Mitochondrial and nuclear DNA defects in *Saccharomyces cerevisiae* with mutations in DNA polymerase γ associated with progressive external ophthalmoplegia

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A number of nuclear mutations have been identified in a variety of mitochondrial diseases including progressive external ophthalmoplegia (PEO), Alpers syndrome and other neuromuscular and oxidative phosphorylation defects. More than 50 mutations have been identified in *POLG*, which encodes the human mitochondrial DNA (mtDNA) polymerase γ, PEO and Alpers patients. To rapidly characterize the effects of these mutations, we have developed a versatile system that enables the consequences of homologous mutations, introduced *in situ* into the yeast mtDNA polymerase gene *MIP1*, to be evaluated *in vivo* in haploid and diploid cells. Overall, distinct phenotypes for expression of each of the *mip1*-PEO mutations were observed, including respiration-defective cells with decreased viability, dominant-negative mutant polymerases, elevated levels of mitochondrial and nuclear DNA damage and chromosomal mutations. Mutations in the polymerase domain caused the most severe phenotype accompanied by loss of mtDNA and cell viability, whereas the mutation in the exonuclease domain showed mild dominance with loss of mtDNA. Interestingly, the linker region mutation caused elevated mitochondrial and nuclear DNA damage. The cellular processes contributing to these observations in the mutant yeast cells are potentially relevant to understanding the pathologies observed in human mitochondrial disease patients.

INTRODUCTION

Pol γ, the DNA polymerase responsible for replication and repair of mitochondrial DNA (mtDNA), is encoded by the nuclear genes *POLG* in humans (1) and *MIP1* in yeasts (2). Mutations in the human *POLG* gene are associated with several mitochondrial disorders including the human neuromuscular disorder, progressive external ophthalmoplegia (PEO), Alpers syndrome, male infertility, sensory ataxia and neuropathy (3). Although PEO arises most frequently because of mutations in *POLG* (4), it can also result from mutations in two other nuclear-encoded genes that function in the mitochondria: the adenine nucleotide translocator *ANT1* (5,6) and the mtDNA helicase encoded by *TWINKLE* (7).

PEO is characterized by the accumulation of multiple point mutations and deletions of mtDNA. Autosomal dominant (adPEO) and recessive (arPEO) forms of the disease generally present between 18 and 40 years of age. PEO causes a progressive weakening and paralysis of the external eye muscles resulting in droopy eyelids and the inability to look right and left. The disease is often accompanied by dysphagia, variable weakness of neck and limb muscles, and can be accompanied by depression or avoidant personality (8,9). In certain cases, as with the Y955C pedigrees, the disease progresses to Parkinsonism (10). Alpers syndrome, another mitochondrial disease associated with mutations in *POLG*, is a fatal childhood mitochondrial disease affecting the brain and liver, which is caused by mtDNA depletion with...
symptoms including progressive cerebral degeneration, hepatic failure, progressive spastic quadriaparesis and electron-transport failure (11,12).

To date, more than fifty mutations in POLG have been found to contribute to PEO, Alpers and other mitochondrial diseases (3) (Human DNA Polymerase Gamma Mutation Database, http://dir-apps.niehs.nih.gov/polg/). These mutations give rise to a myriad of diseases ranging from Alpers syndrome in children, ataxia in patients in the late teens to mid-life and autosomal recessive and dominant forms of PEO, a mid- to late-onset disease. The sheer spectrum of symptoms and phenotypes resulting from genetic defects in this one gene illustrates the complexity of mitochondrial diseases and how little we know in regard to the prediction and assessment of patient management. Currently, when a new POLG mutation is identified, it is impossible to know whether an individual or their offspring are at risk for developing a mitochondrial disease such as PEO or Alpers syndrome. Thoroughly investigating the in vitro and in vivo properties of each of these mutations in human cells or patients involves considerable time, effort and expense. Therefore, we sought a simplified genetic system in which these mutations, singly or in combination with each other in the same or different genes, can be efficiently screened in vivo.

The suitability of yeast as a model for human mitochondrial studies has been reviewed (13–15). Notable differences in mtDNA structure and replication in yeasts and humans include copy number (~105–106 copies in human cells versus ~20–100 copies in yeast cells), genome size (16.6 kb in humans versus 85.8 kb in yeasts), genome structure (predominantly high molecular weight linear forms in yeast versus circular genomes in human cells) and mechanisms of mtDNA replication (13,14–19). The catalytic subunit of the human mtDNA polymerase shares high homology with the Saccharomyces cerevisiae pol γ (1), but yeast differs by the absence of an associated pol γ processivity factor, p55, which is present in animals (20). Despite these differences, the fundamental genetic and biochemical similarities shared by mitochondria in yeasts and humans make yeast an invaluable tool for the investigation of mitochondrial function in normal and disease states (14,15,21,22).

In the present study, six human POLG mutations associated with either PEO or Alpers syndrome (Fig. 1) were introduced site specifically (23) at the homologous nucleotide positions in the MIP1 gene. These mutations are therefore subject to the normal regulatory control in the nuclear compartment from the endogenous MIP1 promoter in an otherwise unaltered sequence context, as opposed to expression in the cytosol from a plasmid vector. The phenotypic expression of these yeast pol γ mutants correlated well with the extent of the biochemical defects in purified human pol γ mutant proteins and also with the severity of these mutations in PEO patients (24). Most importantly, we observed distinctive behavior in vivo for each of the mutant genes that could not be predicted from the biochemical data using the purified human mutant proteins. Thus, here we demonstrate that S. cerevisiae provides a useful in vivo system for quickly evaluating the biological consequences of identified, or suspected, PEO mutation loci. In addition, we also demonstrate that POLG mutations can lead to an increase in nuclear mutation rates.

