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The mitochondrial protein frataxin prevents nuclear damage

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The mitochondrial protein frataxin helps maintain appropriate iron levels in the mitochondria of yeast and humans. A deficiency of this protein in humans causes Friedreich's ataxia, while its complete absence in yeast (Δ*yfh1* mutant) results in loss of mitochondrial DNA, apparently due to radicals generated by excess iron. We found that the absence of frataxin in yeast also leads to nuclear damage, as evidenced by inducibility of a nuclear DNA damage reporter, increased chromosomal instability including recombination and mutation, and greater sensitivity to DNA-damaging agents, as well as slow growth. Addition of a human frataxin mutant did not prevent nuclear damage, although it partially complemented the Δ*yfh1* mutant in preventing mitochondrial DNA loss. The effects in Δ*yfh1* mutants result from reactive oxygen species (ROS), since (i) Δ*yfh1* cells produce more hydrogen peroxide, (ii) the effects are alleviated by a radical scavenger and (iii) the glutathione peroxidase gene prevents an increase in mutation rates. Thus, the frataxin protein is concluded to have a protective role for the nucleus as well as the mitochondria.

INTRODUCTION

Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disease that causes physical disability at an early age (1). Patients develop multiple symptoms, including gait and limb ataxia, dysarthria, hypertrophic cardiomyopathy, and diabetes mellitus (1–5). The FRDA gene is conserved across evolution, with homologues being found in purple bacteria, Caenorhabditis elegans, Drosophila melanogaster and the yeast Saccharomyces cerevisiae (6–8). The importance of the FRDA gene has been underscored by the recent finding that a knockout of this gene leads to early embryonic lethality in mice (9). The gene codes for frataxin, a protein that helps maintain mitochondrial iron homeostasis (10), and decreased amounts are usually the consequence of GAA triplet repeat expansion within the gene (11–13). High iron levels have been found in affected tissues of patients (14). The primary tissues affected by the disease – neurons and cardiomyocytes – require high amounts of ATP. Since mitochondrial function is impaired as a result of iron overload (15), the various physiological disease symptoms observed have been attributed to lowered ATP production in these energy-intensive tissues (16).

The yeast S. cerevisiae has been used as a model system to study frataxin, which also regulates mitochondrial iron homeostasis in this organism (17,18). Yeast frataxin, encoded by the YFH1 gene, binds iron and is reported to form high-molecular-weight complexes that may help regulate levels of iron in vivo (19). Deletion of YFH1 results in a 10-fold excess of iron accumulation within mitochondria (10,20). In addition, Δyfh1 mutants exhibit a deficiency of complex IV and mitochondrial proteins containing Fe–S clusters (15,20,21) and they are unable to grow on non-fermentable carbon sources such as ethanol and glycerol (21,22). This petite phenotype is considered to be due to alterations in mitochondrial DNA, presumably resulting from free-radical damage associated with increased iron in the mitochondria. In addition, Δyfh1 cells are sensitive to agents that cause oxidative damage and to high concentrations of iron and copper (10,20).

Δyfh1 mutants have also been reported to grow more slowly than other petites on fermentable carbon sources such as glucose (10,20). This slow-growth phenotype led us to investigate the possibility that, in addition to maintaining appropriate iron levels in the mitochondria, frataxin might also have a role in protecting compartments outside the mitochondria. We found that a DNA damage-inducible nuclear gene was highly expressed in cells lacking frataxin and that Δyfh1 mutants are genetically unstable. They were also hypersensitive to killing by agents that damage DNA and/or inhibit nuclear DNA replication. The various effects are likely due to reactive oxygen species (ROS). Similar responses were found in Δyfh1 cells expressing mutant FRDA protein derived from a patient. These responses were specific in that the mutant protein could

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complement the Δyfh1 growth defect and many of the cells retained mitochondrial function. The results demonstrate that a yeast or human-derived mitochondrial protein can have a nuclear protective effect.

RESULTS

Δyfh1 mutants grow much more slowly than isogenic rho^0 strains

The anecdotal reports indicating that Δyfh1 mutants grow slowly (10,20), led us to investigate this phenotype further to better understand the role of the frataxin gene in yeast. Deletion of YFH1 by PCR-mediated gene replacement in strains GK5 and BY4741 resulted in Δyfh1 cells growing more slowly than isogenic YFH1 rho^0 mutants generated by ethidium bromide treatment (Fig. 1A). Since the fresh isolates of Δyfh1 mutants were petite (unable to grow on glycerol plates) and were lacking mitochondrial DNA based on quantitative PCR analysis (23), rho^0 mutants were used as YFH1$^+$ controls. (We also note that some Δyfh1 isolates exhibited extremely slow growth on glycerol; however, there were no differences in the various tests described below.) All Δyfh1 mutants in the current study grew well at 37°C, unlike strains used in another study (10). Δyfh1$^{-/-}$ diploids grown at 30°C exhibited a similar slow-growth phenotype, while heterozygous diploids formed by crossing Δyfh1 and YFH1 rho^0 strains grew at rates comparable to a rho^0 diploid, indicating that the mutation is recessive (Fig. 1B).

The reduced growth rate is likely due to higher levels of iron that can lead to increased levels of ROS (see Discussion). The increase in ROS could also result from higher levels of iron that might be imported into the cytoplasm. For example, FET3 encodes a protein that transports iron into cells and is induced in Δyfh1 strains (18). This led us to examine the growth rates of Δfet3Δyfh1 double mutants, since the Δfet3 mutation prevented petite induction in Δyfh1 cells of another strain background that was less prone to petite formation. We deleted the FET3 and YFH1 genes, in that order, from the GK5 strain. The double-mutant cells were petite and grew at the same rate as Δyfh1 cells (Fig. 1C). Thus, the mitochondrial iron levels that occur in Δyfh1 cells were adequate to cause slow growth. However, these results do not rule out the possibility that other iron-import pathways may exist (23) and contribute to the observed damage (see Discussion).

Δyfh1 mutants are sensitive to a DNA-alkylating agent and a replication inhibitor

The slow-growth phenotype of Δyfh1 mutants might be due to increased spontaneous nuclear DNA damage. If so, exposure to agents that can damage nuclear DNA, such as methyl methanesulfonate (MMS), might exacerbate the growth defect. As shown in Figure 2A, diploid Δyfh1 cells were highly sensitive to MMS, a DNA-alkylating agent producing lesions that are repaired primarily by base excision repair (BER) and recombination, while rho^0 diploids and heterozygous Δyfh1/YFH1 rho^0 diploids were resistant. In contrast, Δyfh1 mutants were not hypersensitive to ultraviolet or gamma radiation (data not shown).

