MOLECULAR AND CELLULAR BIOLOGY, June 2005, p. 5196–5204 Vol. 25, No. 12
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.12.5196–5204.2005


# Oxidative DNA Damage Causes Mitochondrial Genomic Instability in *Saccharomyces cerevisiae*

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Received 7 January 2005/Returned for modification 28 February 2005/Accepted 23 March 2005

**Mitochondria contain their own genome, the integrity of which is required for normal cellular energy metabolism. Reactive oxygen species (ROS) produced by normal mitochondrial respiration can damage cellular macromolecules, including mitochondrial DNA (mtDNA), and have been implicated in degenerative diseases, cancer, and aging. We developed strategies to elevate mitochondrial oxidative stress by exposure to antimycin and $H_2O_2$ or utilizing mutants lacking mitochondrial superoxide dismutase (*sod2*Δ). Experiments were conducted with strains compromised in mitochondrial base excision repair (*ntg1*Δ) and oxidative damage resistance (*pif1*Δ) in order to delineate the relationship between these pathways. We observed enhanced ROS production, resulting in a direct increase in oxidative mtDNA damage and mutagenesis. Repair-deficient mutants exposed to oxidative stress conditions exhibited profound genomic instability. Elimination of Ntg1p and Pif1p resulted in a synergistic corruption of respiratory competency upon exposure to antimycin and $H_2O_2$. Mitochondrial genomic integrity was substantially compromised in *ntg1*Δ *pif1*Δ *sod2*Δ strains, since these cells exhibit a total loss of mtDNA. A stable respiration-defective strain, possessing a normal complement of mtDNA damage resistance pathways, exhibited a complete loss of mtDNA upon exposure to antimycin and $H_2O_2$. This loss was preventable by Sod2p overexpression. These results provide direct evidence that oxidative mtDNA damage can be a major contributor to mitochondrial genomic instability and demonstrate cooperation of Ntg1p and Pif1p to resist the introduction of lesions into the mitochondrial genome.**

The mitochondrial genome of eukaryotic cells encodes integral components of the ATP-producing oxidative phosphorylation pathway. Maintenance of the mitochondrial genome is critical to ensure proper functioning of this organelle in mediating cellular energy demands (2). However, as a consequence of normal aerobic respiration, reactive oxygen species (ROS) are produced and create a highly oxidative environment (18). Upon exposure to ROS, oxidative modification to biomolecules, including DNA, can result (53).

Various defense mechanisms operate in mitochondria to minimize the deleterious effects of oxidative stress and maintain mitochondrial genomic integrity. Primary defenses against oxidative damage include protective proteins and small molecules that scavenge ROS or sequester metal ions. The antioxidant enzyme superoxide dismutase is involved in the conversion of superoxide anion to dioxygen and hydrogen peroxide, which is further detoxified by catalase or peroxidase (24). *Saccharomyces cerevisiae* possesses two genes encoding superoxide dismutase, one of which, Sod2p, specifically localizes to the mitochondrial matrix and is believed to protect mitochondria against oxidative stress (19). Additionally, secondary defenses consist of enzymes that repair the products of oxidatively damaged components (35). If oxidative stress defense mechanisms are compromised or overwhelmed, disease states may ensue. It has been proposed that mitochondrial mutations, presumably resulting from oxidative mitochondrial DNA (mtDNA) damage, contribute to neurodegenerative disorders, cancer, and the aging process in humans (12, 47, 55).

Repair of nuclear and mitochondrial oxidative DNA lesions occurs predominantly through the base excision repair (BER) pathway (37). As demonstrated by mammalian in vitro repair studies, the overall pathway for mitochondrial BER is similar to the short patch pathway of BER in the eukaryotic nucleus (39, 51). BER is initiated by the action of DNA *N*-glycosylases that specifically recognize and excise damaged bases from DNA. Release of the damaged base creates an apurinic/apyrimidinic (AP) site that can be further processed by an *N*-glycosylase-associated AP lyase or a separate, hydrolytic AP endonuclease. Following appropriate 3′ or 5′ end-trimming steps, DNA polymerase inserts the correct base and DNA ligase seals the nick, thereby repairing the damage (4). Mitochondrial BER in *S. cerevisiae* presumably occurs via a similar mechanism and is supported by the observation that several components of the yeast nuclear BER machinery colocalize to mitochondria, including the major AP endonuclease, Apn1p (54), as well as the *N*-glycosylases, Ogg1p (48) and Ntg1p (1, 56), which initiate repair of oxidatively damaged purines and pyrimidines, respectively. In addition to BER, other yeast mitochondrial repair and damage tolerance pathways have been implicated in handling oxidative mtDNA damage, such as recombination (3) as well as recombination-independent functions of proteins such as Abf2p and Pif1p (38).

Pif1p is a 5′-to-3′ DNA helicase that localizes to both the nucleus and mitochondria (27, 28). With respect to its role in mitochondria, Pif1p is important for maintenance, repair, and

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recombination of mtDNA (27). Recently, we demonstrated that the recombination-independent activities of Pif1p cooperate with BER to resist spontaneous oxidative mtDNA damage (38). Our previous analysis of the mitochondrial genomic instability phenotype of *pif1*Δ mutants led us to postulate a role for this unique helicase in mtDNA repair, through governing the rate of mtDNA replication or regulating mtDNA copy number (38). However, the extent to which these proteins cooperate under conditions of chronic oxidative stress is unknown.

When pathways involved in maintaining nuclear genomic integrity are corrupted, many deleterious consequences ensue, including chromosomal aberrations, cellular transformation, and death (9). Additionally, recent studies have implicated ROS in nuclear gross chromosomal rearrangements (22, 49). However, studies examining potentially similar events during the loss of mitochondrial genomic integrity resulting from oxidative stress are limited. *S. cerevisiae* is an ideal model system for delineating the basic molecular mechanisms underlying the maintenance of mtDNA. Yeast are facultative anaerobes; therefore, cells that are devoid of functional mitochondrial respiration (resulting from genomic instability) can still be propagated and studied when a fermentable carbon source, such as glucose, is provided (45). In contrast, respiration-deficient mammalian cells are typically inviable (43).

Although oxidative stress is thought to contribute to mitochondrial mutagenesis, to date very few studies have directly addressed molecular mechanisms by which mtDNA oxidative damage leads to genomic instability or have elucidated the involvement of proteins responsible for maintaining mitochondrial genomic integrity. We have employed strategies to selectively elevate levels of mitochondrial oxidative DNA damage through exploitation of genetic mutants lacking pathways involved in mtDNA maintenance to determine the biological consequences of mitochondrial oxidative stress and to evaluate the relative contributions of these proteins toward prevention of oxidative DNA damage in mitochondria. Our results directly demonstrate that oxidative DNA damage causes mtDNA instability and indicate that Ntg1p and Pif1p are collectively involved in maintaining mitochondrial genomic integrity under conditions of chronic oxidative stress.

## MATERIALS AND METHODS

**Yeast strains.** All yeast strains used in this experiment are derivatives of DBY2006 (α *his3*Δ*200 leu2-3,112 ura3-52 trp1-*Δ*1 ade2*) with the exception of BS127 (α *ade1 ade2 leu2 trp1 ura3 cyhR canR sapR supR gal*). Wild type (WT) refers to the TWO1 strain (DSC0121) (α *his3*Δ*200 leu2-3,112 ura3-52 trp1-*Δ*1 ade2 ntg1*Δ*::KanMX4 pRS316-NTG1* [*URA3 CEN*/*ARS*]), which contains a chromosomal disruption of the *NTG1* locus that is covered by a plasmid containing a wild-type copy of the *NTG1* gene expressed from its own promoter. This strain is functionally wild type due to the plasmid copy of the *NTG1* gene complementing the chromosomal disruption. The following strains are derivatives of TWO1 constructed by standard marker disruption and/or plasmid shuffle as previously described: DSC0122 (*ntg1*Δ), DSC0125 (*pif1*Δ), and DSC0126 (*pif1*Δ *ntg1*Δ) (38).

