was assumed that mutagens induced DNA damage, and the damaged DNA caused the replicative polymerases to make errors during DNA replication. However, it is now understood that most types of DNA damage inhibit DNA replication, and that the cellular response to this inhibition is critical for the induction of mutation. The cellular response to replication blocks in the yeast *Saccharomyces cerevisiae* has been intensively studied and is

¹Corresponding author Floyd E. Romesberg, Department of Chemistry, The Scripps Research Institute, CB262R, 10550 N. Torrey Pines Rd, La Jolla, CA, 92037, Phone: (858) 784-7290, Fax: (858) 784-7472, E-mail: floyd@scripps.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mediated in part by the intra-S checkpoint response [12] which helps to stabilize stalled replication forks, inhibit late origin firing, and induce an S-phase specific increase in the levels of dNTPs [13–16].

In addition, for many types of DNA damage the induction of mutation requires the nonessential specialized translesion synthesis (TLS) polymerases Rev1, Polζ, or Polη, encoded by *REV1*, *REV3* and *REV7*, and *RAD30*, respectively. These genes are part of the RAD6 genetic epistasis group [17–19], which is responsible for post-replication repair (PRR), a process that converts low molecular weight DNA fragments into higher molecular weight DNA after genome replication is complete [20]. PRR likely involves the filling in of DNA single-stranded gaps that are created after synthesis is re-initiated downstream of a replication block [21–23]. However, the TLS polymerases are not required for mutation induced by some mutagens, such as the alkylating agent EMS [24], suggesting that other pathways of induced mutation exist.

Using the complete set of systematic gene deletion mutants created by the *Saccharomyces* Genome Deletion Project [25,26] we have performed a screen for genes required for UV- and MMS-induced mutation. We identified genes that appear to act in two independent pathways: the known mutagenic PRR pathway and a novel pathway that appears to induce mutation at damaged DNA by up-regulating dNTP levels and facilitating TLS by the replicative polymerase Polδ. We discuss the importance of these pathways in inducing mutation and the possibility of inhibiting them to eliminate mutation induced by chemotherapy.

2. Materials and Methods

2.1 General Procedures, Media, and Strains

Compounds used in this study were obtained from commercial sources as follows: Icanavanine sulfate, US Biological; 5-FC, Acros; MMS, Aldrich; EMS, Acros; G418, RPI; and HU, US Biological. Yeast were cultured using standard methods at 30 °C in Yeast extract/Peptone/Dextrose (YPD) or Synthetic Complete (SC). Can^r mutants were selected on SC-Arg media containing 60 mg/L l-canavanine sulfate. *lys2ΔBgl* and *hom3–10* revertants were selected on SC media lacking lysine or threonine, respectively; 5-fluorocytosine (5-FC) resistant colonies were selected on SC media containing 100 µg/mL 5-FC. UV irradiations (UV-C, 254 nm) were performed with a G8T5 germicidal tube (Ushio America, Cypress, CA). Strains used in this study are listed in Table S1. Screening experiments were performed using a 4848 member library of nearly all the nonessential yeast gene deletion strains in the genetic background BY4741 obtained from Open Biosystems (Huntsville, Alabama). Characterization of individual gene deletions identified by the screens were performed in strain RDKY3615 [27] (*MATa ura3–52 leu2Δ1 trp1Δ63 his3Δ200 lys2ΔBgl hom3–10 ade2 Δ 1 ade8 hxt13::URA* kindly provided by R.D. Kolodner, UCSD). Characterization of induced mutation frequencies was performed in strains RDKY3615 and S18-1 (*MATa ade5 lys2::insE trp1–289 his7–2 leu2–3,112 ura3–52* kindly provided by D. Gordenin, NIH/NIEHS). All genomic DNA for strain constructions and mutation spectra determinations was prepared using a standard phenol/chloroform extraction method [28]. Nucleotide pool measurements were determined as described previously [16].

2.2 Screening procedure

The library of yeast deletion mutants was inoculated into YPD in 96-well microplates and incubated at 30 °C for 2 days. Five microliters of the resulting saturated culture of each strain were plated onto YPD, YPD containing 0.015% MMS, and YPD that was subsequently irradiated with UV light (50 J/m²). Plates were incubated at 30 °C for 2 days and photographed. The plates were replica plated onto SC-Arg containing canavanine, incubated at 30 °C for 3 days and photographed to score the number of *CAN1* mutants.

The screen identified 305 deletion strains which qualitatively appeared less mutable than wild type cells. Thirty-eight strains were found to require arginine for growth and were not further characterized, as their isolation was assumed to be an artifact of the SC-Arg media required for Can^r selection. Of the remaining 267 strains, 151 had an apparent UV-induced Can^r forward mutation frequency that was more than 2-fold lower than that observed for wild-type. These 151 library deletion strains (BY4741 strain background) were re-constructed in strain RDKY3615. Genomic DNA from the selected library strains was isolated and used as a template for PCR amplification of the gene specific *kanMX4* cassette flanked by ~50 bases of homology to the upstream and downstream region of the gene. Double stranded DNA cassettes were transformed into RDKY3615 and gene disruptants were selected on G-418 using standard protocols [29]. The MMS- and UV-induced mutation frequencies of multiple, independent transformants were tested using the high throughput mutation assay described above. A representative transformant was selected for further quantitative mutation assays. For each strain, the spontaneous mutation frequencies were re-determined using three independent clones, and the UV-induced mutation frequencies were re-determined using one clone. Of the 151 library deletion strains selected, only 31 mutants had a UV-induced mutation frequency that was 2-fold lower than wild-type cells. The majority of the strains, ~60%, exhibited a UV-induced mutation frequency that was similar to wild-type despite the corresponding BY4741 strains exhibiting significantly reduced frequencies. For about 10% of the library deletion strains examined, PCR amplification with the gene flanking primers resulted in wild-type products rather than the *kanMX4* cassette, indicating that the strains no longer carried the expected gene deletions. For the 31 strains that showed a ≥ 2 -fold reduced induced mutation frequency in the experiment with a single clone, we next re-determined the mutation frequencies using 3 independent clones. A subset of the 31 strains showed induced mutation frequencies within error of wild-type frequencies, and these strains were eliminated from further analysis. Gene deletions were also confirmed at this stage by PCR for the presence of the gene deletion cassette and the absence of wild-type gene. A subset of strains did not pass the PCR confirmation test, and these strains were removed from further analysis. In all, 20 strains were identified with a properly integrated *kanMX4* cassette and ≥ 2 -fold average reduction in the UV-induced mutation frequency as compared to wild type. These 20 strains were subjected to more rigorous analysis by determining UV-induced (36 J/m²) mutation frequencies at other loci using reversion of the *lys2 Δ Bgl* allele to allow growth on media lacking lysine, reversion of the *hom3-10* allele to permit growth in the absence of threonine, and forward mutation of genes involved in resistance to 5-FC.