RESULTS

Choice of yeast mip1-PEO mutations and construction of mutant strains

To evaluate the effects of the human POLG mutations associated with either PEO or Alpers syndrome, we targeted six homologous amino acid codons in the yeast MIP1 gene (Fig. 1A). These residues include four amino acids (Gly725, Arg745, Tyr757 and Ala759) in the conserved regions of the polymerase active site, one amino acid (Leu260) located in the exonuclease domain and one residue (Ile416) in the linker region. As shown in Fig. 1B, the four amino acids in the polymerase region are highly conserved whereas Leu260 in the exonuclease region is conserved in animal cells and in yeast pol γ. Ile416 is generally found as Ala in other species (e.g. Ala467 in human pol γ), with the exception of yeast. A467T is the most common disease mutation in POLG, with an allelic frequency of 0.6% in the Belgian population (25), and is frequently found in Alpers syndrome patients (11,12). We therefore included this allele in our yeast study, despite its limited conservation. The amino acid codon changes were made directly into the chromosomal MIP1 gene by delitto perfetto site-directed mutagenesis, as described in Materials and Methods.

Determination of petite frequencies

To ascertain the effect of these pol γ mutations on mitochondrial function, petite frequencies were determined in the diploid and haploid strains as described in Materials and Methods. As a control for petite production, we also examined the petite formation in haploid and diploid strains bearing an exonuclease-deficient MIP1, as previously described (26). The mip1 exo− strain contains tandem D171A and E173A mutations, introduced by delitto perfetto mutagenesis, in motif I of the exonuclease region in the MIP1 gene on the chromosome. The equivalent mutations in human pol γ totally inactivate the exonuclease function (27). In haploid cells, the mip1 exo− strain produced nearly 100% petite colonies, whereas the heterozygous MIP1/mip1 exo− diploid strain had a petite frequency of <1%, near the background level in wild-type cells (0.15% in MIP1/mip1 exo− cells versus 0.14% in MIP1/MIP1 cells).

In diploid yeast strains bearing mip1-PEO mutant alleles, the petite frequencies in MIP1/mip1-L416T and MIP1/mip1-A759S cells (1.2 ± 1.1 and 2.5 ± 1.6%, respectively) were similar to the background level (0.9 ± 0.6%) observed in the MIP1/MIP1 reference strain. Petite frequencies were slightly but significantly elevated in MIP1/mip1-L260R and MIP1/mip1-G725D cells (7.9 ± 1.7 and 7.6 ± 3.8%, respectively) and greatly elevated in MIP1/mip1-R745H and MIP1/mip1-Y757C cells (36.6 ± 7.0 and 81.8 ± 5.9%, respectively; Fig. 2A). The significant increase in petite formation in the MIP1/mip1 strains bearing the L260R, G725D, R745H and Y757C mutations indicates that these mutant polymerases were exerting a dominant-negative effect (28).

All haploid MIP1 (wild-type) colonies arising from sporulated, dissected diploid MIP1/mip1-PEO heterozygous strains gave petite frequencies (range 4.7–6.8%) that were statistically identical to the petite frequency (6.6 ± 2.9%) determined in
haploid colonies isolated from the \textit{MIP1}/\textit{MIP1} reference strain (Fig. 2B). \textit{Mip1}-I416T haploid cells also had a background petite frequency (8.9 ± 4.5%), whereas \textit{mip1}-A759S cells gave an elevated petite frequency (35.2 ± 9.1%), and the remaining mutants (L260R, G725D, R745H and Y757C) all gave >99.6% petite cells. The latter four mutants also gave a 2:2 segregation of \textit{rho}^{+} to entirely petite colonies upon replication of the YPD (yeast extract, bacto-peptone, dextrose) tetrad dissection plate colonies to YPG (yeast extract, bacto-peptone, glycerol) plates, indicating that cells arising from these \textit{mip1}-PEO tetrads spor(es) became petite during or shortly following sporulation. \textit{[Rho}^{+}\textit{]} cells are respiration-competent cells containing wild-type, fully functional mitochondria; \textit{rho}^{-} cells are respiration-defective ('petite') cells because of mutations in the mtDNA, or mutations in mitochondrial-associated nuclear genes such as \textit{MIP1}; \textit{rho}^{0} cells are respiration-defective cells that are devoid of mtDNA. \textit{Rho}^{+} and \textit{rho}^{0} cells cannot grow on non-fermentable substrate such as glycerol or ethanol.] Petite frequencies subsequently determined from spore colony-derived, single-colony isolates were found to be 3.25 ± 1.34% (\textit{MIP1}), 5.80 ± 2.16% (\textit{mip1}-I416T), 20.74 ± 5.63% (\textit{mip1}-A759S) and 100% for each of the remaining mutants.