*SOD2* gene disruptions were created using a PCR-based gene deletion system as described by Longtine et al. (32). The following strains were generated: the *sod2*Δ strain (DSC0245) (α *his3*Δ*200 leu2-3,112 ura3-52 trp1-*Δ*1 ade2 ntg1*Δ*::KanMX4 sod2*Δ*::TRP1 pRS316-NTG1* [*URA3 CEN*/*ARS*]), the *ntg1*Δ *sod2*Δ strain (DSC0246) (α *his3*Δ*200 leu2-3,112 ura3-52 trp1-*Δ*1 ade2 ntg1*Δ*::KanMX4 sod2*Δ*::TRP1*), the *pif1*Δ *sod2*Δ strain (DSC0247) (α *his3*Δ*200 leu2-3,112 ura3-52 trp1-*Δ*1 ade2 ntg1*Δ*::KanMX4 pif1*Δ*::HIS3 sod2*Δ*::TRP1 pRS316-NTG1* [*URA3 CEN*/*ARS*]), and the *pif1*Δ *ntg1*Δ *sod2*Δ strain (DSC0248) (α *his3*Δ*200 leu2-3,112 ura3-52 trp1-*Δ*1 ade2 ntg1*Δ*::KanMX4 pif1*Δ*::HIS3 sod2*Δ*::TRP1).* During strain construction, all strains were maintained on yeast extract-peptone-glycerol (YPG) medium to sustain mitochondrial respiration competence.

To create a strain that overexpressed Sod2p from its own promoter, BS127 was transformed with 2μm plasmid p42K-TEF (DSC0250) (Dual Systems Biotech).

All strains were grown in standard synthetic dextrose (SD), synthetic glycerol, yeast extract-peptone-dextrose (YPD), or YPG as previously described (46).

**Dichlorofluorescein diacetate assay.** The conditions of the dichlorofluorescein diacetate (DCFHDA) assay were adapted from the protocol of Brennan and Schiestl (8). This assay utilizes the oxidant-sensitive probe DCFHDA to assess intracellular ROS levels. Individual colonies of various strains were used to inoculate 10 ml YPG. Cultures were grown at 30°C in a roller drum until an optical density at 60 nm ($OD_{600}$) of 0.5 was achieved. Cell counts were taken for each sample to normalize reported relative fluorescence values. Cells were washed twice, resuspended in 10 ml distilled $H_2O$, and divided into 1.5-ml aliquots. When applicable, cells were treated with various concentrations of antimycin and $H_2O_2$ for 1 h at 30°C. Next, DCFHDA was added from a 5 mM stock in ethanol to a final concentration of 10 μM. Cells were incubated at 30°C for 30 min. Following treatments, an aliquot of cells was removed, washed, diluted, and plated to determine the fraction of survival. Next, each sample was washed twice and resuspended in 1.5 ml of a solution containing 1% sodium dodecyl sulfate, 2% Triton X-100, 100 mM NaCl, 10 mM Tris, pH 8.0, and 1 mM EDTA. After addition of 0.3 g acid-washed glass beads, samples were vortexed for 10 min. Cells were incubated at room temperature for 10 min and then pelleted. Fluorescence of 200 μl of the supernatant was measured using a Packard Fluoro-count fluorescence multiwell plate reader with a fluorescence excitation of 485 nm and emission at 520 nm. Reported average results were obtained from at least four independent cultures.

**DNA isolation and processing.** Cells were grown in YPG and isolated during log phase. Approximately $2 \times 10^9$ cells were harvested by centrifugation and resuspended in 0.8 ml SorEDTA (0.9 M sorbitol, 0.1 M EDTA [pH 7.4]) containing 25 μl dithiothreitol (1 M) and 100 μl zymolyase 20T (10 mg/ml). Cells were incubated overnight at 37°C. Spheroblasts were resuspended in 0.5 ml Tris-EDTA (50 mM Tris-HCl [pH 7.4], 20 mM EDTA) containing 50 μl of 20-mg/ml proteinase K and incubated at 55°C for 1 h. One hundred microliters of 10% sodium dodecyl sulfate was added, and samples were incubated for 20 min at 65°C. After addition of 300 μl of 5 M potassium acetate, samples were incubated on ice for 30 min. Supernatants from each sample were twice extracted with chloroform. DNA was precipitated with isopropanol, resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8]) and digested with 5 μl RNase A (10 mg/ml) at 37°C for 1 h. Samples were precipitated with ethanol, washed twice, and resuspended in TE buffer. DNA was quantified by fluorometry using the fluorescent dye bisbenzamide (Hoechst 33258) (Sigma DNA quantitation kit, product no. DNA-QF). Fluorometry was performed according to the manufacturer's recommendations.

**Mitochondrial oxidative DNA damage assay.** This electrophoretic assay determines the levels of Ntg1p recognizable lesions present within a coding region of the mitochondrial genome and is an adaptation of a similar method previously used by our group for measuring oxidative nuclear DNA damage (15). Ten-microgram aliquots of genomic DNA were digested with NdeI for 4 h at 37°C. Digestion with this enzyme yields a 4.4-kb fragment containing the mitochondrially encoded *COB1* gene. After digestion, DNA was precipitated, pooled, and quantitated. Ten micrograms of NdeI-digested genomic DNA was subsequently incubated with 5 μg recombinant glutathione *S*-transferase-tagged Ntg1p in a 25-μl reaction mixture containing 15 mM $KH_2PO_4$, pH 6.8, 10 mM EDTA, 10 mM β-mercaptoethanol, and 40 mM KCl. The reaction was performed at 37°C for 30 min (56). Reactions were terminated by heating to 60°C for 5 min. The recombinant Ntg1p used in these experiments was N-terminally tagged with glutathione *S*-transferase and expressed in *Escherichia coli* as described previously (34). Ntg1p was purified by glutathione-agarose chromatography followed by Mono-S fast-performance liquid chromatography as previously described (34).

Restricted DNA samples were resolved on a 1.1% denaturing, alkaline agarose gel (30 mM NaOH, 1 mM EDTA) using alkaline electrophoresis buffer (30 mM NaOH, 2 mM EDTA) and denaturing loading dye (50 mM NaOH, 1 mM EDTA). Electrophoresis was performed for 30 min at 40 V followed by 15 h at 17 V (6). Additionally, an internal standard (loading control) corresponding to a 400-bp segment of the *COB1* gene, generated by PCR amplification, was added to each sample after Ntg1p incubation at a concentration of 0.15 ng per μg of genomic DNA. Transfer and Southern hybridization were performed according to the method of Sambrook and Russell (42) with the following modifications. Transfer was performed utilizing alkaline transfer buffer (20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.4 N NaOH). After 24 h of transfer, DNA was UV cross-linked to nylon membranes and hybridized at 65°C in

rapid-hyb buffer (Amersham Biosciences). A 400-bp PCR-generated fragment of the *COB1* gene was labeled with [$\alpha$-$^{32}$P]dATP using randomly primed synthesis. Band intensity was determined by phosphorimager analysis and normalized to the *COB1* loading control. The number of Ntg1p-recognized lesions were determined as previously described (5). Average values reported were obtained from at least four independent experiments.

**Erythromycin resistance mitochondrial mutation frequency assay.** The conditions of the erythromycin resistance assay were adapted from the protocol of Chi and Kolodner (10). Five milliliters of YPG medium was inoculated with individual colonies from the indicated strains, and the cultures were grown at 30°C until an $OD_{600}$ of 2 to 4 was reached. Cultures were diluted and plated onto YPG grown at 30°C for 4 to 5 days to obtain single colonies. Ten to fifteen individual colonies were used to inoculate separate 10-ml cultures of YPG grown at 30°C for 48 h. Samples were plated onto YPG containing 1 mg/ml erythromycin and grown at 30°C for 6 to 7 days. Additionally, a small sample of each culture was removed to determine the total number of respiration-competent cells by plating onto YPG. Mutation frequencies were calculated as follows: number of erythromycin-resistant colonies/total number of colonies. Potential jackpot cultures were statistically identified as outliers by using the Grubbs test (21). Reported average frequencies are calculated from 30 to 45 independent cultures, and standard error of the mean (SEM) is indicated.