2.3 Quantitative determination of mutation frequency

To characterize spontaneous mutation, cells from saturated cultures (grown for 24 h) were harvested by centrifugation, washed and resuspended in saline, and plated on media containing canavanine to identify Can^r mutants and on SC-Arg to determine the number of viable cells. To characterize induced mutation, similarly prepared cells were plated on SC-Arg plates and irradiated with varying doses of UV light. Cells were allowed to recover for 6 h at 30 °C after which they were washed off the SC-Arg plates and re-plated onto Can^r selective media to identify mutants and onto SC-Arg to determine the number of viable cells. Colonies were counted after three days at 30 °C. Spontaneous mutation frequencies were determined by fluctuation analysis using the method of the median [30] from 9 independent cultures determined 2–3 times. Induced mutation frequencies were determined by dividing the number of Can^r mutants observed by the number of viable cells and subtracting the contribution of the preexisting, *i.e.* spontaneous, mutations. Induced frequencies were determined from 3–8 independent cultures. Mutation frequencies at other loci were examined similarly using appropriate media for selection of mutants and SC for determination of viability.

2.4 Determination of Mutation Spectrum

Mutation spectra of canavanine resistant (Can^r) clones were determined by DNA sequencing of the entire 1.8 kb *CAN1* gene amplified from mutant colonies obtained from deletion strains in the RDKY3615 genetic background. Mutants were collected from plates generated during determination of induced mutation frequencies. Colonies were re-streaked to canavanine containing media, and genomic DNA was extracted. The primers used for PCR amplification were 5'-GTA AAC CGA ATC AGG GAA TCC and 5'-CTA CTC CGT CTG CTT TCT TTT C; and the resulting PCR product was sequenced using 5'-CGA AAT GGC GTG GAA ATG TG and 5'-CAA TGT AGA AGG TTA AGA TAC GG.

3. Results

3.1 The induced mutagenesis screen

To identify non-essential genes involved in induced mutation, we performed a qualitative screen for deletion mutants that are unable to induce mutations in the *CAN1* gene in response to UV or MMS treatment. Mutations in *CAN1*, typically substitutions or insertions/deletions, confer the easily selectable phenotype of canavanine resistance (Can^r) [31]. Strains were spotted onto permissive media, treated to induce DNA damage, and transferred to Can^r selective media. Growth of each strain was evaluated and compared on the permissive and selective plates. Three different phenotypes were observed: (1) sensitivity to DNA damage, (2) little to no sensitivity to DNA damage and wild-type-like or greater growth on selective media, and (3) little to no sensitivity to damage and less than wild-type-like growth on selective media (Figure S1). Two hundred sixty-seven strains exhibiting the latter phenotype in both the MMS- and UV-induced mutation screens were identified and tentatively assigned as deficient in inducing mutation in the *CAN1* gene (Table S2).

3.2 Quantitative determination of mutation frequencies

We next validated the reduced mutation phenotype of the identified strains by determining quantitative mutation frequencies under both spontaneous and UV-induced conditions. We found that 151 of the 267 identified strains had an apparent UV-induced Can^r forward mutation frequency that is more than 2-fold lower than wild-type, with similar trends observed for the spontaneous mutation frequencies (Table S2).

We next examined whether the observed decreases in mutation frequencies resulted from disruption of the expected open reading frame. Deletion mutants corresponding to the 151 library strains were re-constructed in strain RDKY3615. Analysis of Can^r mutation in these strains identified 20 with a UV-induced mutation frequency at least 2-fold lower than wild-type (Table S3). Included among these 20 strains are those previously implicated in induced mutation, including *RAD5*, *RAD6*, *RAD18*, *REV1*, *REV3*, *REV7*, *POL32*, and *SRS2*. These constitute all of the key non-essential components of mutagenic PRR, and as they have already been well characterized, they were not characterized further here.

For the 12 remaining genes, we next examined UV-induced reversion of the +4 *lys2ΔBgl* and +1 *hom3-10* frameshift alleles, as well as spontaneous and UV-induced forward mutation of genes conferring resistance to the toxic cytosine analog fluorocytosine (5-FC) (Table 1 and 2). Resistance to 5-FC may be acquired through point mutations in *FCY1*, a cytosine deaminase or *FCY2*, a purine-cytosine permease (unpublished data). These assays are differentiated according to whether mutation results in a gain-of-function (*lys2ΔBgl* and *hom3-10*) or loss-of-function (*CAN1* and 5-FC), and together these assays allowed us to differentiate between strains that are truly defective for induced mutation and those that are only apparently defective due to artifacts, such as polyploidy (multiple copies of a loss-of-function allele will result in apparently reduced mutation frequencies [32]). Eight deletion strains exhibited decreased

mutation frequency in the loss-of-function assays but increased frequencies in the gain-of-function assays. Further analysis confirmed that these strains are deficient in the regulation of chromosome number and they will be described elsewhere.