Confocal microscopy for detection of mtDNA
As this study involved the use of \textit{mip1} mutant strains, the mitochondrial petite mutants that arose were almost certainly due to mutations occurring in mitochondrial-encoded genes, deletions of mtDNA or depletion of mtDNA (ultimately resulting in \textit{rho}^{0} cells). To differentiate between these possibilities, we examined the cells by confocal fluorescence microscopy following 4,6-diamidino-2-phenylindole hydrochloride (DAPI) staining of cytoplasm, indicative of mtDNA. Diploid cells were transformed with plasmid pDO10 that expresses the mitochondria-specific marker

![Figure 1](image1.png)

\textbf{Figure 1.} Mutational changes introduced into \textit{MIP1}. (A) Locations of targeted mutations in the \textit{S. cerevisiae MIP1} gene, with the corresponding human \textit{Pol \gamma-PEO} mutations shown in parentheses. L304R and A467T are arPEO mutations; G923D, R943H, Y955C and A957S are adPEO mutations. (B) Wild-type pol \gamma amino acid alignments from various species. PEO-associated mutant amino acid positions relevant to this study are highlighted by bold underlined text and solid triangles; amino acids conserved in at least half of the species listed are shown in uppercase. Refer to Figure 2 in Ropp and Copeland (1), for extended amino acid alignments and the locations of the exonuclease and polymerase motifs, indicated earlier.

![Figure 2](image2.png)

\textbf{Figure 2.} Petite frequencies in diploid and haploid mutant strains. (A) Petite frequencies in \textit{MIP1}/\textit{MIP1} and \textit{MIP1}/\textit{mip1}-PEO diploid strains. (B) Petite frequencies in \textit{MIP1} and \textit{mip1}-PEO haploid strains. Asterisks indicate significant differences from the wild-type controls.
Cox4p- green fluorescent protein (GFP) and sporulated and dissected to obtain haploid pDO10-bearing strains. Each of the pDO10-bearing diploid and haploid strains was treated with the DNA-specific dye DAPI and examined using confocal fluorescence microscopy. As expected, all $\rho^+$ diploid (MIP1/MIP1 and MIP1/mip1-Y757C) cells and haploid MIP1 and mip1-Y757C cells are shown, illustrating the $\rho^+$ phenotypes observed: $\rho^+$ or $\rho^-$ in MIP1/MIP1, MIP/mip1-Y757C and MIP1 cells versus $\rho^-$ in mip1-Y757C cells. The red spots present in the cytoplasm in some of these cells that does not co-localize with the GFP signal are nuclear DNA; the nuclear DNA is not always present in the images, depending on the confocal plane examined. DIC, differential imaging contrast.

### Figure 3
Confocal microscopy images of S. cerevisiae cells containing mitochondria labeled with Cox4p-GFP and DNA stained with DAPI. Representative images from diploid MIP1/MIP1 and MIP1/mip1-Y757C cells and haploid MIP1 and mip1-Y757C cells are shown, illustrating the $\rho^+$ phenotypes observed: $\rho^+$ or $\rho^-$ in MIP1/MIP1, MIP/mip1-Y757C and MIP1 cells versus $\rho^-$ in mip1-Y757C cells. The red spots present in the cytoplasm in some of these cells that does not co-localize with the GFP signal are nuclear DNA; the nuclear DNA is not always present in the images, depending on the confocal plane examined. DIC, differential imaging contrast.

Quantitative PCR analysis of mtDNA copy number and mtDNA damage

In order to test the hypothesis that a defective polymerase leads to mutations affecting oxidative phosphorylation (OXPHOS), leading to the generation of reactive oxygen species (ROS) and subsequent mtDNA damage, quantitative PCR (QPCR) was used to examine the integrity of the mitochondrial and nuclear genomes. This assay is well established and has been successfully used to identify endogenous lesions in nuclear and mtDNAs of several organisms including yeast (29–31). Although mtDNA in S. cerevisiae consists of a collection of linear fragments in a state of constant recombination (16,17,19), we observed only a single band of the
expected fragment length from the (non-rho\(^0\)) wild-type and mutant strains. This result indicates that with respect to the QPCR analyses, the template mtDNA was essentially of uniform structure, rather than a collection of recombinogenic intermediates of indiscriminate length.

Quantitative amplification of short targets was used to determine the copy number of mitochondrial genomes, whereas quantitative amplification of long targets was used to determine the integrity of nuclear and mtDNAs in each of the diploid and haploid strains (Fig. 4). QPCR analysis of the small (298 bp) mitochondrial fragment (31) resulted in no detectable PCR products in the haploid L260R, G725D, R745H and Y757C \(mip1\) strains (confirming the absence of mtDNA in these cells) and no loss of mtDNA in the \(mip1\)-I416T, \(mip1\)-A759S or \(MIP1\) haploid strains (data not shown). In the diploid strains, QPCR analysis of the 298 bp fragment indicated that the amount of mtDNA was significantly reduced in the \(MIP1/mip1\)-Y757C (\(~50\%\) of control, \(P = 0.04\)) and \(MIP1/mip1\)-R745H (\(~20\%\) lower than control, \(P = 10^{-6}\)) strains, relative to the \(MIP1/MIP1\) reference strain (Fig. 4B). The remaining diploid strains had normal amounts of mtDNA (Fig. 4B).

The relative amplification of the 6.95 kb mtDNA fragment in the mutant strains compared with the amplification of the reference wild-type controls gives information regarding the presence of DNA damage that blocks the PCR polymerase (31). We found (Fig. 4A) that the \(mip1\)-I416T haploid cells had \(~0.7\) lesions per 10 kb per strand, corresponding to \(~12\) additional lesions per mitochondrial genome (85.8 kb) when compared with \(MIP1\) wild-type cells (\(P = 0.008\)). Although the \(mip1\)-A759S cells appeared to have slightly less mtDNA damage relative to \(MIP1\) cells (Fig. 4A), this difference was not significant (\(P = 0.64\)).