**Fluorescence microscopy.** Individual colonies were used to inoculate 5 ml of YPD or SD where indicated. One-milliliter aliquots were removed during late-logarithmic phase growth ($OD_{600}$, ~0.5 to 0.6) for microscopic evaluation. Cells were washed twice and resuspended in 70% ethanol. One microliter of 1-mg/ml diaminophenylindole (DAPI) was added, and cells were incubated at room temperature for 5 min. Cultures were washed twice before examination by microscopy at magnification ×100.

**Petite-mutant induction assay and cell viability.** The basis for the petite-mutant assay is that growth of yeast cells in YPG (glycerol-containing) medium requires mitochondrial respiration, whereas growth on YPD (glucose-containing) medium can occur in the absence of mitochondrial respiration. To determine the rate of spontaneous petite formation over time, cultures of various yeast strains were grown to near-saturation (stationary phase) in 5 ml of YPG medium to maintain respiration competence. The culture was then diluted to an $OD_{600}$ of 0.005 into 100 ml SD medium with appropriate nutritional supplements as required by each strain. Samples from the experimental cultures were removed immediately (time zero) and every 24 h thereafter during growth at 30°C with agitation (200 rpm) on a rotary shaker. Identical samples from each culture were diluted and plated onto YPD and YPG plates and allowed to grow at 30°C for 2 to 5 days until colonies formed. The number of colonies on each plate was determined (100 to 1,000 CFU/plate in all experiments), and the percentage of respiration-competent cells was calculated as follows: (number of colonies on YPG/number of colonies on YPD) × 100.

Aliquots of cells were streaked onto YPD plates at each time point to assess cell viability. Plates were allowed to grow at 30°C for 24 h and then viewed at ×40 magnification. Approximately 300 colonies were counted and assessed as viable (formed colony of greater than five cells) or nonviable (failed to form colony of greater than five cells). Additionally, cells were analyzed by DAPI staining and fluorescence microscopy at each time point as described above.

**Exposure to oxidative stress agents.** Indicated strains were grown to mid-logarithmic phase ($OD_{600}$ of ~0.5). Cells were washed twice and resuspended in deionized $H_2O$ together with the indicated concentrations of antimycin and $H_2O_2$. Cells were incubated at 30°C for 1 h. After treatment, cells were washed twice then resuspended in deionized $H_2O$. Cells were subsequently analyzed for petite formation, viability, and mitochondrial DNA status as appropriate.

## RESULTS

**Oxidative stress can be selectively targeted to mitochondria.** In order to assess the biological endpoints of chronic oxidative stress in mtDNA repair-deficient mutants, we developed a system to selectively enhance mitochondrial oxidative stress utilizing endogenous and exogenous sources of ROS. To induce conditions of oxidative stress, cells were exposed to antimycin in combination with $H_2O_2$. Antimycin, a drug that selectively inhibits respiratory complex III, has been previously shown to enhance mitochondrial superoxide production in isolated organelles (7) as well as in vivo (41). To enhance ROS levels via endogenous mechanisms, mutants with a deleted mitochondrial superoxide dismutase (*SOD2*) gene were created. Through its enzymatic scavenging of superoxide anion radicals, Sod2p mediates protection of yeast against oxygen toxicity in the mitochondria (20). To determine the extent to which the levels of intracellular ROS could be manipulated, the oxidant-sensitive fluorescent probe DCFHDA was utilized (57). Both *sod2*Δ and *ntg1*Δ *sod2*Δ strains displayed an approximately threefold increase in the amount of fluorescence over that of their isogenic parental strains (Fig. 1), indicating that *sod2* mutants display a significant increase in intracellular ROS levels. The level observed in *sod2*Δ deletion strains was approximately equivalent to treatment with 10 μg/ml antimycin, a concentration that results in about 80% survival (data not shown). When antimycin treatment was used in combination with $H_2O_2$ exposure, there was a synergistic increase in ROS levels compared to treatment with either agent alone (Fig. 1). This result suggests that the mitochondrial specificity of antimycin can be exploited to elevate the level of mitochondrial ROS when used in combination with $H_2O_2$, resulting in a model system that can be used to study mitochondrial oxidative stress. However, it should be noted that the proportion of ROS present in the mitochondria is unknown.

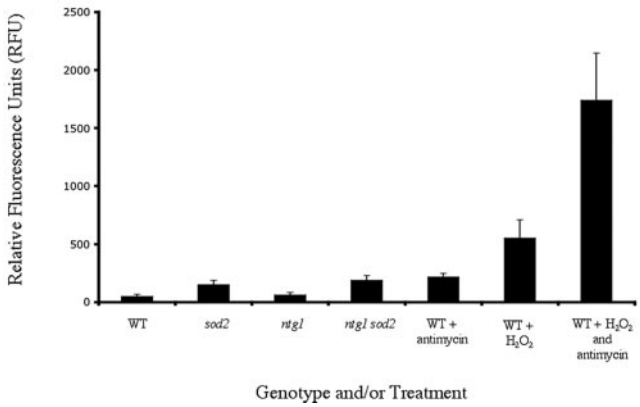


FIG. 1. Increased ROS levels in *sod2*Δ mutants and cells treated with antimycin and/or $H_2O_2$. Fluorometric results of DCFHDA fluorescence on WT, *sod2*Δ, *ntg1*Δ, and *ntg1*Δ *sod2*Δ mutants are presented. The WT strain was also treated with 10 μg/ml antimycin and/or 10 mM $H_2O_2$ (see Materials and Methods). The ordinate represents relative fluorescence units normalized for the number of cells assayed. Each bar value represents the mean of at least four independent measurements, and each error bar represents the standard error of the mean.

**Mitochondrial oxidative stress results in a direct increase in oxidative mtDNA damage.** To investigate the relationship between oxidative stress and oxidative mtDNA damage, we utilized a mitochondrial gene-specific DNA damage detection assay that measures the amount of Ntg1p-recognizable lesions in a 4.4-kb fragment of the mitochondrial genome. Total cellular DNA was obtained using a method that minimizes introduction of adventitious oxidative DNA damage (15). DNA was subsequently treated with Ntg1p, a BER DNA glycosylase with associated AP lyase activity that primarily recognizes and cleaves DNA containing oxidative pyrimidine base damage and abasic sites (56). Under the conditions of denaturing agarose gel electrophoresis, such cleavage products produce DNA single-strand breaks at sites of unrepaired base damage recog-

TABLE 1. Ntg1p-recognized DNA lesions in the *COB1* locus and overall mitochondrial genome

| Genotype | Ratio of band intensities of Ntg1p-treated to these untreated samples[a] | No. of lesions per 4.4-kb *COB1* fragment[b] | No. of lesions per genome[c] |
|---|---|---|---|
| WT | 0.96 ± 0.05 | 0.043 | 1 |
| *ntg1* | 0.90 ± 0.04 | 0.11 | 2 |
| *pif1* | 0.71 ± 0.02 | 0.357 | 7 |
| *ntg1 pif1* | 0.52 ± 0.02 | 0.654 | 12 |
| *sod2* | 0.87 ± 0.06 | 0.154 | 3 |
| *ntg1 sod2* | 0.65 ± 0.03 | 0.43 | 8 |

[a] Values represent the average of ratios derived from a minimum of four independent blots plus or minus the SEM.
[b] Values calculated using the Poisson expression: $S = -\ln P_0$ (S, lesions per 4.4-kb *COB1* fragment; $P_0$, ratio of band intensities of Ntg1p-treated to untreated samples).
[c] Values were extrapolated from calculation of lesions per 4.4-kb *COB1* fragment.

nized by Ntg1p (15). When probed with the mitochondrial *COB1* gene, the extent of hybridization signal decrease is proportional to the amount of Ntg1p-recognizable lesions present within this locus and can be extrapolated to estimate the number of lesions per mitochondrial genome (5).