We also examined the effect of the replication inhibitor hydroxyurea (HU), which blocks ribonucleotide reductase function. As shown in Figure 2B, no growth was observed when diploid Δyfh1 cells were pronged to 200 mM HU, whereas there was confluent growth of YFH1 rho^0 cells. The heterozygous diploid Δyfh1/YFH1 rho^0 was resistant to 200 mM HU, demonstrating that the sensitivity was Δyfh1-dependent. Comparable results were obtained with haploid Δyfh1 cells (data not shown). Thus, the Δyfh1 mutation can sensitize cells to agents that affect DNA metabolism.

A nuclear DNA damage-inducible promoter is constitutively activated in Δyfh1 mutants

While MMS is commonly used to induce lesions in DNA, it can also damage proteins by methylation (24). To resolve if the sensitivity to this agent is due to nuclear DNA damage, a DNA-damage response reporter assay was used (25). The plasmid YEplac112DIN1placZ, containing the promoter of the DNA damage-inducible gene DIN1 (RNR3) fused to the Escherichia coli lacZ gene, was introduced into Δyfh1 and YFH1 rho^0 cells. Activation of the DIN1 promoter in response to DNA damage is indicated by the appearance of blue colonies on Xgal plates (Fig. 3A). Since Δyfh1 colonies patched onto medium containing Xgal formed a deep blue color while rho^0 patches remained white (Fig. 3A), there appears to be substantial spontaneous nuclear damage in this mutant.

Δyfh1 strains exhibit increased chromosome instability, including recombination

The above results demonstrating that nuclear DNA damage arises when there is a deficiency of frataxin led us to examine effects on chromosome stability. We compared illegitimate mating in Δyfh1 mutants versus wild-type cells. This mating occurs primarily as a result of loss of chromosome III, although it also can be due to chromosome arm loss or recombination (26–28). It is detected by the ability of haploid cells of the same mating type to give rise to diploids or by MATa/MATα diploids being able to mate with haploids.

Patches of Δyfh1 and rho^0 strains were replica-plated onto lawns of mating testers. As expected, haploid Δyfh1 MATα strains mated with the MATa tester to give a confluent mating patch. However, as shown in Figure 4, many papillae were observed on plates containing the MATα tester. Diploid Δyfh1 cells also showed an increased frequency of papillae when given the opportunity to mate with either MATa or MATα tester strains. We conclude that the Δyfh1 defect leads to a decrease in chromosome stability.

To further address the genetic consequence of a frataxin deficiency, we examined recombination in Δyfh1 mutants using the sister chromatid exchange (SCE) assay described by Fasullo et al. (29). YFH1 was deleted from strain YB146 containing the SCE cassette and isogenic rho^0 isolates were obtained following ethidium bromide treatment (see Materials and Methods). Recombination rates were estimated by determining the frequencies of recombination in seven independent isolates (see Materials and Methods) and using the method of the median to determine the rates of recombination (30). The spontaneous recombination rates were approximately 6-fold

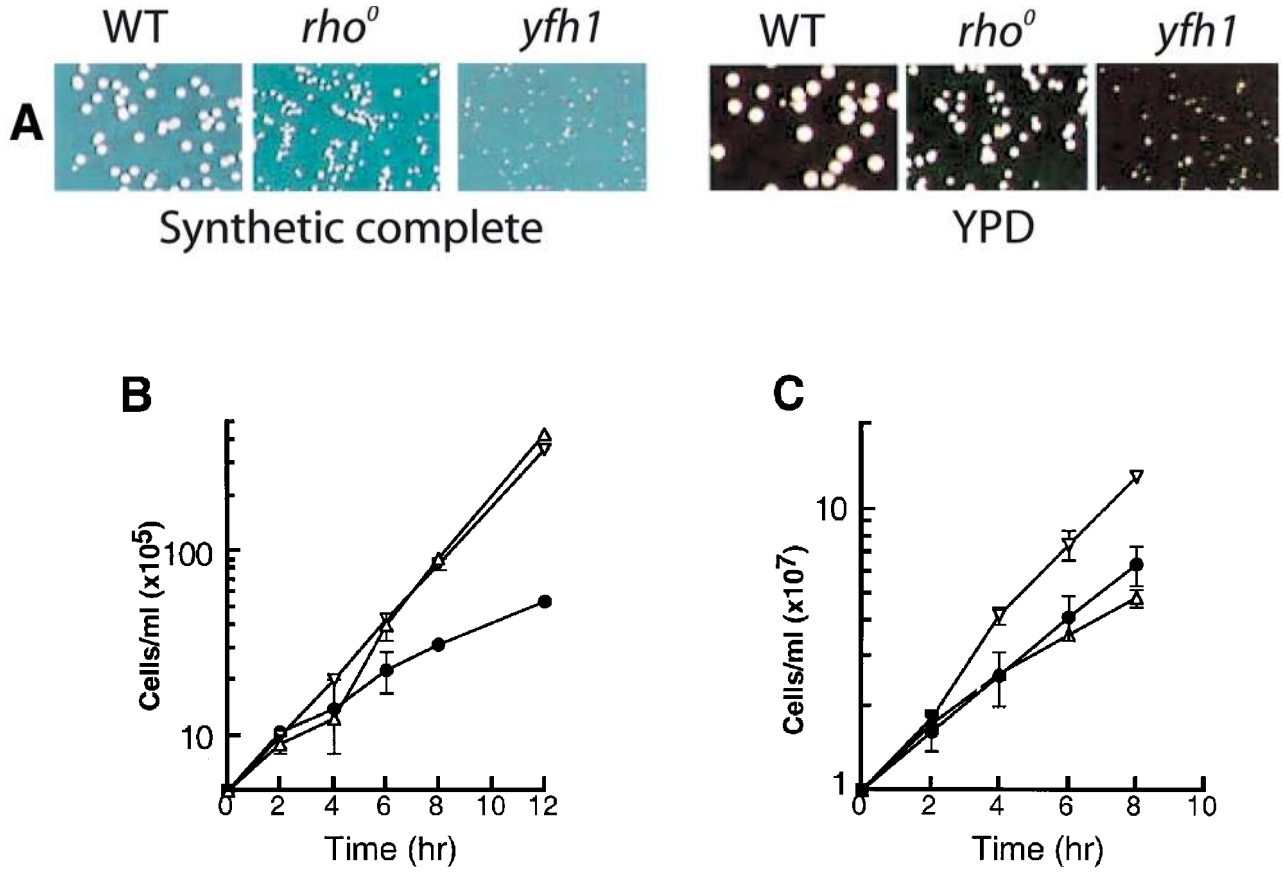

Figure 1. Growth of Δyfh1 strains in different media. (A) WT, rho^0 and Δyfh1 were grown on SC and YPD media to characterize the slow-growth phenotype of Δyfh1 strains. (B) Diploid strains YFH1/YFH1 rho^0 (∇) YFH1/Δyfh1 rho^0 (Δ) and Δyfh1/Δyfh1 (•) were analyzed for growth rate in YPD media. (C) Haploid strains Δyfh1 (•), fet3 Δyfh1 (Δ) and YFH1 rho^0 (∇) were analyzed for growth rate in YPD media.