3.3 Genes involved in induced mutation

Of the four remaining genes whose deletion results in reduced mutation in both gain- and loss-of-function assays (*KAP123*, *VPS3*, *FYV6* and *RNR4*), *KAP123* and *VPS3* were not further characterized. For *KAP123*, the observed decreases in mutation frequency were restricted to the loss-of-function assays and were generally smaller than for the other identified genes. For *VPS3*, which encodes a protein involved in sorting and processing of soluble vacuolar proteins [33], wild-type mutation rates were observed when more time was allowed between UV irradiation and Can^r selection (Figure S2), suggesting that the apparently reduced mutation rates are an artifact associated with phenotypic lag. This recovery of induced mutation was not observed for any of the other deletions strains identified. The remaining two genes, *FYV6* and *RNR4*, appear to play a significant and general role in induced mutation.

The only previously proposed role of *FYV6* that is obviously related to DNA damage tolerance or repair is regulation of non-homologous end joining [34]. However, this function is unlikely to be related to the role of *FYV6* in induced mutation as *yku70Δ*, *lif1Δ*, *dnl4Δ* and *mre11Δ* strains, all of which are known to be defective in non-homologous end joining, showed wild-type mutation rates (as expected since they were not isolated in the screen, data not shown). Thus, we determined whether *FYV6* functions in the same pathway(s) as the known TLS polymerases. Genetic epistasis analysis using UV induced mutation of *CAN1* shows that *FYV6* and *REV3* are not epistatic, suggesting that at least some of the mutations facilitated by Fyv6 are independent of Polζ (Figure 1A). Analysis of the Can^r mutation spectra (Figure 2) showed that mutations induced in *fyv6Δ* are similar to those induced in wild-type cells ($p > 0.05$) and clearly distinct from those induced in *rev3Δ* cells ($p < 0.02$) [35]. We also found that unlike deletion of *REV3*, deletion of *FYV6* results in a small but significant reduction in EMS-induced mutagenesis (Figure 1J).

Rnr4 encodes one of the small subunits of RNR and is induced by DNA damage [36,37]. Previously, Brendel and co-workers demonstrated that deletion of *RNR4* reduces UV-induced mutation and the authors suggested that this resulted from reduced translesion synthesis by Polζ [38]. We first examined the UV sensitivity and mutability of a *rad1Δ rnr4Δ* mutant to determine whether Rnr4 is involved in NER, the primary repair mechanism responsible for removing UV-lesions. While the *rnr4Δ rad1Δ* double mutant was not significantly more sensitive to UV light (2.5 J/m²) than the *rad1Δ* mutant (as expected since the *rnr4Δ* single mutant is not sensitive to UV; Figure S3), it had a significantly lower UV-induced mutation frequency (Figure 1B). Thus, in the absence of NER, Rnr4 still appears to be required for the efficient induction of mutation.

rnr4Δ is also not epistatic with either *rev1Δ* or *rev3Δ* (Figure 1C and 1D). In fact, the reduction in induced mutation associated with deleting *RNR4* is similar in *rev3Δ* cells and wild-type cells, suggesting that, like Fyv6, Rnr4 facilitates at least some mutations independently of mutagenic PRR. Deletion of *RAD30* has no effect on UV-induced mutation in either the wild-type or the *rnr4Δ* strain (Figure 1E), thus no genetic interaction may be inferred. In addition, we were unable to construct the *rnr4Δ pol32Δ* mutant, which is consistent with a synthetic lethal interaction reported previously [39]. However, while indirect, the HU data presented below suggest that Pol32 is not required for Rnr4-mediated mutation. Also as was observed with *fyv6Δ*, the UV-induced Can^r mutation spectrum of *rnr4Δ* is clearly distinct from *rev3Δ* ($p < 0.01$), but similar to wild-type cells ($p > 0.05$) (Figure 2). Furthermore, in contrast to *rev1Δ*, *rev3Δ* or *rad30Δ* cells ([24] and Figure S4), EMS induces virtually no Can^r mutations in *rnr4Δ* cells (Figure 1J). Given the phenotypic similarities, it is not surprising that *FYV6* and

RNR4 are epistatic for mutation at *CAN1* (Figure 1F), possibly with the exception of high UV-irradiation conditions. These data indicate that *RNR4* and *FYV6* function in a pathway for induced mutation that is distinct from mutagenic PRR. However, the smaller effect of *FYV6* deletion, relative to *RNR4* deletion, suggests that Fyv6 participates in only a subset of the mutations induced by Rnr4.

Lastly, we examined the genetic interactions between *RNR4* and *RAD18*. The double mutant was viable but grew much more slowly than either single mutant. In addition, we found that the *rnr4Δ rad18Δ* mutant is synergistically more sensitive to UV, MMS, or EMS exposure than either single mutant (Figure 3A). These results suggest that Rnr4 functions to help tolerate both spontaneous and induced DNA damage that may also be processed by PRR.

3.4 The contribution of replicative polymerase proofreading to spontaneous and induced mutation

Since *FYV6* appears to be involved in only a subset of the mutations mediated by Rnr4, we focused further efforts on the characterization of *RNR4*. At least some, and perhaps most of the mutations facilitated by Rnr4 are independent of the conventional TLS polymerases, suggesting that perhaps one or both of the replicative polymerases is responsible for the mutations induced by *RNR4*. To examine this possibility we characterized the *pol3-1* and *pol2-4* strains, in which the exonuclease proofreading activities of Polδ and Polε, respectively, have been disrupted. Both mutants exhibit increased spontaneous mutation (Table 1); and as reported previously [40,41], the effect is much larger with Polδ. In each case, deletion of *RNR4* reduces the spontaneous hypermutability by ~3-fold (Table 1). The fact that deletion of *RNR4* reduces mutation in both exonuclease deficient strains, but not in the wild-type strain, suggests that proofreading reduces the contribution of *RNR4* to spontaneous mutation.