Experiments were conducted with strains previously shown to be compromised in mitochondrial BER (*ntg1*Δ) and oxidative mtDNA damage resistance (*pif1*Δ) under conditions of chronic oxidative stress achieved by elimination of Sod2p, a major mitochondrial ROS-scavenging enzyme. A small, but reproducible increase (~2-fold) in Ntg1p-recognizable lesion levels was observed in the *ntg1*Δ null mutant strain (Table 1). In contrast, higher levels (sevenfold) of Ntg1p-recognizable lesions were detected in the *pif1*Δ mutant strain, and this increase was further enhanced (greater than additive) in the *pif1*Δ *ntg1*Δ double mutant strain (Table 1). Such increases in the levels of Ntg1p-recognizable lesions when considered with previously published reports (38) demonstrating a similar pattern of increase in mtDNA mutagenesis and petite induction in these strains suggest that Ntg1p and Pif1p are important mediators for the prevention of oxidative mtDNA damage and mutagenesis. In addition, simultaneous elimination of Ntg1p and Sod2p (*ntg1*Δ *sod2*Δ mutant) results in a greater than additive increase in the number of oxidative lesions present within the mitochondrial genome (Table 1). It is important to note these experiments cannot be performed with the *pif1*Δ *sod2*Δ double mutant and the *ntg1*Δ *pif1*Δ *sod2*Δ triple mutant strain because such petite mutants either possess corrupt mtDNA genomes ($rho^-$) or lack mtDNA altogether ($rho^0$). Together, these results indicate that the mitochondrial genome is a major target of oxidative stress resulting in the generation of deleterious oxidative DNA lesions.

**Oxidative mtDNA damage results in increased mtDNA point mutagenesis.** Erythromycin is an antibiotic that specifically inhibits mitochondrial translation (11). Resistance to the drug can be acquired through specific point mutations in the mitochondrial *rib2* and *rib3* loci, which encode the two mitochondrial rRNAs (14, 50). Therefore, acquisition of erythromycin resistance is a convenient, direct measurement of mtDNA point mutagenesis (10). Using this assay, we determined mtDNA mutation frequencies in *ntg1*Δ and *sod2*Δ single-mutant strains as well as in the corresponding double mutant. The *ntg1*Δ mutant displays a slight increase in mutation frequency (Table 2) as previously reported (38). The *sod2*Δ mutant also exhibits an increased mitochondrial mutation frequency (~5-fold) that increases synergistically when *NTG1* is also deleted (~15-fold). This result strongly supports an in vivo biological function for the repair of mutagenic, oxidative mtDNA damage by Ntg1p. Additionally, it indicates a direct relationship between enhanced mitochondrial oxidative stress, oxidative DNA damage, and mtDNA mutagenesis.

**Mitochondrial genomic integrity is lost by corrupting mtDNA damage resistance pathways.** Petite mutants caused by mtDNA dysfunction can be distinguished into two phenotypic subclasses: $rho^-$ and $rho^0$. $rho^-$ mutants possess mtDNA, albeit in an altered form that results from large deletions followed by amplification of remaining sequences. Such mutants are incapable of carrying out normal mitochondrial respiratory function (40). $rho^-$ and $rho^0$ subclasses are distinguishable from each other by microscopic analysis of DAPI-stained, respiration-compromised cells revealing the presence ($rho^-$) or absence ($rho^0$) of mtDNA.

We sought to determine the rho status of mutants compromised in DNA damage resistance mechanisms in order to ascertain the relative contribution of oxidative DNA damage to mitochondrial genomic stability. Wild-type, *ntg1*Δ, *sod2*Δ, and *ntg1*Δ *sod2*Δ mutants were all characterized as being rho positive ($rho^+$) under late-log-phase growth conditions (Fig. 2). Both *pif1*Δ and *ntg1*Δ *pif1*Δ were characterized as a mixed population of $rho^+$ and $rho^-$ cells due to the fact that these strains exhibit a high rate of spontaneous petite formation as previously reported (38). The *pif1*Δ *sod2*Δ double mutant was characterized as $rho^-$ due to the presence of mtDNA and a complete inability to grow in YPG (Fig. 2). The loss of mtDNA genomic integrity is even more severe in the *ntg1*Δ *pif1*Δ *sod2*Δ triple mutant, since these cells exhibit a rapid and total loss of mtDNA (Fig. 2). These data, when taken together with the assessment of oxidative mtDNA damage levels, suggest that oxidative mtDNA damage directly contributes to cellular petite formation and complete loss of mtDNA.

**Oxidative stress causes a loss of mitochondrial respiration capacity in cells with compromised mtDNA damage processing.** We have previously reported that eliminating both Ntg1p and Pif1p results in a synergistic increase in spontaneous petite formation (38). Therefore, it was of interest to determine whether a similar synergistic effect occurs in response to induced, acute oxidative stress. For this purpose, WT, *ntg1*Δ, *pif1*Δ, and *ntg1*Δ *pif1*Δ mutants were exposed to antimycin and $H_2O_2$, a combination of agents that enhances mitochondrial oxidative stress. Following such treatment, the percentage of

TABLE 2. Frequency of erythromycin-resistant mutants in *ntg1 sod2* double-mutant strains

| Genotype | Mutation frequency[a] | Fold effect[b,c] |
|---|---|---|
| WT | 7.83E−09 | 1.00 |
| *ntg1* | 1.63E−08 | 2.07 ± 0.08 |
| *sod2* | 4.60E−08 | 5.61 ± 0.38 |
| *ntg1 sod2* | 1.28E−07 | 16.42 ± 1.09 |

[a] Calculated from the results of plating 30 to 45 independent cultures.
[b] Calculated relative to the wild-type strain.
[c] SEM reported.