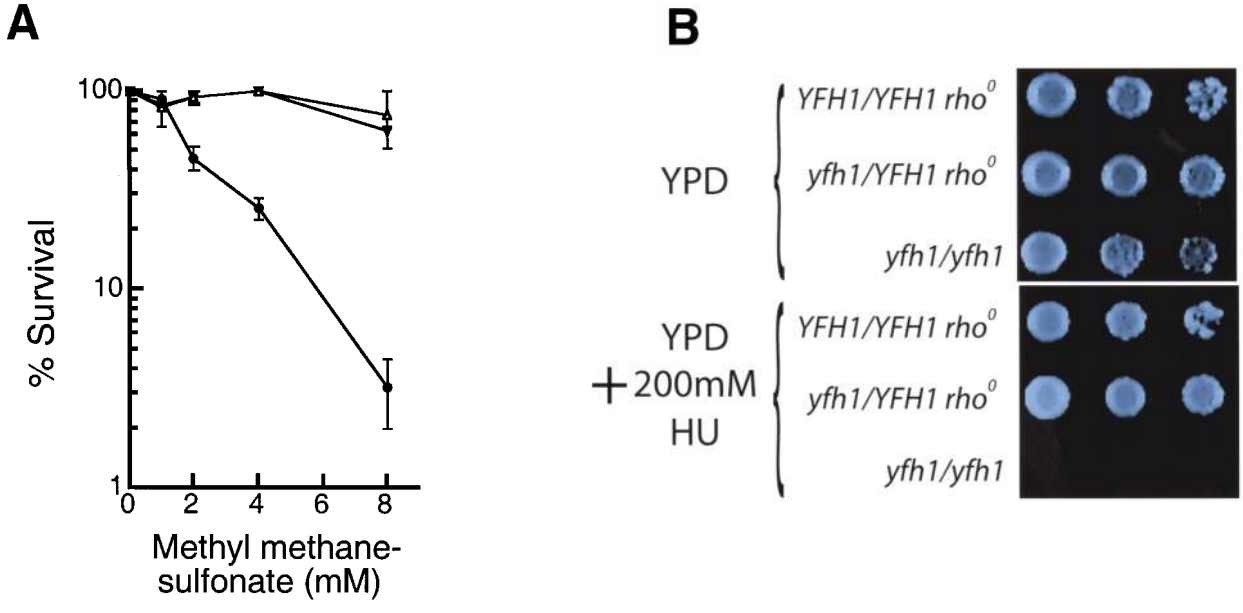

Figure 2. Sensitivity to DNA metabolic and damaging agents. (A) Diploid strains YFH1/YFH1 rho^0 (∇), YFH1/Δyfh1 rho^0 (Δ), and Δyfh1/Δyfh1 (•) were exposed to methyl methanesulfonate at different concentrations for 15 minutes. Cells were washed and plated onto YPD media to determine survival. (B) Diploid strains were dilution-pronged onto YPD and YPD with 200 mM hydroxy urea (HU).

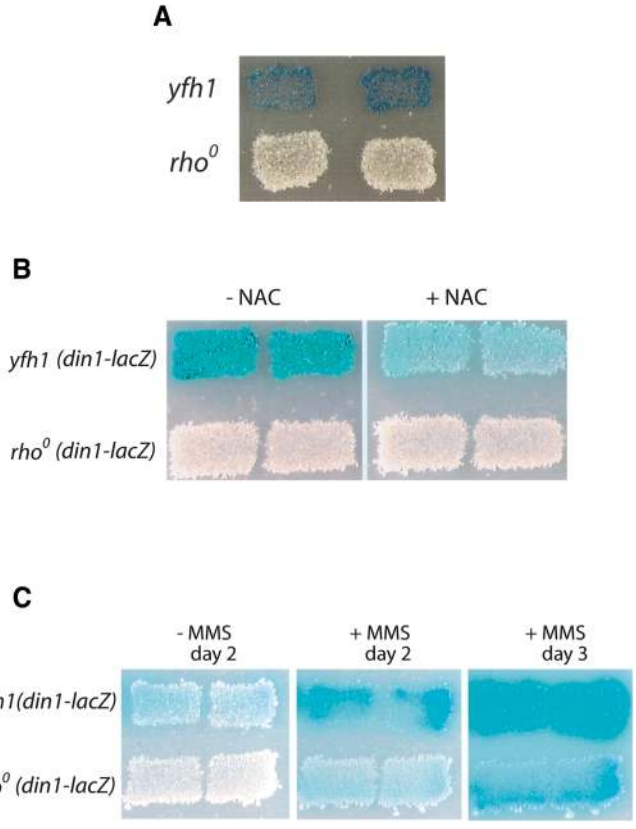

Figure 3. Constitutive din1 expression and its alleviation by a radical scavenger. (A) Strains bearing the din1–lacZ plasmid were lightly replica-plated onto Xgal plates to determine DIN1 induction, which is indicated by blue color. (B) The radical scavenger N-acetylcysteine (NAC) reduces the appearance of blue color on Xgal plates in Δyfh1 strains. (C) NAC does not prevent induction of DIN1 in Δyfh1 strains by 1 mM methyl methanesulfonate (MMS). rho^0 strains bearing the plasmid also induce DIN1 in response to MMS. Maximal color is observed on day 3.

higher in a Δyfh1 mutant as compared with the control rho^0 strain (Table 1). We conclude that the absence of frataxin can lead to various kinds of nuclear genome instability (including mutation, as described below).

Table 1. Rates of sister chromatid recombination in a petite and a frataxin deletion strain

Strain	Recombination rate ($\times 10^7$)[a]	Fold increase
rho^0	7.5 (3–22)	5.7
Δyfh1	43 (32–115)	

[a]Confidence interval in parentheses.

The toxic effects of supplemental iron are reversible by a free-radical scavenger

FRDA is a disease that appears to be associated with iron imbalance and high levels of iron accumulation in the mitochondria as the disease progresses. The yeast frataxin mutant also accumulates iron within the mitochondria, and high concentrations of iron added to the medium inhibit the growth of Δyfh1 cells. The presence of 5 mM iron in the medium was inhibitory to the Δyfh1 mutants in the strain backgrounds used in this study, and, as shown in Figure 5A, there was a 4–5 order-of-magnitude reduction in survival in Δyfh1 mutants compared with rho^0 control cells.

Since the toxic effects of iron have been associated with products generated through iron-catalyzed Fenton chemistry, a free-radical scavenger might reverse this sensitivity. To test this, we used N-acetylcysteine (NAC), a commonly used free-radical scavenger that is not an iron chelator (31, 32). Serial dilutions of cells were exposed to 5 mM iron with and without 20 mM NAC added to the plates [this concentration of NAC was found to be optimal (33) (data not shown)]. As shown in Figure 5B, survival increased 2–4 orders of magnitude, indicating that much of the iron toxicity could be reversed by NAC. NAC also improved the growth of Δyfh1 cells in standard yeast growth media (data not shown).