In the case of both UV- or EMS-induced mutation, disabling Polδ proofreading has a large effect, increasing mutation up to 70-fold. Interestingly, this hypermutability is dependent on *RNR4* (Figure 1G and 1K); with the *pol3-1 rnr4Δ* double mutant showing UV- and EMS-induced mutation rates that are indistinguishable from those of wild-type cells. The mutation frequency of the *pol3-1 rnr4Δ* strain is not fully reduced to the level of the *rnr4Δ* strain which is consistent with the observation that deletion of *RNR4* does not completely abolish the damage-mediated increase in dNTP levels (see below). Alternatively, this behavior may reflect an ability of exonuclease deficient Polδ to replicate through a lesion even in the absence of increased dNTP levels. In contrast, to *RNR4*, the hypermutability that results from rendering Polδ exonuclease deficient does not depend on Polζ as deletion of *REV3* has no effect (Figure 1H and 1L). Deletion of *REV3* in a *pol3-1 rnr4Δ* double mutant likewise has no effect on induced mutation (Figure 1G and 1H). Surprisingly, disabling Polε proofreading has no effect on UV- or EMS-induced mutation in wild-type cells (Figure 1I and 1K). Likewise, disabling Polε proofreading in *rnr4Δ* strains has no effect on UV-induced mutation (Figure 1I), however, it does result in a small increase in EMS-induced mutation (Figure 1K). These data suggest that *RNR4* interacts specifically with Polδ, and that even in the absence of proofreading, Polε contributes little to induced mutation, and even then, only in the absence of *RNR4*.

To further understand the relationship between polymerase proofreading and induced mutation, we examined the effect of rendering each polymerase exonuclease deficient in a *rad18Δ* mutant lacking PRR (Figure 3B). Disabling Polε proofreading had no effect on the sensitivity of the *rad18Δ* strain to UV, MMS, or EMS. Again, in stark contrast, disabling Polδ proofreading strongly suppresses the sensitivity of the *rad18Δ* strain to all three damaging agents. These data suggest that Polδ acts upstream of PRR and that disruption of Polδ proofreading specifically facilitates a pathway involving Rnr4 that is distinct from PRR, but that acts to repair or tolerate the same lesions.

3.5 Deletion of *RNR4* reduces intracellular nucleotide pools

RNR4 mutants are known to progress more slowly through S-phase and show decreased RNR activity [37], which was suggested to result from reduced nucleotide pools in *rnr4* cells [36, 37]. To determine whether deletion of *RNR4* reduces dNTP levels, we analyzed the amount of dNTPs that are present in wild-type and *rnr4* strains, both in undamaged cells and in cells treated with 30 J/m² UV light or 0.025% MMS (Figure 4 and Figure S5). The basal levels of dNTPs were the same in wild-type and *rnr4*Δ strains. However, while dNTP levels were elevated ~7-fold following UV-damage in wild-type cells, they were only elevated ~3-fold in *rnr4*Δ strains. Moreover, MMS damage induced a greater than 6-fold dNTP increase in wild-type cells, but only ~2-fold in *rnr4*Δ cells. This suggests that *RNR4* may contribute to induced mutation by helping to mediate increases in dNTP levels when DNA is damaged.

3.6 Hydroxyurea inhibits induced mutagenesis

Hydroxyurea (HU) is a small molecule chemotherapeutic that is thought to inhibit RNR and thus reduce intracellular dNTP concentrations [42]. To confirm that decreased nucleotide pools can reduce mutation, and to determine whether induced mutation may be inhibited by a small molecule, we examined the effects of HU. Remarkably, HU significantly inhibits UV-induced mutation in a dose-dependent manner (Figure 5A). For example, at 100 mM HU, the Can^r mutation frequency per viable cell after 67 J/m² of UV light is reduced by ~100-fold, virtually to spontaneous levels. A similar reduction in UV-induced mutation frequency was observed with *lys2ΔBgl* reversion (Figure 5A), implying that HU inhibits the formation of both point mutations and frameshift mutations. In addition, HU also potently inhibits EMS-induced mutation (Figure S6), and its inhibition of UV-mutation is independent of *RAD30*, *POL32*, and *REV3* (Table 3), further suggesting that HU specifically inhibits the same pathway that is mediated by Rnr4.

At high concentrations (~200 mM), HU induces S-phase arrest of *S. cerevisiae* [43], thus it is possible that an increase in repair during a prolonged S-phase is responsible for the effect of HU. To determine whether the HU-mediated inhibition of mutation (which occurs at concentrations as low as 10 mM), results from a prolonged S-phase, the UV-induced mutation frequency with or without HU was determined in an S-phase arrest deficient *rad53*Δ mutant. (Examining the same effect with the *rnr4*Δ mutant was not possible, since the *rnr4*Δ *rad53*Δ double mutant is inviable [36].) With or without Rad53, HU induces a profound reduction in mutation, suggesting that a checkpoint-mediated delay in S-phase is not required (Figure 5B).

To further support the suggestion that the anti-mutagenic effect of HU is mediated through reduced dNTP levels, we examined the effect of HU in the absence of *SML1*. Sml1 is a protein inhibitor of RNR, and nucleotide levels are known to be elevated in *sml1*Δ strains [44,45]. The anti-mutagenic effect of HU was clearly suppressed in the *sml1*Δ strain, consistent with the effect being mediated by a reduction in nucleotide pools (Table 3).