QPCR analysis of nuclear DNA damage

Upon analysis of the large chromosomal DNA fragment (9.35 kb) in the haploid strains, each of the \(rho^0\) strains had surprisingly better amplification and thus less DNA damage than the basal level present in the \(MIP1\) reference strain (Fig. 4D). In contrast, the \(mip1\)-I416T strain had significantly elevated levels of nuclear DNA damage, 0.39 lesions per 10 kb per strand (\(P = 0.0012\)). No DNA damage was evident in the nuclear DNA of any of the heterozygous \(MIP1/mip1\)-PEO diploid strains when compared with the \(MIP1/MIP1\) reference strain, with the exception that the DNA from the \(MIP1/mip1\)-Y757C mutant amplified slightly better, indicating less DNA damage than the basal level present in the wild-type control (data not shown).
Determination of nuclear mutation rates

Nuclear mutation rates in the haploid strains were determined by measuring the reversion of the lys2-InsE-A14 frameshift reporter and forward mutations in the CAN1 gene (Table 1). Can1 cells have a defective plasma membrane-bound arginine permease and are thus resistant to the toxic arginine analog canavanine (32). As the can1 assay requires Arg⁺ strains, we first confirmed that like the parent strain, each of the mutant haploid strains was Arg⁺, regardless of mitochondrial respiratory status.

There were no significant differences in the Lys⁺ reversion frequency in mip1-PEO versus MIP1 cells, with the exception that Lys⁺ revertants occurred slightly more frequently in mip1-I416T cells (2.21 ± 0.075 × 10⁻⁷) than in the remaining strains (range 0.76–2.05 × 10⁻⁷). The elevated frequency of mip1-I416T Lys⁺ revertants probably reflects the increased level of nuclear DNA damage observed in this strain by QPCR (Fig. 4A).

In accordance with previous reports describing increased mutational rates in rho⁻ cells (33,34), we found that the can1 forward mutation rate was elevated ~2.3–3.3-fold in each of the rho⁻ mip1 strains relative to the MIP1 reference strain, but not in the rho⁻ strains (Table 1). The larger size of the CAN1 gene, encompassing a 1773 bp open-reading frame, permits the more sensitive detection of mutational events than the measurement of reversions targeted to a run of 14 adenosines in lys2-InsE-A14. Interestingly, the strains in which the can1 mutation rate was elevated (L260R, G725D, R745H and Y757C mip1 cells) were also those which had decreased amounts of nuclear DNA damage, suggesting that DNA lesions had been fixed mutationally.

Determination of cellular viability

To judge the overall health of the various strains, the viability of each of the haploid and diploid strains was assessed over time (Fig. 5). There was no significant loss of viability over a 4 week period, with the exception that after storage at 4°C for approximately 5 days, the rho⁻ haploid L260R, G725D, R745H and Y757C mip1 strains began to die at a rate of ~2.5–2.8%/day. Reduced viability in stationary phase rho⁻ yeast cells versus non-rho⁻ cells has been reported (35) and could reflect reduction and depletion of intracellular ATP levels (36). Mitochondrial oxidative energy failure can also contribute to increased senescence (37).

DISCUSSION

We have been investigating biochemical and molecular defects associated with PEO, including the characterization of human PEO mutant pol γ proteins in vitro (24,38,39). To complement and to understand the consequences of these mutations in vivo, here we introduced analogous human pol γ PEO mutations into the yeast pol γ gene MIP1 (Fig. 1). Four of the mutations chosen for study (G725D, R745H, Y757C and A759S) are homologs of adPEO mutations previously characterized in biochemical assays by our group (24) and are located in the polymerase domain of DNA pol γ. Also included in the current study were two mutations (L260R and I416T) corresponding to human arPEO mutations that are located in the exonuclease and linker domains of the polymerase, respectively. In this study, we have addressed the consequences of these mip1 mutations on cellular and mitochondrial function in vivo in S. cerevisiae. For each of the mutations, heterozygous (MIP1/mip1) diploid and mip1 haploid yeast strains were constructed. We found a diverse range of phenotypes in mip1-PEO mutant cells, including increases in DNA damage and mutation rates, depletion of mtDNA and reduced cellular viability in mtDNA-depleted cells (Table 2; to facilitate cross-comparisons, most of the data are summarized in a single table).

We previously demonstrated that the Y955C and R943H adPEO mutant polymerases, containing mutations in the polymerase active site, are the most biochemically compromised having very low processivity and low catalytic activity, whereas the G923D and A957S adPEO mutants show only a moderate reduction in the polymerase activity (24). These observations are consistent with the severity of the disease observed in patients, with the Y955C and R943H mutations being associated with the most severe adPEO clinical phenotypes and the G923D and A957S mutations resulting in milder symptoms. In yeast, the homologous Y757C and R745H mip1 mutations also resulted in the highest petite frequencies in MIP1/mip1 diploid cells, followed by the G725D and the L260R (exonuclease domain) mutants (Fig. 2A). The elevated petite frequencies in these MIP1/mip1 cells indicate that the L260R, G725D, R745H and Y757C mutant polymerases act in a dominant-negative manner (28). Although petite frequencies were not significantly elevated in MIP1/mip1 cells bearing the polymerase-domain A759S mutation, petite frequencies were dramatically elevated in mip1-A759S haploid cells.