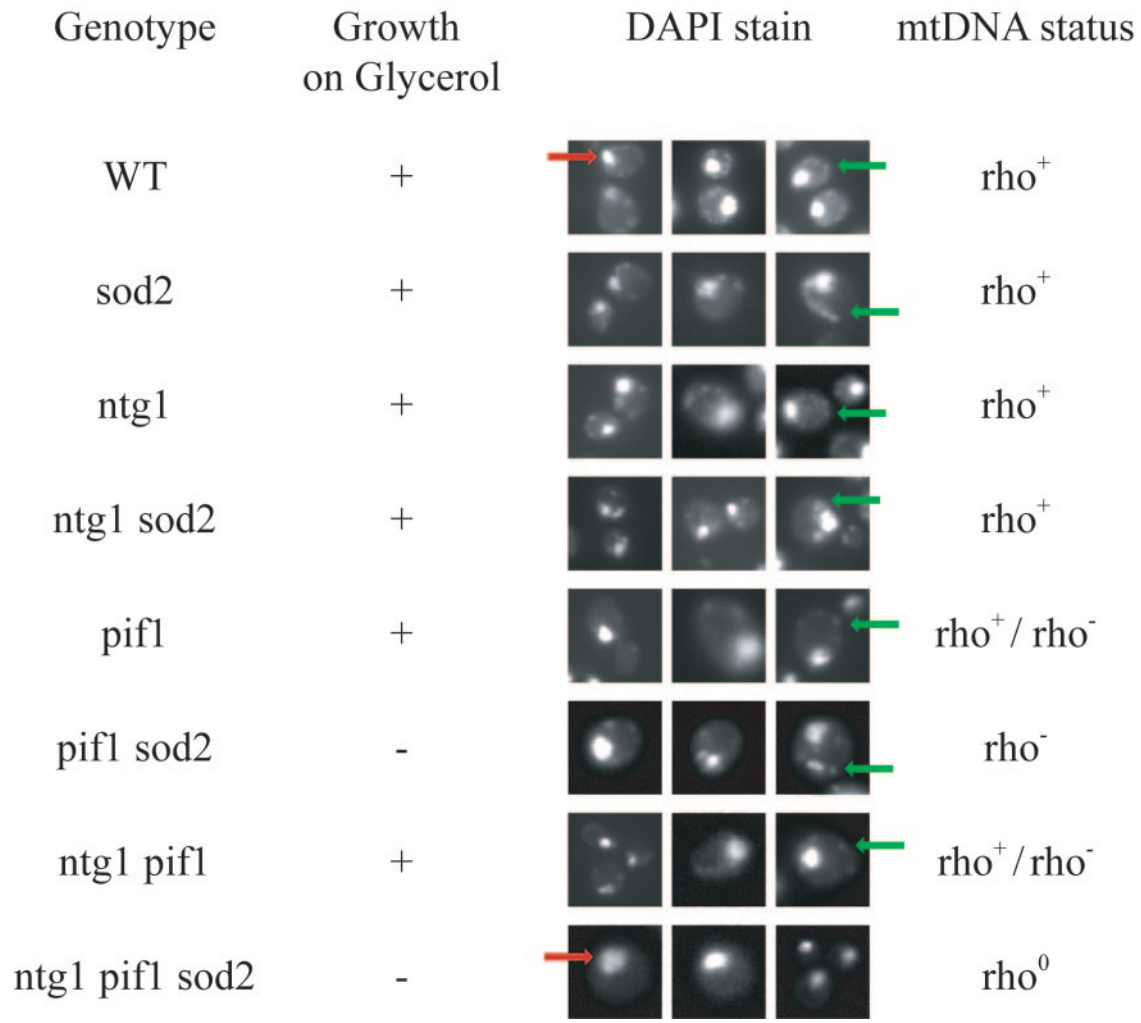


FIG. 2. rho status in mtDNA damage resistance mutants. Strains were characterized based upon their ability (respiration competent, $rho^+$) or inability (respiration incompetent, $rho^-$ and $rho^0$) to grow on YPG. $rho^-$ and $rho^0$ strains were distinguished by the presence ($rho^-$) or absence ($rho^0$) of mtDNA, which can be visualized by microscopic examination of DAPI-stained nuclear (red arrows) and mitochondrial (green arrow) DNA. mtDNA staining in both $rho^+$ and $rho^-$ late-log-phase cells is revealed by a network of punctate dots located at the cell periphery (25). Cells shown are representative of approximately 500 DAPI-stained cells microscopically evaluated for each strain (see Materials and Methods).

petite mutants observed in both *ntg1*Δ and *pif1*Δ single mutants exposed to oxidative stress does not significantly change (Fig. 3). In contrast, the *ntg1*Δ *pif1*Δ double-mutant strain displayed a synergistic petite induction in response to oxidative stress (Fig. 3), indicating that Ntg1p and Pif1p together provide resistance to induced oxidative DNA damage. Additionally, these data suggest that the ability of oxidative mtDNA damage to cause genetic instability can be enhanced under conditions of induced mitochondrial oxidative stress by exposure to exogenous agents such as antimycin and $H_2O_2$.

**Pif1p is a critical mediator of mitochondrial genomic stability under conditions of oxidative stress.** The above results indicate that Ntg1p-mediated mtDNA excision repair, Sod2p-mediated ROS scavenging, and Pif1p-mediated functions are all important events involved in maintaining mitochondrial genomic integrity. In order to assess the relative contribution of each of these components in preserving mtDNA stability, we assessed the length of time required for a yeast cell with these systems compromised to change from a respiration-competent ($rho^+$) phenotype to a respiration-compromised (petite) phenotype. Mitochondrial respiration incompetence resulting from mtDNA damage can be considered an early, measurable event in the processes leading to a genome-wide corruption of mtDNA. Experiments were conducted in stationary-phase cultures in order to maximize the relative contribution of Sod2p, which has been shown to be induced under stationary-phase conditions, to counteract increases in ROS (13). Additionally, stationary-phase cells are a particularly useful model system for the study of damage that occurs during oxidative stress and disease states because this allows for time-dependent damage accumulation in these nondividing cells (30, 31).

WT cells and the *ntg1*Δ single mutant maintained mitochondrial genomic integrity throughout the 12-day course of the experiment (Fig. 4). The *ntg1*Δ *sod2*Δ double mutant converted into a predominantly petite population more rapidly than the *sod2*Δ single mutant alone (Fig. 4, day 10 versus day 12, respectively). The *pif1*Δ mutant displayed a rapid loss of mitochondrial genomic integrity, which was further enhanced when

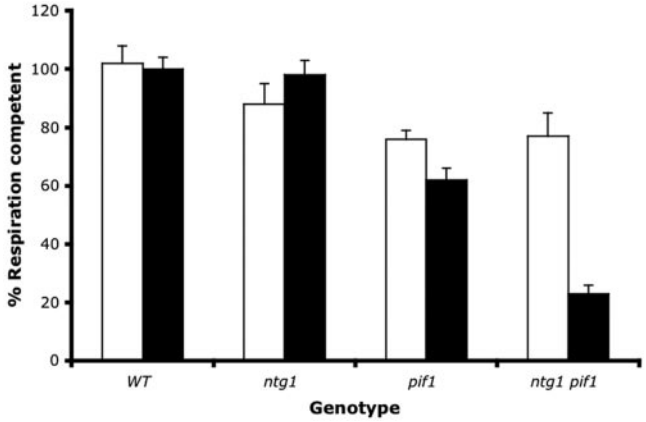


FIG. 3. Antimycin plus $H_2O_2$ synergistically enhances petite-mutation formation in *ntg1*Δ *pif1*Δ double mutants. Petite-mutation induction assays with WT and *ntg1*Δ, *pif1*Δ, and *ntg1*Δ *pif1*Δ mutants before (open bars) or after (closed bars) exposure to antimycin (1 μg/ml) and $H_2O_2$ (10 mM) as described above (see Materials and Methods). The ordinate represents the percentage of respiration-competent cells. Each bar value represents the mean of at least four independent measurements, and each error bar indicates the standard error of the mean.

simultaneously deleted for *NTG1*. Strikingly, the *ntg1*Δ *pif1*Δ double mutant completely converted into a respiration-incompetent population within 4 days (Fig. 4). The rapid loss of respiration competency in cells compromised for Pif1p function reveals its important contribution to the maintenance of mitochondrial genomic stability.

**Oxidative stress induces complete loss of mtDNA in stable rho$^-$ cells and is preventable by superoxide distmutase (Sod2p).** Because oxidative mtDNA damage appears to be a major contributor to mitochondrial genomic instability, it was of particular interest to determine whether ROS could cause a stable rho$^-$ cell line, with its complement of mtDNA damage resistance pathways intact, to undergo a complete loss of mtDNA. The strain BS127 is able to stably maintain a nonfunctional (rho$^-$) mitochondrial genome comprised of multiple *ori5* repeats (33). When BS127 cells were exposed to 1 μg/ml antimycin and 10 mM $H_2O_2$, the cells converted to a rho$^0$ phenotype after 8 to 10 generations of outgrowth as determined by microscopic analysis of DAPI-stained mtDNA (Fig. 5B). Isogenic cells overexpressing Sod2p from a 2μm plasmid maintained their mitochondrial genomes despite receiving equivalent exposures to mitochondrial oxidative stress (Fig. 5D). These results indicate that the loss of mitochondrial genomic integrity can be directly attributed to ROS present in mitochondria.

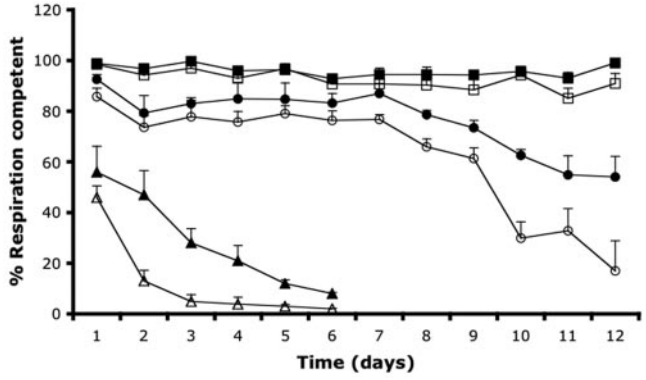


FIG. 4. Temporal relationship between corruption of mtDNA damage resistance pathways and loss of respiration competency. Results from petite-mutant induction assays are shown for the indicated strains grown to stationary phase and monitored for respiration competency over 12 days as described in Materials and Methods: WT (filled squares) and *ntg1*Δ (open squares), *sod2*Δ (filled circles), *ntg1*Δ *sod2*Δ (open circles), *pif1*Δ (filled triangles), and *ntg1*Δ *pif1*Δ (open triangles) mutants. The ordinate represents time (days) after initial culture inoculation, and the abscissa represents the percentage of respiration-competent cells measured as described above (see Materials and Methods). Each value was derived from at least four independent measurements. Error bars show standard errors of the mean. The lower half of each error bar was eliminated for clarity.