4. Discussion

To identify proteins that are required for induced mutagenesis, we carried out a screen for non-essential genes in *S. cerevisiae* that when absent, render cells less able to mutate. The screen identified all of the key non-essential PRR genes previously known to be involved in mutation, *RAD5*, *RAD6*, *RAD18*, *REV1*, *REV3*, *REV7*, *POL32*, and *SRS2*. The screen also identified *FYV6* and *RNR4*, which appear to cooperate in a second, distinct pathway of induced mutation. Fyv6 has no sequence homology to a known protein and its identification in high-throughput screens for mutants sensitive to the antifungal K1 killertoxin [46] and mutants with altered telomere length [47] suggest no obvious function. While it has been suggested that Fyv6 plays a role in regulation of non-homologous end joining in stationary cells [34], this activity does

not appear to contribute to the protein's role in induced mutation. However, *FYV6* has also been isolated in a high-throughput screen for genes required for resistance to DTT [48]. This screen also identified the genes encoding thioredoxin, which is an essential electron donor for RNR. Given the role of RNR in this pathway, one plausible role for Fyv6 is that it interacts in some manner with thioredoxin to help regulate RNR activity. Nonetheless, Fyv6 appears to play a role in only a subset of the mutations induced by Rnr4.

RNR4 encodes one of the four subunits of RNR. We find that deletion of *RNR4* results in an up to 10-fold reduction in UV-induced mutation. Previously, Rnr3, another subunit of RNR has been implicated in MMS-induced mutation. Specifically, the inhibition of the TORC1 pathway, which controls transcription of *RNR3* via Rad53, also results in a reduction of mutation in response to MMS [49]. These data suggest that the effect on induced mutation may be generalized to other subunits of the RNR complex. We also demonstrated that *RNR4* is required for UV- and MMS-induced increases in dNTP levels, consistent with the ability of the protein to stimulate TLS as proposed previously [38]. Moreover, our data suggest that RNR4 functions in a previously unknown second pathway of induced mutation that involves TLS by the replicative polymerase Pol δ . While this pathway contributes significantly to UV and MMS-induced mutation, it appears to be the major pathway responsible for EMS-induced mutation.

Interestingly, Pol δ has been previously implicated in TLS across an abasic site *in vivo*, at least when the abasic site is located within a single-stranded region of a plasmid [50]. Moreover, the hypermutability associated with rendering Pol δ exonuclease deficient has been shown to depend on the activity of the S-phase checkpoint [51]. The hypermutability is significantly diminished by deletion of *DUN1*, which encodes a substrate of Rad53 that regulates RNR activity, and this hypermutability is not affected by deletion of *REV3*. These data are consistent with an S-phase checkpoint-mediated upregulation of dNTP levels for Pol δ mediated TLS.

A model consistent with all of the data is presented in Figure 6. In the case of UV-damage, the majority of photo-lesions are repaired by NER, but unrepaired photolesions, or lesions induced by other types of mutagens (i.e. MMS or EMS) that are not repaired prior to S-phase, cause replication forks to stall and the induction of the intra-S checkpoint response. As part of this response, Rnr4 is upregulated and the resulting increase in dNTPs facilitates error-prone TLS by Pol δ , perhaps specifically during lagging strand synthesis where Pol δ is thought to predominantly function [52,53]. This pathway is also favored by disabling Pol δ exonuclease proofreading activity, presumably because this activity competes with TLS. If TLS does not occur, genome replication is completed by error-free or mutagenic PRR, perhaps involving gap filling after re-initiation of synthesis downstream of the blocking lesion. This explains why factors that favor Pol δ TLS, including disabling its exonuclease activity or the presence of *RNR4*, strongly reduce the damage sensitivity of PRR deficient cells.

The data does not rule out a contribution of Rnr4 to Pol ζ -mediated mutagenic PRR, and examining this issue will require additional studies. What is clear from the data is that the Pol ζ and Pol δ pathways are distinct and that the Pol δ pathway relies on Rnr4, but not on Pol ζ . Both pathways, Rnr4/Pol δ and mutagenic PRR, appear to contribute to mutation induced by many types of DNA damage, including UV and MMS, but the Rnr4/Pol δ pathway alone appears to play an important role in EMS-induced mutagenesis. It seems likely that UV- and MMS-induced damage are more potent blocks of replication, and thus are more likely to require the specialized activities of the conventional TLS polymerases, while EMS induces damage that may be more easily replicated through by Pol δ , at least in the presence of elevated levels of dNTPs. The effects of other mutagens may similarly partition between these two pathways depending on the ability of Pol δ at elevated levels of dNTPs to synthesize through the associated lesion.

In addition to facilitating lesion bypass, the presence of elevated dNTP levels may also reduce the fidelity of Pol δ acting on undamaged templates and may result in the induction of undirected mutations. In fact, elevated nucleotide pools have been associated previously with reduced fidelity of DNA replication possibly by making competitive the rate at which a mispair is extended with the rate at which it is excised [54]. Additional work is required to deconvolute the contributions of lesion directed and undirected mutation mediated by the Rnr4/Pol δ pathway.

The ability of HU to inhibit Rnr4/Pol δ -mediated mutation is particularly interesting, as it suggests that if similar mechanisms are conserved in human cells, the mutations induced during chemotherapy might be inhibited. The potential therapeutic utility of such an approach depends on the proportion of mutations mediated by Rnr4/Pol δ as opposed to PRR, which is currently under investigation. Whatever the result, the data suggest that there are a finite number of pathways, perhaps only two, that independently induce mutation. Thus, HU combined with an inhibitor of Pol ζ , might represent a therapeutic combination that efficiently eliminates all forms of chemotherapy-induced mutation. Studies directed towards reaching these goals promise to provide an unprecedented opportunity to understand genome instability in eukaryotic cells and also to intervene in mutation, and thus in the development and progression of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dinesh Kumar and Andrei Chabes (Umeå University) for performing the dNTP measurements. Financial support for this work was provided by the National Institutes of Health (GM68569 to F.E.R.) and a graduate fellowship from the California Breast Cancer Research Program (11GB-0004 to E.T.L.).