Upon isolation from the rho⁺ diploid MIP1/mip1 parent strains, the haploid L260R, G725D, R745H and Y757C cells were virtually 100% petite (Fig. 2B). The rapid rate at which these haploid cells lost functional mitochondria during the period of colony formation from growth of the microdissected haploid spores, less than ~20 generations, indicated that the polymerase activity of these mutant pol γ molecules was severely compromised. The suspicion that these cells had become rho⁻ was confirmed by the absence of cytoplasmic DAPI staining by confocal fluorescence microscopy (Fig. 3) and by QPCR (Fig. 4A). Overall, the yeast petite frequency data agreed remarkably well with both the biochemical activity of the purified human pol

<table>
<thead>
<tr>
<th>Table 1. Nuclear mutation rates of haploid mip1 mutants</th>
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<td>MIP1 mutant allele</td>
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</tr>
<tr>
<td>can1</td>
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<td>Wild-type</td>
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γ mutant proteins and the severity of the PEO clinical phenotypes (Table 2) (24).

Not unexpectedly, the quantity of mtDNA was reduced in MIP1/mip1 cells bearing the potent Y757C and R745H dominant-negative mutant alleles (Fig. 4B) and was absent in each of the entirely petite, rho0 L260R, G725D, R745H and Y757C haploid strains (Fig. 4A). The Y757C heterozygous diploid strain also had elevated amounts of damaged mtDNA (Fig. 4C). This is consistent with the Mip1p-Y757C enzyme acting as a mutator DNA polymerase when replicating mtDNA. The analogous human Y955C pol γ enzyme has a 45-fold decrease and a 2-fold decrease in the misinsertion fidelity in exonuclease-deficient and -proficient backgrounds, respectively (38). Additionally, large mtDNA deletions are detected as the consequence of replication stalling and altered fidelity in PEO patients heterozygous for this mutation. The other two adPEO mutations in humans did not have a significant alteration in replication misinsertion fidelity, similar to what we observed with the yeast G725D and A759S mutations in MIP1.

The L304R mutation in the human pol γ is located in the exonuclease region, close to the conserved motif II that includes the active site for exonucleolytic function. This

<table>
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<th>Table 2. Summary of mip1-PEO results from S. cerevisiae</th>
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<td>Mutant allele: yeast (human)</td>
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<tr>
<td>Wild-type</td>
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<td>L260R</td>
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<td>I416T</td>
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<td>G725D</td>
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<td>Y757C</td>
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<td>A759S</td>
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The downward and upward arrows depict the degree change relative to the wild-type level.

Clinical phenotype in heterozygous PEO patients. From Graziewicz et al. (24). Wild-type PEO is autosomal dominant PEO.

Cell viability in stationary-phase cells after 28 days at 4°C, determined by exclusion of Trypan blue dye. (QPCR), mtDNA.
mutation in humans causes arPEO and was originally identified as a compound heterozygous mutation in trans with A467T. The Leu304 amino acid is conserved in yeast as the Leu260 residue. The mild dominant-negative character of the mip1-L260R mutant in heterozygous diploid cells, as well as the complete loss of mitochondrial respiratory activity in L260R haploid cells, was unexpected, given that this mutation was originally described as an autosomal recessive mutation (4). The loss of mitochondrial respiratory activity in the haploid L260R yeast mutant suggests either a complete loss of polymerase function, possibly due to misfolding, or a strong mutator due to inactivation of the exounuclease activity. Although no biochemical data are available for this mutant enzyme, the location of this mutation would be predicted to affect either exounucleolytic proofreading or general folding of the protein. A mutation affecting overall folding of the protein would most likely be recessive, whereas an exounucleolytic deficient pol γ in a MIP1/mip1 diploid strain would be complemented by the wild-type copy to proofread errors, similar to the findings in the heterozygous exounuclease-deficient pol γ mice (40). Consistent with this idea, the mip1 exo− mutant described in Results (Determination of Petite Frequencies) gives >99% petite colonies in haploid cells but <1% petites in diploid cells, indicative of a recessive mutation within the exounuclease domain. Thus, a more plausible explanation in yeast for the relative severity of the L260R mutation, compared with the L304R mutation in humans, is that this mutation not only inactivates the exounuclease active site but also does not allow the enzyme to readily dissociate from the mispair termini, preventing the wild-type pol γ protein from gaining access to the replication fork.

The A467T mutation is the most common disease mutation in the human POLG gene. This mutation is found as an autosomal recessive mutation in PEO, Alpers, ataxia and neuro-pathy diseases, suggesting a partial loss of function or protein interaction by this mutation. In yeast, the analogous I416T mutant displayed only a mild phenotype in haploid cells and wild-type-like phenotype in heterozygous diploid cells (Table 2), which is consistent with A467T acting as an arPEO mutation. The observation of elevated mtDNA damage by the I416T mutant was intriguing. In vitro biochemical analysis of the human A467T mutation suggests that the enzyme has an altered conformation that retains only 4% of polymerase activity and has lost the ability to interact with the p55 accessory subunit (41). The loss of p55 binding by this mutation in humans indicates that this part of the pol γ linker region is responsible for accessory subunit interaction and is consistent with deletion and mutagenesis studies in catalytic subunit of the Drosophila pol γ (42,43). In animal cells, the pol γ accessory subunit is a DNA-binding factor and is required for highly processive mtDNA synthesis (44). The yeast pol γ lacks an associated subunit and must have evolved to carry out processive DNA synthesis without an associated accessory factor (20). This is consistent with a shorter linker region in the Mip1p protein when compared with the pol γ in animal cells (1). Thus, the analogous Ile416 residue in Mip1p must function in another capacity besides accessory subunit interaction; however, this does not preclude effects on folding or interaction with other replication factors such as the DNA helicase, single-stranded DNA-binding protein and DNA repair proteins. In T7 DNA polymerase, the closest member in the family A class of DNA polymerases to the pol γ sequences, this part of the linker region is responsible not only for thioredoxin binding but also for binding the helicase–primase and single-stranded DNA-binding proteins (45). An altered conformation in the I416T mutant, like the A467T human enzyme, may prevent the polymerase from assembling with repair proteins required for base excision repair and could affect the removal of base damage caused by endogenous sources of ROS. Mitochondria contain an efficient base excision repair pathway for removal of this damage, which relies on pol γ for the DNA re-synthesis step and for removal of the 2-deoxyribose-5-phosphate moiety from the 5′ end of the apurinic/apyrimidinic endonuclease-cleaved DNA (46). A loss of interaction of mtDNA repair proteins would be consistent with the increase in mtDNA damage detected by the QPCR analysis in the I416T haploid strain. The observation that this level of mtDNA damage is restored to wild-type levels in MIP1/mip1 diploid cells again emphasizes the recessive nature of this mutation.