The ability of NAC to reverse the growth-inhibitory effects of exogenous iron suggested that the DNA instability and sensitivity to DNA-damaging agents of Δyfh1 mutants were mediated by an excess of free radicals. To test this, cells were examined for inducibility of the din1–lacZ construct in the presence and absence of 20 mM NAC. As shown in Figure 3B, lacZ expression was reduced on plates containing NAC. To

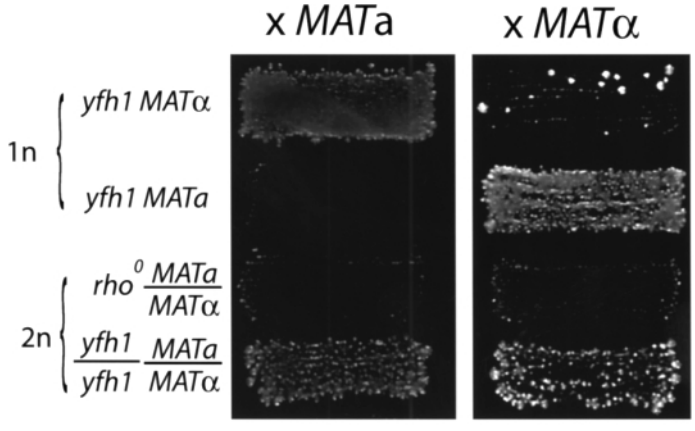

Figure 4. Illegitimate mating tests. Strains were grown as patches on YPD media and lightly imprinted on a lawn of mating-type testers on media lacking the appropriate amino acids in order to score illegitimate mating.

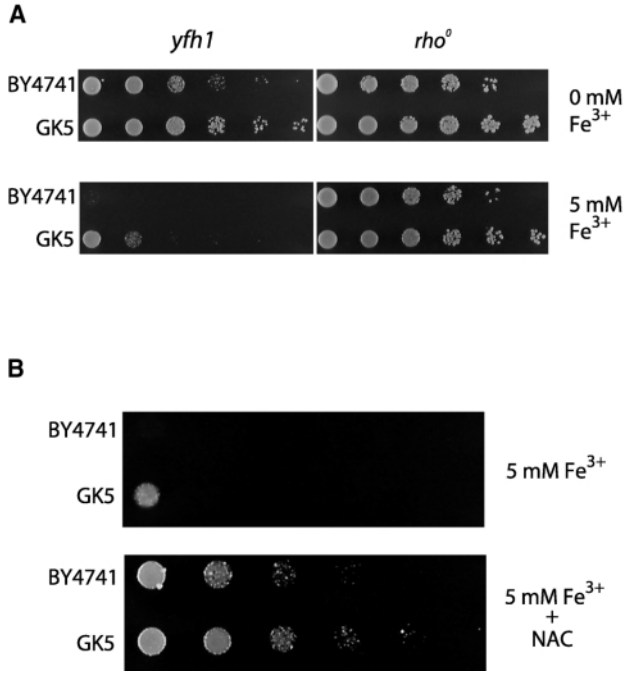

Figure 5. Sensitivity to iron and alleviation by NAC. (A) Strains were dilution-pronged onto SC media with 5 mM iron ($FeCl_3$). Δyfh1 strains show severe inhibition of growth at this concentration of iron. (B) The radical scavenger NAC alleviates the growth inhibition of Δyfh1 strains on 5mM iron.

control for the possibility that NAC might generally inhibit the appearance of the blue color, the effect of NAC on MMS induction of din1–lacZ was examined. The concentration of MMS employed (1 mM) was not lethal to Δyfh1 cells, but could induce DNA damage and turn on DIN1 expression. As seen in Figure 3C, NAC did not prevent MMS-induced din1–lacZ expression in either the Δyfh1 or the rho^0 cells. Thus, we conclude that the spontaneous nuclear damage in Δyfh1 cells is caused by free radicals and that protection against this damage can be provided by a free-radical scavenger.

Δyfh1 cells produce increased amounts of hydrogen peroxide

The nuclear damage in Δyfh1 cells could result from excess hydrogen peroxide (H_2O_2) production caused by high iron concentrations within the mitochondria (see Fig. 8A). Since H_2O_2 is diffusible, it should be detectable external to the cells. To test this, logarithmically growing cells were incubated in phosphate buffer and the appearance of H_2O_2 in the buffer was determined using an Amplex Red Hydrogen Peroxide Assay Kit (see Materials and Methods). As shown in Figure 6, the level of H_2O_2 was 2–4-fold higher in Δyfh1 strains compared with the control cells. There was no apparent difference between the rho^0 or $rho^+ YFH1^+$ strains.

A glutathione peroxidase, Δyfh1 double mutant exhibits high mutation rates

Since H_2O_2 appeared to be a source of DNA damage in the Δyfh1 mutant, we anticipated that deletion of the peroxidase gene, GPX1, might increase genome instability, including the appearance of mutations. The GPX1 and YFH1 genes were deleted, and canavanine resistance (can^R) mutation rates were determined by the method of the median (30) in Δgpx1, Δyfh1, Δgpx1Δyfh1 and rho^0 mutants (see Materials and Methods). There was no apparent difference in growth rates between Δyfh1 and Δgpx1Δyfh1 double mutants. Forward mutation of the arginine permease resulting in resistance to canavanine is frequently used to examine endogenous sources of mutation (34). As shown in Table 2, the mutation rates of the Δgpx1, Δyfh1 and rho^0 mutants are comparable to each other. In contrast, there is a dramatic increase in mutation rate in the Δgpx1Δyfh1 double mutant. These results support the view that

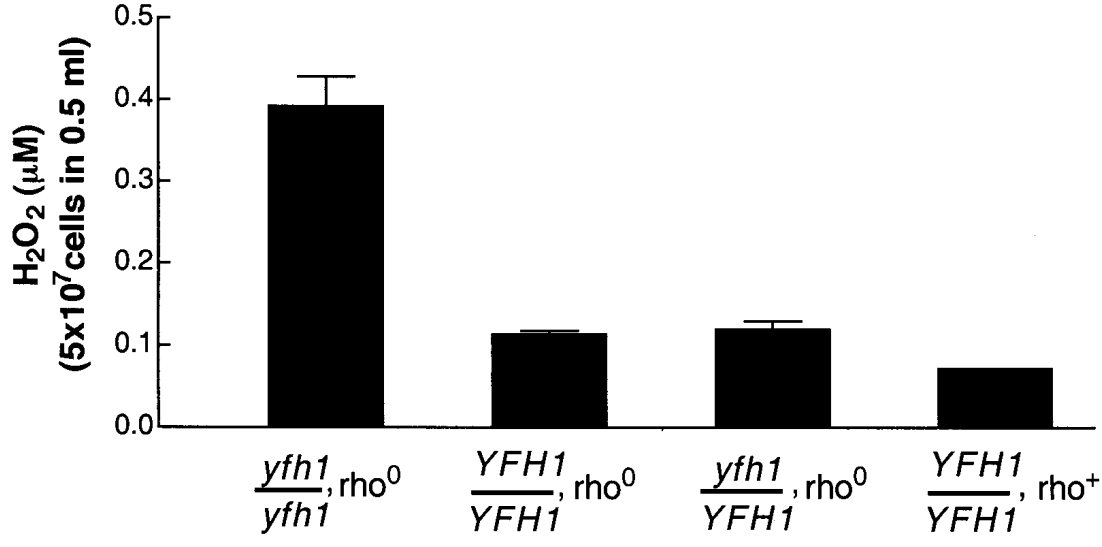

Figure 6. Hydrogen peroxide (H_2O_2) detection in cell supernatants. H_2O_2 was estimated in supernatants of diploid strains that were suspended in PBS and incubated for 40 minutes.