LITERATURE CITED

1. Friedberg, EC.; Walker, GC.; Siede, W. DNA Repair and Mutagenesis. Washington, D.C: ASM Press; 1995.
2. Trifunovic A, Hansson A, Wredenberg A, Rovio AT, Dufour E, Khvorostov I, Spelbrink JN, Wibom R, Jacobs HT, Larsson NG. Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. *Proc. Natl. Acad. Sci. USA* 2005;102:17993–17998. [PubMed: 16332961]
3. Fishel R, Lescoe MK, Rao MR, Copeland NG, Jenkins NA, Garber J, Kane M, Kolodner R. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* 1993;75:1027–1038. [PubMed: 8252616]
4. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991;253:49–53. [PubMed: 1905840]
5. Mitelman, F. Catalog of Chromosome Aberration in Cancer. New York: Wiley Liss; 1991.
6. Casazza AM, Fairchild CR. Paclitaxel (Taxol): mechanisms of Resistance. *Cancer Treat Res* 1996;87:149–171. [PubMed: 8886452]
7. Volk EL, Schneider E. Wild-Type Breast Cancer Resistance Protein (BCRP/ABCG2) is a Methotrexate Polyglutamate Transporter. *Cancer Res* 2003;63:5538–5543. [PubMed: 14500392]
8. Tonetti DA, Jordan VC. Possible mechanisms in the emergence of tamoxifen-resistant breast cancer. *Anticancer Drugs* 1995;6:498–507. [PubMed: 7579553]
9. Loeb LA, Loeb KR, Anderson JP. Multiple mutations and cancer. *PNAS* 2003;100:776–781. [PubMed: 12552134]
10. Greene MH. Is cisplatin a human carcinogen? *J. Natl. Cancer Inst* 1992;84:306–312. [PubMed: 1738180]
11. Sjoblom T, Parvinen M, Lahdetie J. Germ-cell mutagenicity of etoposide: induction of meiotic micronuclei in cultured rat seminiferous tubules. *Mutat. Res* 1994;323:41–45. [PubMed: 7508565]

12. Kolodner RD, Putnam CD, Myung K. Maintenance of genome stability in *Saccharomyces cerevisiae*. *Science* 2002;297:552–557. [PubMed: 12142524]
13. O'Neill BM, Szyjka SJ, Lis ET, Bailey AO, Yates JR III, Aparicio OM, Romesberg FE. Psy2 and Pph3 form a phosphatase complex required for Rad53 dephosphorylation and replication fork restart during recovery from DNA damage. *Proc. Natl. Acad. Sci. USA* 2007;104:9290–9295. [PubMed: 17517611]
14. Lambert S, Carr AM. Checkpoint responses to replication fork barriers. *Biochimie* 2005;87:591–602. [PubMed: 15989976]
15. Branzei D, Foiani M. The DNA damage response during DNA replication. *Curr Opin Cell Biol* 2005;17:568–575. [PubMed: 16226452]
16. Chabes A, Georgieva B, Domkin V, Zhao X, Rothstein R, Thelander L. Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell* 2003;112:391–401. [PubMed: 12581528]
17. Lawrence CW, Das G, Christensen RB. *REV7*, a new gene concerned with UV mutagenesis in yeast. *Mol. Gen. Genet* 1985;200:80–85. [PubMed: 3897794]
18. Lemontt JF. Mutants of yeast defective in mutation induced by ultraviolet light. *Genetics* 1971;68:21–33. [PubMed: 17248528]
19. Minesinger BK, Jinks-Robertson S. Roles of RAD6 Epistasis Group Members in Spontaneous Pol ζ -Dependent Translesion Synthesis in *Saccharomyces cerevisiae*. *Genetics* 2005;169:1939–1955. [PubMed: 15687278]
20. di Caprio L, Cox BS. DNA synthesis in UV-irradiated yeast. *Mutat Res* 1981;82:69–85. [PubMed: 7022172]
21. Lopes M, Foiani M, Sogo JM. Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. *Mol Cell* 2006;21:15–27. [PubMed: 16387650]
22. Heller RC, Marians KJ. Replication fork reactivation downstream of a blocked nascent leading strand. *Nature* 2006;439:557–562. [PubMed: 16452972]
23. Waters LS, Walker GC. The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G2/M phase rather than S phase. *PNAS* 2006;103:8971–8976. [PubMed: 16751278]
24. Prakash L. Effect of Genes Controlling Radiation Sensitivity on Chemically Induced Mutation in *Saccharomyces cerevisiae*. *Genetics* 1976;83:285–301. [PubMed: 17248715]
25. Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, et al. Functional Characterization of the *S. cerevisiae* Genome by Gene Deletion and Parallel Analysis. *Science* 1999;285:901–906. [PubMed: 10436161]
26. Giaever G, Chu AM, Ni L, Connelly C, Riles L, et al. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* 2002;418:387–391. [PubMed: 12140549]
27. Chen C, Kolodner RD. Gross chromosomal rearrangements in *Saccharomyces cerevisiae* replication and recombination defective mutants. *Nat. Genet* 1999;23:81–85. [PubMed: 10471504]
28. Amberg, DC.; Burke, DJ.; Strather, JN., editors. *Methods in Yeast Genetics*. Cold Spring Harbor, NY: CSHL Press; 2005.
29. Longtine MS, McKenzie A III, Demarini DJ, Shah NG, Wach A, Brachet A, Philippsen P, Pringle JR. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 1998;14:953–961. [PubMed: 9717241]
30. Lea DE, Coulson CA. The distribution of the numbers of mutants in bacterial populations. *J. Genet* 1949;49:264–285.
31. Grenson M, Mousset M, Wiame JM, Bechet J. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. I. Evidence for a specific arginine-transporting system. *Biochim Biophys Acta* 1966;127:325–338. [PubMed: 5964977]
32. Pinto I, Winston F. Histone H2A is required for normal centromere function in *Saccharomyces cerevisiae*. *EMBO J* 2000;19:1598–1612. [PubMed: 10747028]
33. Raymond CK, O'Hara PJ, Eichinger G, Rothman JH, Stevens TH. Molecular analysis of the yeast VPS3 gene and the role of its product in vacuolar protein sorting and vacuolar segregation during the cell cycle. *J. Cell Biol* 1990;111:877–892. [PubMed: 2202738]