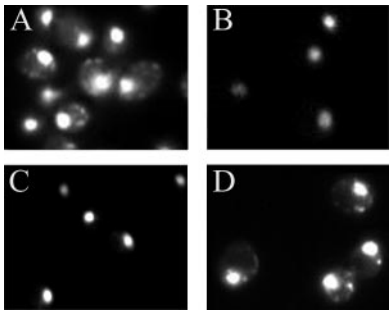


FIG. 5. Complete loss of mtDNA caused by mitochondrial ROS. Fluorescence microscopy images of DAPI-stained BS127 (stable rho$^-$) cells before or after treatment with 1 μg/ml antimycin and 10 mM $H_2O_2$ followed by 8 to 10 generations of outgrowth (see Materials and Methods). (A) BS127 prior to ROS exposure. (B) BS127 after ROS exposure. (C) BS127 with empty 2μm plasmid after ROS exposure. (D) BS127 with 2μm plasmid expressing Sod2p after ROS exposure.

## DISCUSSION

To gain insight into how eukaryotic cells resist damage to the mitochondrial genome, we utilized two strategies to elevate mitochondrial oxidative stress using both endogenous and exogenous sources of ROS. In response to conditions of mitochondrial oxidative stress, eukaryotic cells display a range of measurable, biologically relevant endpoints, including oxidative mtDNA damage and mutagenesis, which contribute to corruption of mitochondrial genome integrity. Strikingly, strains simultaneously missing three components of pathways involved in preventing and repairing oxidative mtDNA damage exhibit a rapid and total loss of mtDNA, revealing the requirement for maintaining mitochondrial genome integrity in the context of oxidative stress. Furthermore, to directly demonstrate whether mitochondrial oxidative stress causes loss of mitochondrial genomic stability, a stable rho$^-$ strain completely lost mtDNA (i.e., converted to rho$^0$) upon exposure to antimycin and $H_2O_2$ (Fig. 5).

In addition to demonstrating that oxidative mtDNA damage directly contributes to loss of mitochondrial genome integrity, we assessed the relative contributions of three pathways involved in maintenance of mitochondrial genome integrity: Sod2p-mediated ROS scavenging, Ntg1p-mediated mitochon-

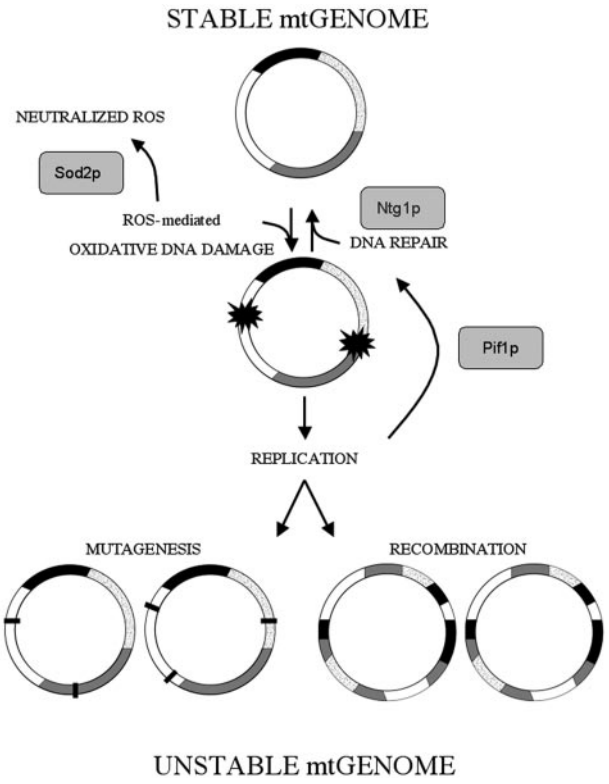


FIG. 6. Cooperative nature of mtDNA damage resistance pathways in maintaining mitochondrial genomic integrity. Sod2p functions to prevent ROS-mediated introduction of oxidative mtDNA damage (black stars) through its ROS-scavenging properties, thereby contributing to mitochondrial genomic integrity. Similarly, Ntg1p functions in the removal of oxidative lesions from the mitochondrial genome. The role of Pif1p in maintenance of mitochondrial genomic integrity is complex, including roles in replication timing, recombination, and perhaps other functions. Together, these pathways form a balance of activities that maintains mitochondrial genomic stability. Buffering capacities for genomic stability are present such that when one pathway is eliminated, the remaining pathways can compensate to various extents. However, if multiple pathways are corrupted, deleterious biological consequences result, including extensive mtDNA damage, mutagenesis (black bars), elimination of respiration competency (not shown), and genomic instability, including increased recombination frequency.

drial BER, and Pif1p-mediated oxidative mtDNA damage resistance. The data presented here suggest that these three pathways operate in a delicate balance to ensure mitochondrial genomic integrity (Fig. 6). Sod2p maintains mitochondrial genomic integrity by preventing oxidative mtDNA damage through its antioxidant catalytic properties. However, if mitochondrial ROS scavenging systems are not operating at maximum capacity, ROS have the potential to damage mtDNA. Oxidative mtDNA base damage can be repaired by mtDNA excision repair activities mediated by Ntg1p, thereby restoring mitochondrial genomic integrity. Pif1p is a 5′-to-3′ DNA helicase that has been implicated in mtDNA maintenance, repair, and recombination (17). In addition to its reported mitochondrial functions, the nuclear functions of Pif1p include inhibition of bidirectional rRNA gene replication (23), telomere length regulation (44), and prevention of gross chromosomal rearrangements (36). Based on its known nuclear activities, it has been postulated that Pif1p may govern the rate of mtDNA replication (3, 38). Additionally, because of the multifunctional nature of Pif1p, it may be involved in several aspects of mitochondrial genomic maintenance. In the context of mitochondrial oxidative stress and subsequent mtDNA damage, Pif1p may inhibit replication progression in order to allow time for repair to occur. In the absence of such a "checkpoint," replication may proceed through unrepaired damage-containing DNA templates, resulting in mutagenesis and stimulating recombination, both of which constitute genomic instability. Pif1p may also promote tolerance of oxidative mtDNA damage through its recombinational activities or other novel mechanisms. Thus, if the balance of these mtDNA damage management pathways is disrupted, deleterious biological endpoints occur, including mtDNA oxidative damage, mutagenesis, and extensive mitochondrial genomic corruption. Additionally, other factors, such as the mismatch repair protein, Msh1p, have been shown to play a role in maintaining mitochondrial genomic stability (10).

By exploiting genetic analysis in *S. cerevisiae*, it is possible to assess the relative contributions of each pathway to maintenance of mitochondrial genome integrity. A range of phenotypes was observed that were dependent upon the combination of DNA damage resistance pathway components eliminated. WT and single pathway-deficient strains display low levels of oxidative mtDNA damage (Table 1) and mtDNA mutagenesis (Table 2) and no overt growth defects and maintain a $rho^+$ mitochondrial phenotype (Fig. 2). These results suggest that Ntg1p, Pif1p, and Sod2p are part of an interactive network of mtDNA damage resistance pathways. In the absence of one pathway, the remaining pathways serve as a buffer to maintain mitochondrial genomic integrity. However, when multiple pathways are compromised, as in the *pif1*Δ *sod2*Δ double mutant and the *ntg1*Δ *pif1*Δ *sod2*Δ triple mutant, genomic instability occurs substantially more rapidly (Fig. 2).