Table 2. Impact of defects in the frataxin (Δyfh1) and glutathione peroxidase (Δgpx1) genes on forward (to can^R) mutation rates

Strain	Mutation rate ($\times 10^7$)[a]
rho^0	2.4 (1.9–4.1)
Δyfh1	5.0 (0.7–8.7)
Δgpx1	2.1 (1.7–3.0)
Δgpx1Δyfh1	27.5 (16.9–52.6)

[a]Confidence interval in parentheses.

an important source of nuclear damage in frataxin mutants is H_2O_2, and that there is a large potential for inducing mutations that is greatly reduced through a peroxidase detoxification mechanism.

Human FRDA mutant protects against petite formation but not nuclear DNA damage

The human FRDA gene product can complement a yeast Δyfh1 mutant when expressed from a glyceraldehyde-3-phosphate dehydrogenase promoter (35). A G130V FRDA mutation was also shown to complement the yeast mutant, whereas W173G FRDA was unable to complement the Δyfh1 defect and cells rapidly became petite and accumulated iron in their mitochondria (35). Given the difference between the mutants, we examined them for their ability to complement the growth defect and to protect cells from HU and MMS in our Δyfh1 strain background. Plasmids bearing the human FRDA gene and its variants (described in 35) were transformed into strain GK5 and the YFH1 gene was subsequently deleted. In this strain background, the FRDA wild-type gene, the G130V mutant and, surprisingly, even the plasmid bearing the W173G mutant could complement the yeast Δyfh1 mutation so that isolates appeared to grow normally and were not petite (Fig. 7A). Upon closer examination, we observed that 70% of the cells within a W173G clone gave rise to petite colonies, suggesting that the strain is partially defective for frataxin function. Similarly, while FRDA and the G130V mutant were able to protect Δyfh1 cells from HU and MMS (Fig. 7A and data not shown), the Δyfh1 cells containing the W173G mutant were strongly growth-inhibited by 200 mM HU and 2 mM MMS (Fig. 7A,B).

We also examined the Δyfh1 cells containing the FRDA plasmids for the impact of HU and MMS on cell cycle progression. Both MMS and HU can cause a G_2 checkpoint arrest, which is detected in yeast as an increase in large budded cells (36,37). Cells were grown on plates containing 200 mM HU or 2 mM MMS. They were examined after 2 days. Approximately 90% of the W173G-bearing cells in HU and 40% in MMS were large-budded. The frequencies of G_2 cells were much lower in the WT strain or the Δyfh1 strain complemented by the human FRDA or G130V mutant (Fig. 7A, B).

The effects of external agents on Δyfh1 cells expressing the W173G frataxin hypomorphic mutation could be due to increased internal lesions. As shown in Figure 7C, the cells with the W173G mutation exhibited a strong din1–lacZ nuclear damage response, whereas there was no induction in cells containing the FRDA gene or the G130V mutant. Furthermore, since the strains containing the human genes were not petite, these results also suggest that the effects of HU and MMS described above for the Δyfh1 strains were not specific to yeast frataxin mutants that also happen to be petite.

DISCUSSION

Frataxin is required for maintenance of iron homeostasis in the mitochondria. While decreases in the amount of protein result in the disease Friedreich's ataxia, the specific reason(s) for the disease state have not been established. Because frataxin normally forms multimeric, high-molecular-weight complexes with iron, the absence of this protein results in a large accumulation of iron in the mitochondria. Results from model systems suggest that this iron overload affects mitochondrial function (21,22). Excess iron in the presence of oxygen can generate superoxide and hydroxyl radicals (21), both of which are ROS (Fig. 8A). High ROS levels could cause damage to mitochondrial DNA and Fe–S-containing proteins, which are radical-sensitive (15,19,20).

While frataxin is a mitochondrial protein, the observation that Δyfh1 cells grow more slowly than isogenic rho^0 petite cells (this study, 10 and 20) led us to investigate a possible role for this protein elsewhere in the cell. The increase in iron-associated reactions in the mitochondria of Δyfh1 mutants could also lead to the appearance of ROS in other cellular compartments (see the model in Fig. 8B). The slow growth did not appear to be associated with increased iron influx, since deletion of the iron transporter gene FET3 did not alter the growth rate; instead, the Δyfh1 defect in iron homeostasis resulted in nuclear chromosomal damage, which might explain the slow-growth phenotype. Other iron-import pathways appear to be activated in the absence of FET3 (23), and might be an additional source of damage via Fenton reactions in the cytoplasm. However, direct measurements of iron in Δyfh1 mutants have only revealed increases in the mitochondria (10,20), suggesting that they are the major source of increased ROS, such as H_2O_2.

The Δyfh1 mutation greatly increased sensitivity to the DNA-methylating agent MMS and the DNA-synthesis inhibitor HU. Although MMS can damage proteins in addition to DNA (24), the major impact on survival is generally considered to result from lesions in chromosomal DNA. Moreover, we found that there was a high spontaneous induction of the DNA damage-responsive promoter of the RNR3 gene. This induction could be due to blocks in DNA synthesis caused by oxidized bases (38) or intermediates produced during repair of oxidized DNA by base excision repair, such as abasic sites or nicks. The enhanced sensitivity to HU might be due to a combination of elevated levels of damage, along with a reduction in pools of dNTPs. Further evidence for increased levels of chromosomal damage in Δyfh1 cells was demonstrated by the increase in illegitimate mating that results from chromosome instability, primarily chromosome loss (26–28).