34. Wilson TE. A Genomics-Based Screen for Yeast Mutants With an Altered Recombination/End-Joining Repair Ratio. *Genetics* 2002;162:677–688. [PubMed: 12399380]
35. Kozmin SG, Pavlov YI, Kunkel TA, Sage E. Roles of *Saccharomyces cerevisiae* DNA polymerases Pol ϵ and Pol ζ in response to irradiation by simulated sunlight. *Nucleic Acids Res* 2003;31:4541–4552. [PubMed: 12888515]
36. Huang M, Elledge SJ. Identification of RNR4, encoding a second essential small subunit of ribonucleotide reductase in *Saccharomyces cerevisiae*. *Mol. Cell. Biol* 1997;17:6105–6113. [PubMed: 9315670]
37. Wang PJ, Chabes A, Casagrande R, Tian XC, Thelander L, Huffaker TC. Rnr4p, a novel ribonucleotide reductase small-subunit protein. *Mol. Cell. Biol* 1997;17:6114–6121. [PubMed: 9315671]
38. Strauss M, Grey M, Henriques JA, Brendel M. RNR4 mutant alleles *psa3-1* and *rnr4 Δ* block induced mutation in *Saccharomyces cerevisiae*. *Curr Genet* 2007;51:221–231. [PubMed: 17287963]
39. Pan X, Ye P, Yuan DS, Wang X, Bader JS, Boeke JD. DNA integrity network in the yeast *Saccharomyces cerevisiae*. *Cell* 2006;124:1069–1081. [PubMed: 16487579]
40. Morrison A, Bell JB, Kunkel TA, Sugino A. Eukaryotic DNA Polymerase Amino Acid Sequence Required for 3' to 5' Exonuclease Activity. *PNAS* 1991;88:9473–9477. [PubMed: 1658784]
41. Morrison A, Johnson AL, Johnston LH, Sugino A. Pathway correcting DNA replication errors in *Saccharomyces cerevisiae*. *EMBO J* 1993;12:1467–1473. [PubMed: 8385605]
42. Krakoff IH, Brown NC, Reichard P. Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. *Cancer Res* 1968;28:1559–1565. [PubMed: 4876978]
43. Swaminathan S, Kile AC, MacDonald EM, Koepp DM. Yra1 is required for S phase entry and affects Dia2 binding to replication origins. *Mol. Cell. Biol* 2007;27:4674–4684. [PubMed: 17452447]
44. Chabes A, Domkin V, Thelander L. Yeast Sml1, a Protein Inhibitor of Ribonucleotide Reductase. *J. Biol. Chem* 1999;274:36679–36683. [PubMed: 10593972]
45. Zhao X, Muller EG, Rothstein R. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Mol Cell* 1998;2:329–340. [PubMed: 9774971]
46. Page N, Gerard-Vincent M, Menard P, Beaulieu M, Azuma M, Dijkgraaf GJP, Li H, Marcoux J, Nguyen T, Dowse T, Sdicu A-M, Bussey H. A *Saccharomyces cerevisiae* Genome-Wide Mutant Screen for Altered Sensitivity to K1 Killer Toxin. *Genetics* 2003;163:875–894. [PubMed: 12663529]
47. Gathbonton T, Imbesi M, Nelson M, Akey JM, Ruderfer DM, Kruglyak L, Simon JA, Bedalov A. Telomere length as a quantitative trait: genome-wide survey and genetic mapping of telomere length-control genes in yeast. *PLoS Genet* 2006;2:e35. [PubMed: 16552446]
48. Rand JD, Grant CM. The Thioredoxin System Protects Ribosomes against Stress-induced Aggregation. *Mol. Biol. Cell* 2006;17:387–401. [PubMed: 16251355]
49. Shen C, Lancaster CS, Shi B, Guo H, Thimmaiah P, Bjornsti M-A. TOR Signaling is a determinant of cell survival in response to DNA damage. *Mol. Cell. Biol* 2007;27:7007–7017. [PubMed: 17698581]
50. Avkin S, Adar S, Blander G, Livneh Z. Quantitative measurement of translesion replication in human cells: evidence for bypass of abasic sites by a replicative DNA polymerase. *Proc. Natl. Acad. Sci. USA* 2002;99:3764–3769. [PubMed: 11891323]
51. Datta A, Schmeits JL, Amin NS, Lau PJ, Myung K, Kolodner RD. Checkpoint-dependent activation of mutagenic repair in *Saccharomyces cerevisiae pol3-01* mutants. *Mol. Cell* 2000;6:593–603. [PubMed: 11030339]
52. Pursell ZF, Isoz I, Lundström E-B, Johansson E, Kunkel TA. Yeast DNA polymerase ϵ participates in leading-strand DNA replication. *Science* 2004;317:127–130. [PubMed: 17615360]
53. Garg P, Stith CM, Majka J, Burgers PMJ. Proliferating Cell Nuclear Antigen Promotes Translesion Synthesis by DNA Polymerase ζ . *J. Biol. Chem* 2005;280:23446–23450. [PubMed: 15879599]
54. Pham PT, Olson MW, McHenry CS, Schaaper RM. The Base Substitution and Frameshift Fidelity of *Escherichia coli* DNA Polymerase III Holoenzyme in Vitro. *J. Biol. Chem* 1998;273:23575–23584. [PubMed: 9722597]

Abbreviations

MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; HU, hydroxyurea..

Figure 1.

Mutation epistasis analysis. (A–I) UV-induced mutation frequencies were determined as described in Materials and Methods. (J–L) EMS-induced mutation frequencies. Frequencies were determined using a method analogous to that for UV-induced mutation frequencies, but with EMS present in SC-Arg plates. Characterization of induced mutation frequencies was performed in strain RDKY3615 for Fig.1 A–F, J and in strain S18-1 for Fig.1 G–I, K, L.

Figure 2.