QPCR provides a sensitive measure of endogenous-free radical damage, with a detection limit of the order of one lesion per 105 nucleotides from as little as 5 ng of total genomic DNA (31). This assay has proven useful in quantifying the level of nuclear DNA damage in yeast from endogenous ROS generated from mitochondria (30). Increased mitochondrial ROS production may lead to increases in nuclear DNA damage and mutations (21,26). Interestingly, when compared with the rho− MIP1 cells, the rho− mip1-I416T haploid cells had significantly elevated levels of nuclear DNA damage and the rho0 L260R, G725D, R745H and Y757C haploid cells had decreased levels of nuclear DNA damage. Mitochondria are the major endogenous source of ROS that can produce base damage. The persistence of mtDNA damage in rho− cells can lead to extensive mtDNA damage (47,48), ultimately culminating in a mitochondrial catastrophe with complete loss of mtDNA and OXPHOS capacity (49,50). Hydrogen peroxide is a relatively long-lived, neutral and thereby freely diffusible membrane-permanent oxidant that could account for the increased nuclear DNA damage observed in the mip1-I416T cells (Fig. 4D), as well as the dramatically increased mtDNA damage in mip1-I416T haploid cells (Fig. 4A). This conclusion is in agreement with our previous work using yeast as a system to understand the biology of Friedreich’s ataxia, which is another human mitochondrial disorder (30). It is probable that the nuclear DNA damage arose during the interval in which the cells became rho−, because of compensatory increases in OXPHOS activity and ROS production (49). Subsequently, as the cells progressed to the rho0 state, cessation of OXPHOS activity and lowered production of ROS, combined with fixation of DNA damage, could account for the decreases in DNA damage and elevated mutational rates observed in the nuclei of rho0 cells. For example, error-prone translesion DNA synthesis of nuclear DNA has been described in rho0 cells to account for an increase in nuclear mutations (34).

The decrease in cellular viability observed in aging rho0 cells was striking. Tranciková et al. (35) also observed decreased
viability in stationary phase $\rho^0$ yeast cells, but found no relationship between physiological production of ROS and viability loss. These authors also demonstrated that depletion of intramitochondrial ATP by simultaneous inhibition of respiration and ADP/ATP transport induced viability loss comparable to that induced by chronological aging and that ectopic expression of an anti-apoptotic protein extended the survival of $\rho^0$ cells. Collectively, these data suggest that the reduced cellular viability observed in our $\rho^0$ cells was not due to ROS, but due to a reduction or depletion of intracellular ATP levels (36) or some unknown reason.

Numerous laboratories have demonstrated profound effects on the mitochondrion due to disruption of nuclear-encoded genes. For example, we previously identified the $S.\ ceriseise$ gene $\text{POS5}$ as a mitochondrial mutator and determined that it encodes the mitochondrial NADH kinase (26). Additionally, Karihikeyan et al. (21,30,51) have shown that a reduction in the amount of frataxin, a protein that regulates mitochondrial iron levels, through disruption of the YFH1 gene results in an accumulation of mitochondrial damage, as well as an increase in nuclear DNA damage. As well Fontanesi et al. (22) found that mutations in the $S.\ ceriseise$ gene $\text{AAC2}$, equivalent to human adPEO-associated $\text{ANT1}$ gene, lead to defective OXPHOS and affect mtDNA stability. In the current study, we investigated the phenotypic and genetic consequences of mutations in a nuclear gene ($\text{MIP1}$) encoding a mitochondrial protein (mtDNA polymerase). As mentioned in Introduction, various mutations located throughout this one gene ($\text{POLG}$) in humans result in a diverse spectrum of symptoms and phenotypes in PEO, ataxia/neuropathy and Alpers syndrome. In order to better understand the relative severity of individual mutations, it is highly beneficial to have an in vivo system in which mutations, singly and tandemly, can be readily examined. We believe that the yeast model will therefore be a useful tool, in addition to in vitro studies using purified human pol $\gamma$ mutant proteins.

In summary, we have identified a diverse range of phenotypes arising from PEO-associated amino acid substitutions introduced in the yeast pol $\gamma$, which vary from mutation to mutation as summarized in Table 2. These various effects included increases in DNA damage in the mitochondrion and in the nucleus, increases in nuclear mutation rates and mitochondrial petite frequencies, decreased viability in $\rho^0$ cells and segregation of $\rho^0$ cells at or shortly following meiosis. Increases in nuclear DNA damage and mutation rates resulting from mitochondrial dysfunction and resulting oxidative stress are likely to have deleterious consequences in human patients, given the large number of genes that are potential targets. Additionally, mutations affecting mitochondrial function could affect retrograde regulation and thus regulation of nuclear genes, as well as the segregation of mtDNA genomes following mitosis and meiosis (heteroplasmy; homoplasmyn), and may result in reduced cell viability because of necrosis or apoptosis. Overall, we conclude that studies of mitochondrial-associated genetic mutations characterized in vivo in yeast can generate information and hypotheses that can be useful for understanding the molecular defects in humans with disease alleles and permit quick assessment of potential health risks in humans with uncharacterized pol $\gamma$ mutations.