The Δyfh1 mutation does not lead to a general sensitivity to DNA-damaging agents. Neither camptothecin (unpublished), which interferes with topoisomerases, nor ultraviolet (UV) or

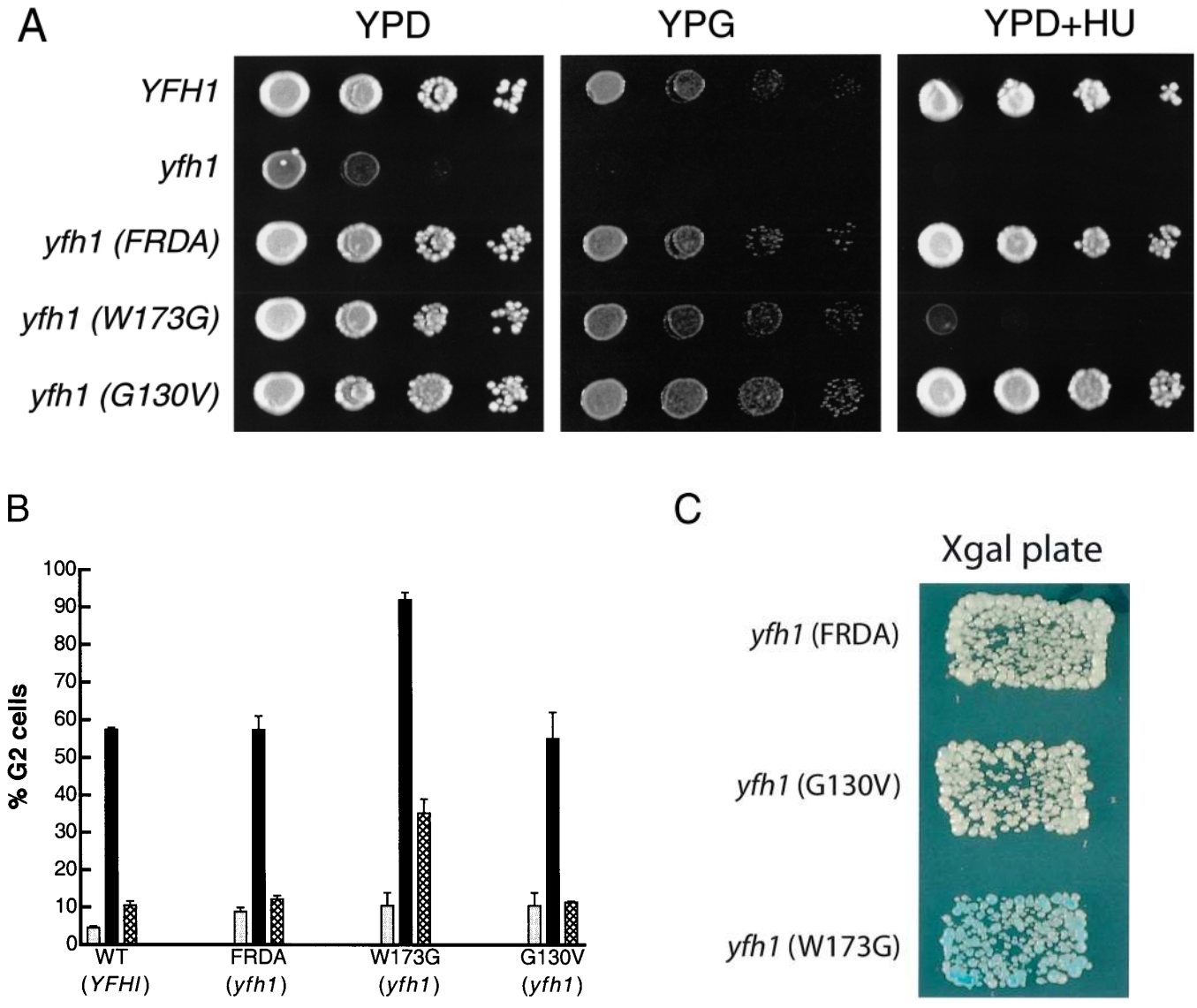

Figure 7. Complementation of Δyfh1 by human FRDA mutants. (A) GK5 wild-type strain, Δyfh1 and Δyfh1 cells with plasmids FRDA, W173G and G130V were dilution-pronged onto YPD- , YPG- and YPD- + HU-containing media. The Δyfh1 yeast strain with W173G shows growth on YPG, indicating that cells retain mitochondrial function, but strong inhibition on YPD + HU plates, indicating effects on nuclear DNA metabolism. (B) A high percentage of cells with W173G were arrested in G_2 following treatment with HU and MMS. Gray bars are for G_2 cells after growth on YPD; black bars correspond to cells grown on YPD + 200 mM HU and hatched bars are for cells grown on YPD + 2 mM MMS. (C) Strains with the W173G human mutant activate the DIN1 gene, as indicated by blue color on Xgal plates.

gamma radiation, which cause several types of DNA lesions, increased killing in Δyfh1 cells. It is interesting that gamma radiation does not seem to have an effect on Δyfh1 cells. Possibly this is due to radiation damage producing radicals that are concentrated in 'tracks' that lead to double strand breaks. This contrasts with the diffuse radical damage mediated by H_2O_2 in Δyfh1 cells. The differences in sensitivity to MMS and UV may indicate that the increased spontaneous lesions are not subject to nucleotide excision repair.

An increase in mutation and/or recombination rates is one of the hallmarks of nuclear DNA damage. The frataxin deficiency resulted in a 6-fold increase in SCE recombination rates. The forward mutation rate to canavanine resistance in Δyfh1 strains was greatly increased when a Δgpx1 mutation was introduced into the Δyfh1 mutant, demonstrating that there is a considerable potential for mutation when frataxin is absent. The increases in chromosome loss, recombination and mutation are consistent with other observations reported here, and collectively demonstrate that the frataxin defect results in nuclear instability.

The protective role that frataxin plays in preventing damage to nuclear DNA is likely due to its control of iron distribution

within the mitochondria. Using the human frataxin gene and mutants, it was possible to distinguish between effects that might be in part related to the mitochondrial deficiency of Δyfh1 mutants versus control of iron distribution. Creation of the Δyfh1 mutation in cells expressing the human frataxin gene or mutants did not lead to petite colonies for the wild-type and G130V alleles used in this study, suggesting that the human proteins could complement the frataxin defect. The Δyfh1 cells expressing the human W173G mutant protein accumulate iron within the mitochondria (35), whereas wild-type and G130V proteins do not. However, the hypomorphic mutant W173G, which results in low amounts of mature frataxin in yeast (35), exhibited partial complementation for loss of mitochondrial function. Also, there was a clear nuclear damage response resulting in din1–lacZ induction and increased growth inhibition by HU and MMS (Fig. 7). The inhibition was due to most cells arresting in the G_2 phase of the cell cycle (Fig. 7A, B and MMS data not shown), where they became swollen and dumbbell-shaped – a characteristic DNA-damage response (39). These results with the W173G mutant support the view that excess iron within the mitochondria is responsible for the nuclear DNA damage reported here. We note that the expression and damage response system that we have developed provides a means for both addressing reduced frataxin function and assessing the nature of human frataxin mutants. By varying the levels of induction using a rheostatable promoter [as has been reported recently for the analysis of human p53 mutant defects in yeast reporter assays (40)], it should be possible to evaluate the extent of functional defect of human frataxin mutants, as well as the importance of various amounts of wild-type protein.