Our results revealed important differences with respect to the relative contributions of each pathway in the maintenance of mitochondrial genomic integrity, with Pif1p mediating a major role. The *pif1*Δ single mutant displays elevated levels of oxidative mtDNA damage (Table 1) and mutagenesis (38); levels that often exceed those found in other double pathway-deficient strains (Table 2, Fig. 4) (38). The most severe phenotypes observed in this study always occurred in the context of a *pif1*Δ mutant with one additional pathway eliminated. For example, substantial levels of oxidative mtDNA damage and elevated mutagenesis are observed in the *ntg1*Δ *pif1*Δ double mutant strain (Table 1) (38). Furthermore, *pif1*Δ *sod2*Δ double mutants exhibit extensive mitochondrial genome instability (Fig. 2). The extreme phenotypic severity of *pif1*Δ mutants may reflect the multifunctional role that Pif1p mediates in response to mitochondrial oxidative stress, including governing replication rates, controlling recombination, and other potential activities, such as influencing the accessibility of mtDNA to repair proteins. The data presented here are consistent with the notion that Pif1p is a major contributor to mitochondrial genomic stability.

By comparing the endpoints of genomic stability in the stains analyzed, a sequence of events is revealed, including a progression from $rho^+$ to $rho^-$ to $rho^0$ that is dependent upon the combination of pathways eliminated (Fig. 2). For example, the

*pif1*Δ *sod2*Δ double mutant maintains its mitochondrial genome, albeit in a compromised (rho$^-$) form. However, when an additional mtDNA damage resistance pathway is eliminated (*ntg1*Δ) and additional mtDNA damage is introduced into the genome, these cells exhibit a rapid and total loss of mtDNA (Tables 1 and 2). One interpretation of these results is that a putative DNA damage threshold level has been surpassed in these strains, leading to progressive genomic instability. While the exact sequence and chronology of events involved in the loss of mitochondrial genomic stability induced by oxidative stress is not entirely clear, several speculations can be made. At moderate levels of oxidative DNA damage and mutation frequencies, such as those observed in the *ntg1*Δ and *pif1*Δ single-mutant strains, it is likely that mtDNA excision repair has reached a maximum capacity (Tables 1 and 2). As a compensatory response, recombination may be stimulated in response to collapsed replication forks in order to tolerate increased levels of oxidative DNA damage. This model is supported by the fact that recombination is involved to some degree for tolerance of oxidative damage in yeast mitochondria (29), as well as the demonstration that recombination rates are substantially increased in the absence of base excision repair in the nuclei of yeast cells (52). It can be postulated that increased recombination of a moderately damaged genome (such as those lacking components of one or more mtDNA damage resistance pathways) could promote illegitimate recombination, thereby resulting in a rearranged genome typical of those present within a rho$^-$ population of cells (16). Once a cell becomes rho$^-$ and enters a state of chronic oxidative stress with increasing, progressive oxidative DNA damage, an eventual complete loss of mtDNA occurs when mtDNA polymerase is unable to extend past a lesion on the template strand. Thus, total loss of mtDNA within the context of a rho$^-$ phenotype can be achieved through the inability of mtDNA polymerase to replicate due to the presence of extensive, unrepairable mtDNA damage. It has also been postulated that extensively damaged mtDNA can be selectively targeted for degradation, which would lead to a rho$^0$ phenotype (26).

To date, very few studies have systematically addressed the contribution of oxidative mtDNA damage to the loss of mitochondrial genomic integrity resulting in defective respiration competency (rho$^-$) and complete loss (rho$^0$) of the mtDNA genome. Yeast provides an attractive eukaryotic model system for addressing the consequences of oxidative stress with regard to mtDNA stability, since mtDNA is dispensable for cell growth. In contrast, loss of mitochondrial genomic stability in human cells is a lethal event; therefore, studies with yeast offer a great advantage over similar studies with mammalian cells. Although petite formation is not a biological outcome associated with mitochondrial defects in humans, it provides an observational window to assess the consequences of mtDNA damage and provides a direct indication of mtDNA functionality. As an increasing number of human diseases are being identified that are associated with mtDNA-linked mutations (55), it is important to understand processes involved in maintaining mitochondrial genomic integrity. Knowledge regarding mechanisms of mitochondrial genome maintenance may provide valuable insights into the generation of mitochondrium-based diseases as well as understanding the contribution of mitochondrial dysfunction in cancer and aging.

## ACKNOWLEDGMENTS

This work was supported by NIH grants ES11163 and CA07341.

We appreciate Thomas O'Rourke for his contribution to strain construction.

## REFERENCES

1. **Alseth, I., L. Eide, M. Pirovano, T. Rognes, E. Seeberg, and M. Bjoras.** 1999. The *Saccharomyces cerevisiae* homologues of endonuclease III from *Escherichia coli*, Ntg1 and Ntg2, are both required for efficient repair of spontaneous and induced oxidative DNA damage in yeast. Mol. Cell. Biol. **19:**3779–3787.
2. **Attardi, G., and G. Schatz.** 1988. Biogenesis of mitochondria. Annu. Rev. Cell Biol. **4:**289–333.
3. **Bessler, J. B., J. Z. Torredagger, and V. A. Zakian.** 2001. The Pif1p subfamily of helicases: region-specific DNA helicases? Trends Cell Biol. **11:**60–65.
4. **Bogenhagen, D. F., K. G. Pinz, and R. M. Perez-Jannotti.** 2001. Enzymology of mitochondrial base excision repair. Prog. Nucleic Acid Res. Mol. Biol. **68:**257–271.
5. **Bohr, V. A., E. H. Chu, M. van Duin, P. C. Hanawalt, and D. S. Okumoto.** 1988. Human repair gene restores normal pattern of preferential DNA repair in repair defective CHO cells. Nucleic Acids Res. **16:**7397–7403.
6. **Bohr, V. A., C. A. Smith, D. S. Okumoto, and P. C. Hanawalt.** 1985. DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell **40:**359–369.
7. **Boveris, A.** 1978. Biochemistry and genetics of yeast. Academic Press, New York, N.Y.
8. **Brennan, R. J., and R. H. Schiestl.** 1998. Chloroform and carbon tetrachloride induce intrachromosomal recombination and oxidative free radicals in Saccharomyces cerevisiae. Mutat. Res. **397:**271–278.
9. **Charames, G. S., and B. Bapat.** 2003. Genomic instability and cancer. Curr. Mol. Med. **3:**589–596.
10. **Chi, N. W., and R. D. Kolodner.** 1994. Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches. J. Biol. Chem. **269:**29984–29992.
11. **Clark-Walker, G. D., and A. W. Linnane.** 1967. The biogenesis of mitochondria in Saccharomyces cerevisiae. A comparison between cytoplasmic respiratory-deficient mutant yeast and chloramphenicol-inhibited wild type cells. J. Cell Biol. **34:**1–14.
12. **Copeland, W. C., J. T. Wachsman, F. M. Johnson, and J. S. Penta.** 2002. Mitochondrial DNA alterations in cancer. Cancer Investig. **20:**557–569.
13. **Costa, V., M. A. Amorim, E. Reis, A. Quintanilha, and P. Moradas-Ferreira.** 1997. Mitochondrial superoxide dismutase is essential for ethanol tolerance of Saccharomyces cerevisiae in the post-diauxic phase. Microbiology **143:**1649–1656.
14. **Cui, Z., and T. L. Mason.** 1989. A single nucleotide substitution at the rib2 locus of the yeast mitochondrial gene for 21S rRNA confers resistance to erythromycin and cold-sensitive ribosome assembly. Curr. Genet. **16:**273–279.
15. **Evert, B. A., T. B. Salmon, B. Song, L. Jingjing, W. Siede, and P. W. Doetsch.** 2004. Spontaneous DNA damage in Saccharomyces cerevisiae elicits phenotypic properties similar to cancer cells. J. Biol. Chem. **279:**22585–22594.
16. **Faye, G., H. Fukuhara, C. Grandchamp, J. Lazowska, F. Michel, J. Casey, G. S. Getz, J. Locker, M. Rabinowitz, M. Bolotin-Fukuhara, D. Coen, J. Deutsch, B. Dujon, P. Netter, and P. P. Slonimski.** 1973. Mitochondrial nucleic acids in the petite colonie mutants: deletions and repetition of genes. Biochimie **55:**779–792.
17. **Foury, F., and A. Lahaye.** 1987. Cloning and sequencing of the PIF gene involved in repair and recombination of yeast mitochondrial DNA. EMBO J. **6:**1441–1449.
18. **Fridovich, I.** 1981. Oxygen and living processes. Springer Verlag, New York, N.Y.
19. **Gralla, E. B., and D. J. Kosman.** 1992. Molecular genetics of superoxide dismutases in yeasts and related fungi. Adv. Genet. **30:**251–319.
20. **Gralla, E. B., and J. S. Valentine.** 1991. Null mutants of *Saccharomyces cerevisiae* Cu,Zn superoxide dismutase: characterization and spontaneous mutation rates. J. Bacteriol. **173:**5918–5920.
21. **Grubbs, F.** 1969. Procedure for detecting outlying observations in samples. Technometrics **11:**1–21.
22. **Huang, M. E., and R. D. Kolodner.** 2005. A biological network in Saccharomyces cerevisiae prevents the deleterious effects of endogenous oxidative DNA damage. Mol. Cell **17:**709–720.
23. **Ivessa, A. S., J. Q. Zhou, and V. A. Zakian.** 2000. The Saccharomyces Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA. Cell **100:**479–489.
24. **Jamieson, D. J.** 1998. Oxidative stress responses of the yeast Saccharomyces cerevisiae. Yeast **14:**1511–1527.
25. **Jensen, R. E., A. E. Hobbs, K. L. Cerveny, and H. Sesaki.** 2000. Yeast mitochondrial dynamics: fusion, division, segregation, and shape. Microsc. Res. Tech. **51:**573–583.