**MATERIALS AND METHODS**

**Yeast strains and media**

Standard media and techniques were used for growth, transformation and genetic manipulation of $S.\ ceriseise$ (52–54). $S.\ ceriseise$ strains E134 (relevant genotype: $\text{MATa lys2-InsE-A14}$) and YH747 (isogenic to E134 but $\text{MATa}$) were kindly provided by Dr Dmitry Gordenin (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA). The lys2-InsE-A14 allele contains a run of 14 adenosines that reverts primarily via $\text{−}1$ frameshift mutations (55).

**Construction of yeast strains with PEO mutations in $\text{MIP1}$**

$S.\ ceriseise$ strain YH747 was used for construction of the $\text{mip1-PEO}$ homologs (Fig. 1); for simplicity, we also refer to the yeast $\text{mip1}$ mutations as ‘PEO’ mutations. Codon changes were introduced directly into the genomic $\text{MIP1}$ gene by delitto perfetto site-directed mutagenesis (23). Delitto perfetto is a two-step, oligonucleotide-based method in which a gene cassette containing yeast selectable markers is temporarily inserted at a pre-selected chromosomal site. Following delitto perfetto mutagenesis, the yeast strain contains only the desired, precise nucleotide changes. This method has the major advantage that the mutant $\text{mip1}$ gene is not expressed from a plasmid or re-introduced elsewhere in the genome and is under the normal endogenous regulatory control. All mutational changes were verified by DNA sequence analysis.

Delitto perfetto mutagenesis resulted in the formation of $\rho^0$ cells, because of the transient disruption of the $\text{MIP1}$ gene. Fully functional mitochondria were re-introduced into the cells by mating them with the $\rho^0$ E134 strain. The resulting $\rho^+$ $\text{MIP1/MIP1}$ and $\text{MIP1/mip1-PEO}$ diploid cells were then sporulated and tetrads were microdissected onto YPD agar plates following standard methods (56), generating the haploid $\text{mip1-PEO}$ mutant strains (Fig. 1). To identify bone fide tetratypes arising from a single ascus, tetrad colonies were replica plated to various test media to (a) verify 2:2 segregation of all selectable markers and mating types; (b) verify 3:1 segregation of red to white colony formation; (c) identify $\text{mip1-PEO}$ mutant cells that gave significantly elevated petite frequencies. In some cases ($\text{mip1-1416T}$ and $\text{mip1-A759S}$ haploid cells), $\text{mip1}$ mutant tetrad colonies were identified by DNA sequence analysis because of the low petite frequencies observed in these mutant strains. Haploid, tetrad-derived mutants identified by elevated petite frequencies (Fig. 2B) were resequenced to confirm the mutational changes in $\text{mip1}$, and all mutant strains were resequenced here and at additional times during the study, to confirm strain identities. At each step in the study, all nutritional markers, haploid or diploid status and mating types were verified. Colony-mating types, which did not correlate with any of the outcomes, were ignored in the analyses. Duplicate sets of each diploid and haploid strain were independently constructed and used for each experiment, to minimize potential effects due to randomly occurring, unidentified secondary mutations. Finally, the haploid $\text{MIP1}$ ‘reference’ strain that was used as a control for all experiments.
involved haploid cells was isolated from the MIP1/mip1-I416T strain; this wild-type strain therefore shared a common ‘pedigree,’ with respect to genetic manipulation, as the diploid-derived mip1-PEO mutant strains.

Petite frequencies

Petite frequencies are defined as the ratio of the number of respiration-deficient cells divided by the total number of cells present in a population. Respiration-deficient rho− or rho0 cells contain defective mitochondria, generally produce smaller (petite) colonies and cannot grow on glycerol-based media, whereas both respiration-competent rho+ cells and petite cells grow on glucose-based media. Therefore, petite frequencies were determined by spreading cells on YPD (dextrose) plates at a dilution that gave single colonies, then replica plating the colonies that arose on these plates to YPD and YPG (glycerol) agar plates, to determine the petite frequencies.

MIP1/MIP1 and MIP1/mip1-PEO diploid strains were maintained as patches on YPD agar plates. Under these ‘steady-state’ conditions, the petite frequency inherent in each strain is expected to be constant. Cells scraped from these patches were diluted in sterile water and spread onto YPD agar plates, giving an average of ~200 well-separated single colonies per plate after 2 days of growth at 30°C. These colonies were then replica plated to YPD and YPG plates and incubated overnight at 30°C, at which time the numbers of colonies were counted and the petite frequencies were calculated. The petite frequencies in the diploid cells were determined in triplicate using single-colony isolates from duplicate isolates of two independently constructed diploid strains, giving a total of 12 determinations for each of the MIP1/MIP1 and MIP1/mip1-PEO diploid strains listed in Table 2.