The strains used in the present study were prone to petite formation when the Δyfh1 gene was deleted, as found in other studies (22), and they were also highly sensitive to low concentrations of iron. These features will be useful in characterizing the effects of various frataxin levels, as evident from the results with the human proteins expressed in this strain. Reports of little or no petite formation in Δyfh1 mutants and lack of induction of oxidative-stress genes (23) could be due to strain background differences. Strain background can markedly influence response to DNA lesions (39). Having a yeast model system that is highly responsive to the Δyfh1 defects is particularly relevant, since FRDA patients express various levels of frataxin.

Since the free-radical scavenger NAC was effective in reducing the impact of the Δyfh1 mutation, we propose that reduced levels of frataxin result in high levels of free radicals that can cause damage in the mitochondria and the nucleus. The consequences in the former are loss of mitochondrial DNA, rendering cells petite (10,22) (this study). The consequences in the latter are decreased chromosome stability and decreased resistance to agents that can cause DNA damage or alter DNA metabolism.

A source of DNA damage could be H_2O_2 production and subsequent radical formation. While previous studies demonstrated increased sensitivity to exogenous H_2O_2, we have established that Δyfh1 cells produce a relatively high level of H_2O_2 (Fig. 6). As depicted in Figure 8A, B, the large excess of iron that is present within the mitochondria of Δyfh1 cells, or possibly is imported into the cytoplasm, could catalyze superoxide radical formation in the presence of oxygen (21). This could lead to the generation of H_2O_2 either spontaneously or by the action of superoxide dismutase (41). It is also formally possible that associated with the frataxin defect is a reduced ability to detoxify H_2O_2. Since H_2O_2 is uncharged (unlike other ROS), it is freely diffusible and can traverse the mitochondrial membranes and enter the nuclear compartment.

The potential for H_2O_2 to cause nuclear DNA damage demonstrated by the increase in mutation rates exhibited by the Δgpx1Δyfh1 double mutants suggests not only that the frataxin deficiency lead to nuclear damage but also that the effects might be greater if there were not peroxidase detoxification. Taken together with the increase in nuclear DNA recombination rates, these results prove that there is nuclear DNA damage caused by the ROS, H_2O_2. Our results of several strong nuclear responses that are ROS-mediated in frataxin mutants appear to differ from reports of a lack of change in global genome expression, particularly induction of genes that respond to oxidative damage (23,42). This lack of induction of oxidative damage response genes might be due to the basal levels being sufficient to deal with the damage. Also, there are several repair mechanisms that deal with oxidative damage, including base excision repair, mismatch repair and recombination (43–45). It is possible that concerted action by all these pathways helps limit the damage in Δyfh1 cells. We are currently in the process of evaluating the role that these repair pathways may play in response to the nuclear DNA damage observed in Δyfh1 cells.

It is also possible that some of the damage to nuclear DNA is caused by radicals transmitted between the membranes of perinuclear mitochondria and the nucleus via lipid peroxides (46). Alternatively, some mitochondria might lose their integrity because of oxidative degradation of the membranes, so that release of radicals could lead to nuclear damage.

There have been reports that FRDA patients can respond positively to free-radical scavengers/antioxidants such as idebenone (47,48). The results with yeast and human cells also lead us to suggest that there is a nuclear component to FRDA that may impact on the development of therapies. It is interesting that in early reports on the phenotypes of cells derived from FRDA patients, there was enhanced sensitivity to DNA-damaging agents (49), analogous to our results with the yeast mutant. Interestingly, there is also an increased frequency of mutations in a 135 bp window upstream from the GAA repeat region within the FRDA gene (11) observed in cells from FRDA patients. An increase in ROS in the nucleus, along with reduced nucleosome organization because of triplex formation (50,51), might account for this increased mutation. A recent report suggesting a link between FRDA and breast cancer (52) further supports the view that damage can occur when there are reduced amounts of frataxin in the mitochondria.

Finally, our findings suggest a novel relationship between mitochondria, mitochondrial proteins and the nucleus. The possibility that mitochondrial processes might impact on nuclear metabolism was previously suggested by the observations that petite mutants have slightly increased nuclear DNA mutation rates (53). Our study is the first to demonstrate that substantial nuclear DNA damage can arise in cells lacking a mitochondria-localized protein. This suggests that other