UV-induced Can^r mutation spectra. Mutation spectra of canavanine resistant clones were determined by DNA sequencing of the *CAN1* gene amplified from mutant colonies obtained after irradiation of cells with 36 J/m² UV-light.

Figure 3.

RAD18 genetic interactions with *RNR4*, *POL2* and *POL3*. Cultures were grown to mid-log phase, and normalized by cell density. 5 μ L drops of five-fold serial dilutions were plated on YPD, YPD subsequently irradiated with (A) 17 J/m² of UV light, and YPD containing 0.0002% MMS or 0.005% EMS; or (B) 25 J/m² of UV light, and YPD containing 0.0008% MMS or 0.01% EMS. Plates were grown for 3 days at 30°C, and then photographed.

Figure 4.

Cellular levels of each dNTP before and after treatment with UV-light in the presence or absence of *RNR4*. Cells from exponential cultures were treated with 30J/m² UV-light and allowed to grow for 2 hours. Cells were then harvested and the dNTP levels determined as described previously [16].

Figure 5.

Inhibition of UV-induced mutation with HU as measured by *CAN1* and *lys2ΔBgl* assays. (A) To determine the number of mutants, cells from saturated cultures were plated on SC-Arg media containing both canavanine and different concentrations of HU and immediately irradiated with 67 J/m² of UV light. To determine the number of viable cells, cells were plated on SC-Arg media containing HU and immediately irradiated with 67 J/m² of UV light. Induced mutation frequencies were determined by dividing the number of Can^r mutants observed by the number of viable cells. (B) Inhibition of UV-induced mutation at *CAN1* by HU. Deletion of *RAD53* requires deletion of *SML1*. The *sml1Δ* mutant was examined as a control for the *sml1Δ rad53Δ* double mutant. Mutation frequencies were determined as described above.

Figure 6.

Model of induced mutation. The **X** in the template represents a lesion and the box in the newly synthesized DNA represents a mutation. See discussion for details.

Table 1Spontaneous mutation frequencies^a

Genotype	Can ^r × 10 ⁻⁸	5-FC ^r × 10 ⁻⁸
wt	21.6	19.7
<i>vps3Δ</i>	16.7	52.0
<i>rev1Δ</i>	9.97	1.70
<i>rev3Δ</i>	7.89	2.40
<i>rev7Δ</i>	8.07	4.60
<i>pol32Δ</i>	25.2	8.70
<i>rrr4Δ</i>	14.3	5.50
<i>srs2Δ</i>	9.71	15.9
<i>kap123Δ</i>	23.3	9.98
<i>fyy6Δ</i>	16.6	40.7
wt ^b	25.9	nd
<i>rrr4Δ</i> ^b	26.8	nd
<i>pol3-1</i> ^b	1130	nd
<i>pol3-1 rrr4Δ</i> ^b	399	nd
<i>pol2-4</i> ^b	128	nd
<i>pol2-4 rrr4Δ</i> ^b	40.9	nd

^a Spontaneous mutation frequencies were determined by fluctuation analysis using the method of the median from 9 parallel cultures. Values reported are an average of 2–3 determinations.

^b rates were determined in strain S18-1 background (see Table S1 in Supporting Information)

nd not determined

Table 2UV-induced mutation frequencies^a

Genotype	Can ^r ×10 ⁻⁶	5-FC ^r ×10 ⁻⁶	<i>lys2ΔBgl</i> ×10 ⁻⁷	<i>hom3-10</i> ×10 ⁻⁷
wt	151±21	222±30	18.1±2.3	10.0±1.8
<i>vps3Δ</i>	1.30±1.3	114±22	12.3±2.3	9.72±4.1
<i>rev1Δ</i>	5.15±1.0	35.4±11	0.93±0.5	2.54±1.2
<i>rev3Δ</i>	5.79±1.3	39.7±5.8	1.36±1.0	1.12±0.1
<i>rev7Δ</i>	6.90±0.6	49.6±33	0.72±0.4	2.50±1.6
<i>pol32Δ</i>	19.4±5.3	34.9±12	1.15±0.6	5.62±2.0
<i>mnr4Δ</i>	29.8±6.4	82.0±16	5.34±2.7	8.92±1.6
<i>srs2Δ</i>	67.4±34	28.2±5.9	9.31±3.5	7.72±5.4
<i>kap123Δ</i>	72.1±11	94.3±22	19.4±1.6	12.9±1.3
<i>fyy6Δ</i>	75.9±12	180±5.0	12.1±2.3	6.89±1.4

^aUV-induced mutation frequencies were determined using a minimum of 3 parallel cultures.

Table 3

Genetic dependence of the anti-mutagenic effect of HU

Genotype	Mutation Frequency ^a		
	0 mM($\times 10^{-6}$)	25 mM($\times 10^{-6}$)	50 mM($\times 10^{-6}$)
wt	221 $\pm$ 22(1.00) ^b	36.2 $\pm$ 11(0.17)	11.1 $\pm$ 1.1(0.06)
<i>rev3</i> Δ	2.89 $\pm$ 0.9(1.00)	0.47 $\pm$ 0.3(0.16)	0.28 $\pm$ 0.3(0.10)
<i>rad30</i> Δ	68.3 $\pm$ 18(1.00)	5.14 $\pm$ 1.7(0.08)	3.41 $\pm$ 1.0(0.05)
<i>pol32</i> Δ	4.16 $\pm$ 0.7(1.00)	0.47 $\pm$ 0.6(0.11)	slow growth ^c
<i>sml1</i> Δ	331 $\pm$ 76(1.00)	131 $\pm$ 25(0.42)	58.5 $\pm$ 11(0.16)

^a Induced mutation frequency at the HU dose indicated following treatment with 50 J/m² UV light.

^b Figures in parenthesis represent the relative rate for each strain. Induced mutation frequencies at 25 and 50 mM HU were normalized by the mutation frequency at 0 mM HU for each strain.

^c Strain showed a significant sensitivity to 50 mM HU; induced mutation frequencies were not determined.