Petite frequencies in MIP1 and mip1-PEO haploid strains were determined initially using newly derived haploid colonies arising from freshly dissected diploid cells grown on the YPD tetrad dissection plates, giving the first possible determination of the petite frequencies in these haploid cells. These haploid cells were diluted in water and spread onto YPD plates, giving an average of ~286 well-separated single colonies per plate after 2 days of growth at 30°C. These colonies were then replica plated to YPD and YPG plates and incubated overnight at 30°C, at which time the numbers of colonies were counted and the petite frequencies were calculated. The petite frequencies in these haploid cells were determined using the colonies arising from four tetrads each from two independently constructed diploid strains, giving a total of eight determinations for each of the MIP1 and mip1-PEO haploid strains listed in Table 2. Finally, as for the diploid strains, haploid petite frequencies were also determined from single-colony isolates. Cells from the colonies on the YPD tetrad dissection plates were diluted and spread onto YPD, giving single colonies; individual colonies were then picked, diluted in water and spread onto YPD plates, again giving single colonies. These YPD plates were then replica plated to YPD and YPG plates for the determination of petite frequencies. These analyses were done in triplicate from each of the duplicate independently constructed and dissected strains, giving six determinations per haploid strain.

Cellular viability

Cellular viability was determined by growing each strain to stationary phase in YPD medium and allowing the cells to age chronologically (57) at 4°C for 28 days. Cellular viability was periodically assessed by the ability of living yeast cells to exclude a 0.4% Trypan blue solution (Invitrogen Corporation, Carlsbad, CA, USA). On average, ~300 cells were counted for each of the duplicate strains, per viability determination.

Nuclear mutation rates

To assess chromosomal mutation frequencies, the reversion frequency of the frameshift reporter lys2-InsE-A14 (55) and the forward mutational rate in the wild-type CAN1 gene (32) were determined in the haploid MIP1 and mip1-PEO strains. Briefly, for each assay, single-colony isolates from the duplicate independently constructed strains (five colonies per strain; 10 colonies total per measurement) were scraped from YPD plates and suspended in water. Dilutions of these cells were plated on YPD plates to determine total cell counts, whereas the remaining cells were plated on either SD-Lys (synthetic dextrose, minus lysine) or the lys2-InsE-A14 assay or SD-Canavamine (synthetic dextrose, minus arginine, containing 0.006% canavanine sulfate) agar plates. The numbers of Lys+ and CanR colonies were recorded after 5 days of growth at 30°C. Mutation rates were calculated using median values according to Lea and Coulson (58), using Eq. (37) \[ r = \frac{\ln(n)}{m} - 1.24, \]

where \( r \) is the median value, and \( r = m/n \) values calculated from Microsoft Excel-derived polynomial best-fit plots prepared from subranges of the data provided in Table 3 of their paper. Mutation rates (mutants/generation) were then calculated using \( n/m \), where \( n \) is the average number of cells per colony, determined from the YPD plates.

Confocal microscopy

Confocal microscopy images were collected using a Zeiss LSM 510 laser-scanning microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). To visualize mitochondria, diploid strains were transformed with plasmid pDO10 (kindly provided by Dr Janet Shaw, University of Utah), which expresses a protein containing the mitochondrial targeting sequence of Cox4p fused in-frame to the N-terminus of GFP (59). Haploid pDO10-expressing cells were obtained by sporulation and dissection of tetrads. Mitochondrial and nuclear DNAs were visualized by staining live cells with 1 μg/ml of DAPI (Invitrogen Corporation) at 30°C for 30 min. Vital staining with DAPI emphasizes the staining of mtDNA relative to nuclear DNA (60). DAPI-stained cells were mounted directly in Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA, USA) and examined by confocal microscopy. To better visualize the overlap of mitochondria and mtDNA
in the confocal merged images, the color of the DAPI channel in the images was set to red.

**QPCR for mitochondrial copy number and genome integrity**

QPCR was used to detect mitochondrial copy number and the integrity of both the nuclear and the mitochondrial genomes of wild-type and mip1-PEO mutant yeast. This gene-specific assay has been successfully used to identify lesions in nuclear and mtDNAs of several organisms treated with different DNA damaging agents (29–31,51,61). QPCR is based on the premise that DNA lesions present in the template DNA block the progression of the polymerase such that the damaged template will not participate in the PCR reaction. Thus, the higher the levels of lesions present in the DNA, the less the final PCR product. Amplification of samples of interest is then compared with the reference control and the relative amplification is calculated. These values are next used to estimate the average number of lesions per 10 kb of the genome, using a Poisson distribution (29,31), where the lesion frequency per strand of the target product is given as $-\ln(A_M/A_{WT})$, where $A_M$ is the amplification of the mutant and $A_{WT}$ is the amplification of the wild-type control. Amplification of small mtDNA fragments (100–300 bp) is also conducted and is used to normalize the lesion frequency (obtained with amplification of the large fragment) to the mtDNA copy number; for details, see Santos et al. (31). A ‘negative’ number of lesions is obtained when amplification of the sample of interest is higher than that of matched control. This phenomenon can sometimes occur by means of induced repair activity in the treated sample (48,62).

QPCR was conducted using primers that amplified small (298 bp) and large (6949 bp) fragments of the mitochondrial-encoded COX1 gene, as well as a 9347 bp region of nuclear DNA encompassing genes PFK2 and HFA1 on chromosome XIII (31). Template DNA was isolated from cultures grown to stationary phase in YPD liquid medium and was quantitated as previously described (31). Results presented here are the mean of two sets of PCR for each target gene of two independently derived strains. Student’s unpaired t-test was performed to evaluate statistical significance. In all cases, the QPCR data values are relative to the mean values of amplification determined in the wild-type (MIP1 or MIP1/ MIP1) reference strains.

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**Conflict of Interest statement.** None declared.