26. **Kang, D., and N. Hamasaki.** 2002. Maintenance of mitochondrial DNA integrity: repair and degradation. Curr. Genet. **41:**311–322.
27. **Lahaye, A., S. Leterme, and F. Foury.** 1993. PIF1 DNA helicase from Saccharomyces cerevisiae. Biochemical characterization of the enzyme. J. Biol. Chem. **268:**26155–26161.
28. **Lahaye, A., H. Stahl, D. Thines-Sempoux, and F. Foury.** 1991. PIF1: a DNA helicase in yeast mitochondria. EMBO J. **10:**997–1007.
29. **Ling, F., H. Morioka, E. Ohtsuka, and T. Shibata.** 2000. A role for MHR1, a gene required for mitochondrial genetic recombination, in the repair of damage spontaneously introduced in yeast mtDNA. Nucleic Acids Res. **28:**4956–4963.
30. **Longo, V. D., E. B. Gralla, and J. S. Valentine.** 1996. Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Mitochondrial production of toxic oxygen species in vivo. J. Biol. Chem. **271:**12275–12280.
31. **Longo, V. D., L. L. Liou, J. S. Valentine, and E. B. Gralla.** 1999. Mitochondrial superoxide decreases yeast survival in stationary phase. Arch. Biochem. Biophys. **365:**131–142.
32. **Longtine, M. S., A. McKenzie III, D. J. Demarini, N. G. Shah, A. Wach, A. Brachat, P. Philippsen, and J. R. Pringle.** 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:**953–961.
33. **Lorimer, H. E., B. J. Brewer, and W. L. Fangman.** 1995. A test of the transcription model for biased inheritance of yeast mitochondrial DNA. Mol. Cell. Biol. **15:**4803–4809.
34. **Meadows, K. L., B. Song, and P. W. Doetsch.** 2003. Characterization of AP lyase activities of Saccharomyces cerevisiae Ntg1p and Ntg2p: implications for biological function. Nucleic Acids Res. **31:**5560–5567.
35. **Moradas-Ferreira, P., V. Costa, P. Piper, and W. Mager.** 1996. The molecular defences against reactive oxygen species in yeast. Mol. Microbiol. **19:** 651–658.
36. **Myung, K., C. Chen, and R. D. Kolodner.** 2001. Multiple pathways cooperate in the suppression of genome instability in Saccharomyces cerevisiae. Nature **411:**1073–1076.
37. **Nilsen, H., and H. E. Krokan.** 2001. Base excision repair in a network of defence and tolerance. Carcinogenesis **22:**987–998.
38. **O'Rourke, T. W., N. A. Doudican, M. D. Mackereth, P. W. Doetsch, and G. S. Shadel.** 2002. Mitochondrial dysfunction due to oxidative mitochondrial DNA damage is reduced through cooperative actions of diverse proteins. Mol. Cell. Biol. **22:**4086–4093.
39. **Pinz, K. G., and D. F. Bogenhagen.** 1998. Efficient repair of abasic sites in DNA by mitochondrial enzymes. Mol. Cell. Biol. **18:**1257–1265.
40. **Pon, L., and G. Schatz.** 1991. Biogenesis of yeast mitochondria, p. 333–406. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast Saccharomyces: genome dynamics, protein synthesis, and energetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Rasmussen, A. K., A. Chatterjee, L. J. Rasmussen, and K. K. Singh.** 2003. Mitochondria-mediated nuclear mutator phenotype in Saccharomyces cerevisiae. Nucleic Acids Res. **31:**3909–3917.
42. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual of research procedures. Cold Spring Harbor Laboratory Press, Cold Spring, N.Y.
43. **Scheffler, I. E.** 1999. Mitochondria. Wiley-Liss, New York, N.Y.
44. **Schulz, V. P., and V. A. Zakian.** 1994. The Saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. Cell **76:**145–155.
45. **Shadel, G. S.** 1999. Yeast as a model for human mtDNA replication. Am. J. Hum. Genet. **65:**1230–1237.
46. **Sherman, F.** 1991. Getting started with yeast. Methods Enzymol. **194:**3–21.
47. **Singh, K. K.** 1998. Mitochondrial DNA mutations in aging, disease and cancer. Springer, New York, N.Y.
48. **Singh, K. K., B. Sigala, H. A. Sikder, and C. Schwimmer.** 2001. Inactivation of Saccharomyces cerevisiae OGG1 DNA repair gene leads to an increased frequency of mitochondrial mutants. Nucleic Acids Res. **29:**1381–1388.
49. **Smith, S., J. Y. Hwang, S. Banerjee, A. Majeed, A. Gupta, and K. Myung.** 2004. Mutator genes for suppression of gross chromosomal rearrangements identified by a genome-wide screening in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **101:**9039–9044.
50. **Sor, F., and H. Fukuhara.** 1983. Complete DNA sequence coding for the large ribosomal RNA of yeast mitochondria. Nucleic Acids Res. **11:**339–348.
51. **Stierum, R. H., G. L. Dianov, and V. A. Bohr.** 1999. Single-nucleotide patch base excision repair of uracil in DNA by mitochondrial protein extracts. Nucleic Acids Res. **27:**3712–3719.
52. **Swanson, R. L., N. J. Morey, P. W. Doetsch, and S. Jinks-Robertson.** 1999. Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:**2929–2935.
53. **Valentine, J. S., D. L. Wertz, T. J. Lyons, L. L. Liou, J. J. Goto, and E. B. Gralla.** 1998. The dark side of dioxygen biochemistry. Curr. Opin. Chem. Biol. **2:**253–262.
54. **Vongsamphanh, R., P. K. Fortier, and D. Ramotar.** 2001. Pir1p mediates translocation of the yeast Apn1p endonuclease into the mitochondria to maintain genomic stability. Mol. Cell. Biol. **21:**1647–1655.
55. **Wallace, D. C.** 1999. Mitochondrial diseases in man and mouse. Science **283:**1482–1488.
56. **You, H. J., R. L. Swanson, and P. W. Doetsch.** 1998. Saccharomyces cerevisiae possesses two functional homologues of Escherichia coli endonuclease III. Biochemistry **37:**6033–6040.
57. **Zhu, H., G. L. Bannenberg, P. Moldeus, and H. G. Shertzer.** 1994. Oxidation pathways for the intracellular probe 2′,7′-dichlorofluorescein. Arch. Toxicol. **68:**582–587.