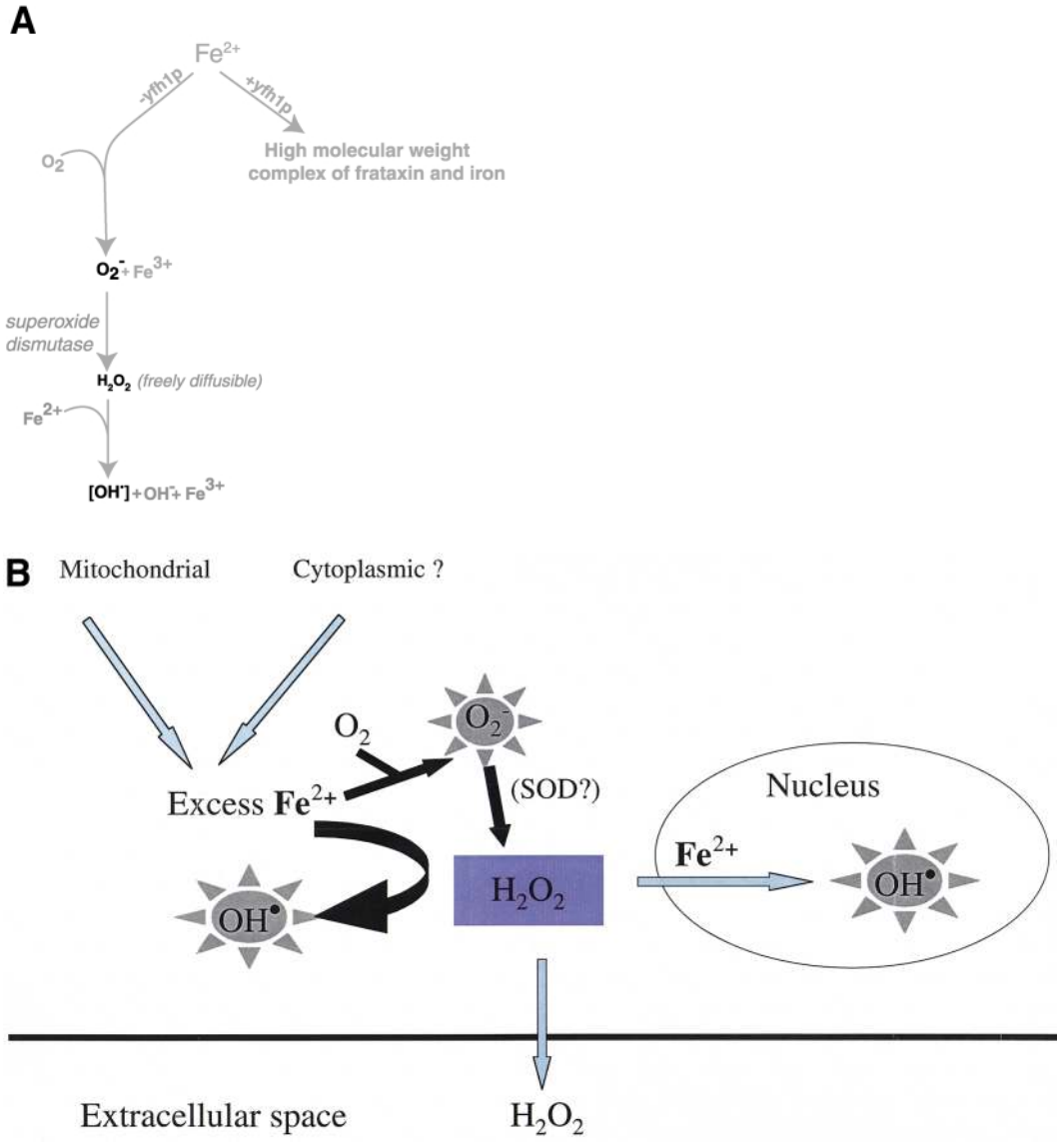

Figure 8. Model for H_2O_2-mediated damage. (A) Superoxide radicals are generated by the action of excess iron on oxygen. Superoxide can be converted to H_2O_2 either spontaneously or by superoxide dismutase. Because the H_2O_2 is freely diffusible, it can traverse membranes and damage nuclear DNA. The presence of frataxin may prevent this cascade of events by sequestering the iron. (B) This model depicts H_2O_2 diffusion across membranes into the nucleus and extracellular medium.

mitochondrial proteins may also play a protective role with regard to nuclear damage.

MATERIALS AND METHODS

Yeast strains and general genetic and molecular methods

Yeast cells were grown at 30°C on standard YPD (yeast extract, peptone, dextrose) or synthetic complete media (SC) (54). Yeast transformation and gene disruption were performed according to the methods of Gietz and Schiestl (55). Oligonucleotides for YFH1 gene disruption were purchased from Life-Technologies. Primers containing 60 bases of homology to YFH1 and 18 bases of homology to the vector PFA6MX4 were used. The following strains were employed for this study: GK5 (MATα ade5–1 his7–2 leu2–3, 112 trp1–289 ura3–52) derived from ALE100 (CG379) (56), BY4741 (MATa his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) (57) and YB146 (MATa ura3–52 his3–Δ200 ade2–101 lys2–801 trp1–Δ1 gal3$^-$ rp1::[his3-Δ3′::Hocs his3-Δ5] (29). Diploid strains were constructed by mating GK5 with ALE100. Isogenic rho^0 strains were obtained by ethidium bromide treatment (58,59).

Din1–lacZ induction assays. A din1–lacZ damage-induction reporter system was derived by amplifying the DIN1 (RNR3) promoter region from genomic DNA (primers DINSTR: ggatccagaaggaaacactcaagggtgttg and BtDin: ctgcagatt-tattgctgctgctattcttgcttg). The PCR product was digested with

BamHI and PstI and cloned into BamHI/PstI-cut YEplac112 (2 μm TRP1) to give plasmid YEplac112DIN1p. The lacZ-containing PstI fragment from plasmid pMC1871 (Pharmacia) was cloned into the PstI site of YEplac112DIN1p to produce YEplac112DIN1placZ. This plasmid was used to monitor chromosomal damage. Xgal plates were prepared by buffering SC medium with 0.1 M sodium phosphate buffer and adding 1.6 ml Xgal (50 mg/ml) (Promega). Patches of cells grown on SC–TRP media were lightly imprinted onto Xgal plates and analyzed after 2–3 days of incubation at 30°C.

Illegitimate mating. Illegitimate mating, which provides an indirect measure of loss of chromosome III, as well as less common recombination events (26–28) was assayed in the following manner. Strains were grown on YPD plates. About 5×10^6 cells of mating-type tester strains (MATa and MATα) with multiple genetic markers were resuspended in 1 ml YPD broth and spread onto plates lacking appropriate amino acids to select for mating. Patches grown on YPD plates were lightly imprinted over the tester strains and examined for the presence of colonies after 2–3 days of incubation.

Growth rate, survival, mutation/recombination rate and H_2O_2 measurements

Growth. To evaluate growth characteristics, log-phase cells were inoculated into YPD broth at $\leq 1 \times 10^7$ cells/ml. All growth-rate measurements were done in duplicate using a hemocytometer with independent clonal isolates. The effects of iron and HU were determined using serial dilution pronging onto YPD plates containing HU or onto SC plates containing $FeCl_3$. Approximately 5×10^6 cells were resuspended in 200 μl of water in a 96-well microtiter dish and serial 10-fold dilutions were pronged onto appropriate plates. Plates were analyzed after 2 days of incubation at 30°C. The free radical scavenger NAC was added to media at a final concentration of 20 mM.

MMS survival curves. Diploid strains were grown overnight in YPD medium, diluted to approximately 1×10^7 cells/ml and treated with MMS. Treated cells were incubated at room temperature for 15 minutes, washed, diluted and spread onto YPD agar plates. Plates were examined after 3 days of incubation.

Mutation and recombination rate measurements. Single colony isolates of the various strains were patched on YPD plates and grown for 2 days. The entire patch was harvested and resuspended in 250 μl of water. Ten microliters of the cells were diluted appropriately and plated on YPD plates to estimate the total number of cells present in the patch. 100 μl of cells were plated on selective plates. The colonies were counted after 3 days' incubation at 30°C. The mutation rates were determined by the method of the median (30).

H_2O_2 analysis. Log-phase cells were inoculated into YPD broth at $\leq 5 \times 10^6$ cells/ml and triplicate cultures were grown for 6 hours. Cells were washed and resuspended in 0.5 ml PBS and incubated at 30°C for 40 minutes (there was no increase in H_2O_2 beyond 30 minutes). The cells were then spun down and the PBS supernatant was passed through a Millex HA (Millipore) 0.45 μm filter. Triplicates of each supernatant were assayed for the presence of H_2O_2 by using the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes Inc.) and assay conditions specified by the kit. Horseradish peroxidase reacts with H_2O_2 and Amplex Red to generate resorufin, which is detected with a fluorimeter. The horseradish peroxidase and Amplex Red reagent (in DMSO) were added to water to make the final assay solution, which was then added to the cell supernatant. The three independent cultures were assayed and the results were averaged.

Note added in proof

It is interesting to note, in light of our observation that frataxin can prevent nuclear damage, that there may be a role for frataxin in protecting cells from tumorigenesis based on the recent report by Shoichet et al. (2002) Frataxin promotes antioxidant defense in a thiol-dependent manner resulting in diminished malignant transformation in vitro. Hum. Mol. Genet., 11, 815–821.

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