Overexpression of DNA Polymerase Zeta Reduces the Mitochondrial Mutability Caused by Pathological Mutations in DNA Polymerase Gamma in Yeast

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Abstract
In yeast, DNA polymerase zeta (Rev3 and Rev7) and Rev1, involved in the error-prone translesion synthesis during replication of nuclear DNA, localize also in mitochondria. We show that overexpression of Rev3 reduced the mtDNA extended mutability caused by a subclass of pathological mutations in Mip1, the yeast mitochondrial DNA polymerase orthologous to human Pol gamma. This beneficial effect was synergistic with the effect achieved by increasing the dNTPs pools. Since overexpression of Rev3 is detrimental for nuclear DNA mutability, we constructed a mutant Rev3 isofrm unable to migrate into the nucleus: its overexpression reduced mtDNA mutability without increasing the nuclear one.

Introduction
In yeast, DNA polymerase zeta (Pol zeta) and Rev1 are involved in the error-prone translesion synthesis (TLS) during replication of nuclear DNA: mutations in any of these polymerases lead to a decrease of mutagenesis in nuclear genome either in normal conditions or in conditions known to increase DNA damage such as UV treatment [1]. The yeast Pol zeta is composed by a catalytic subunit encoded by REV3 and an accessory subunit encoded by REV7. Biochemically, Pol zeta is a poorly processive enzyme [2], lacks of a 3'-5' exonucleolytic proofreading activity [3] and is able to extend terminally mismatched primers [4–6]. From these properties derives its propensity to generate mutations. On the contrary, Pol zeta is very inefficient in inserting nucleotides opposite lesions in vitro, suggesting that a distinct polymerase should act in this first step and that Pol zeta can efficiently extend from primer-terminal base pairs containing mismatches or lesions [6].

Rev1 encodes for a deoxyuridyl transferase that preferentially incorporates G opposite to an abasic site [7]. Also, Rev1 interacts in vitro with Pol zeta through a Rev3-Rev1 binding and this interaction strongly stimulates the activity of Pol zeta and, probably, contributes to its targeting to the replication fork [8].

Recently, it was found that Pol zeta and Rev1 localize in mitochondria and that they participate in the mitochondrial DNA mutagenesis, since disruption of REV3, REV7 or REV1 resulted in a strongly decreased frequency of −1 frameshift mutations in mtDNA [9]. On the contrary, it was shown that deletion of REV3, REV7 or REV1 resulted in increased frequencies of both spontaneous and UV-induced mitochondrial erythromycin resistance, due to base substitutions [10]. On the basis of their results, these authors speculated that, in absence of the TLS by Rev1 and/or Pol zeta, the mitochondrial DNA polymerase gamma (Pol gamma), the mitochondrial replicase, could create mutations in efforts to bypass lesions that block the replication fork [10]. As a consequence, Pol zeta and Rev1, which in the nucleus are responsible with other DNA polymerases for the error-prone bypass of DNA lesions, in the mitochondrial compartment function in a less mutagenic pathway [10].

Pol gamma is a protein conserved in fungi and animals. Structurally, human Pol gamma is a heterotrimer composed by one catalytic subunit, encoded by POLG, and two accessory subunits, encoded by POLG2 [11]. The catalytic subunit contains an N-terminal 3'-5' exonuclease domain (residues 170–440), a C-terminal polymerase domain (residues 440–475 and 785–1239) and a spacer region encompassing residues 475–785 [12]. To date, more than 150 pathological mutations in POLG have been identified in severe mitochondrial disorders (http://tools.niehs.nih.gov/polg/) and, among them, a few mutations have been recently described in stavudine induced toxicity [13,14], in valproate induced hepatotoxicity [15,16] and in breast tumorigenesis [17]. This places POLG as a major locus for mitochondrial diseases and disorders.

In yeast the DNA polymerase gamma, encoded by MIPI gene, is composed by a sole catalytic subunit [18]. Thanks to the similarity between human Pol gamma and Mip1 (approximately 43%), yeast has been used to validate the role of human putative pathological mutations, to understand the biochemical consequences associated to these mutations, to study the pharmacogenetics of drugs such as valproate and stavudine, and to find mechanisms able to rescue the detrimental effects of Mip1 mutations [16,19–28]. Since a therapy has not been yet developed for mitochondrial diseases caused by POLG mutations, a major goal of the research involving yeast/animal models is to find new...
S. cerevisiae retained [29]. These large deletions make mtDNA irreversibly high frequency (approximately 10^3). In addition, deletions do not transitions in the mitochondrial gene encoding the 21 S rRNA, in to erythromycin is acquired through specific transversions or mitochondrial but not cytoplasmic translation. In fact, resistance rho−37.

MIP1 caused by mutations in another gene which is functionally among which a study showing that overexpression of Rev3 led to the discovery that increased levels of dNTP pools reduced the damages caused by mutations in POLG, we analyzed this finding has had impact also in studies on human DNA polymerase gamma as well as by the activity of some proteins were overexpressed with this aim, function in a biological process. Several researches are reported together with DNA polymerase gamma, are present in mitochondria, and produce colony of small size, called petite, rho−2 cells.

Extended mutability is indeed a series of large deletions (generally >70%) that arise spontaneously, and partially randomly, in the mtDNA, without involvement of point mutations. Mutations in a large group of nuclear genes, encoding for proteins directly or indirectly involved in mtDNA replication, recombination, stability and maintenance (reviewed in [30]) produce increase of petite mutability. mtDNA point mutations can be also easily measured as frequency of spontaneous mutants resistant to erythromycin (EryR mutants), an antibiotic that inhibits mitochondrial but not cytoplasmic translation. In fact, resistance to erythromycin is acquired through specific transversions or transitions in the mitochondrial gene encoding the 21 S rRNA, in particular at 1950, 1951, 1952 and 3993 positions [31–34]. EryR mutants arise spontaneously at a frequency of 10^-7–10^-8. EryR mutability is influenced by replication fidelity of DNA polymerase gamma as well as by the activity of some enzymes involved in mtDNA repair and/or recombination [34–37].

In an attempt to investigate about genetic conditions that might reduce the damages caused by mutations in POLG, we analyzed the effect of deletion or overexpression of TLS polymerases that, together with DNA polymerase gamma, are present in mitochondria, Pol zeta [Rev3-Rev7] and Rev1. Genetic tests based on gene overexpression are widely used in yeast to assess a role of a gene function in a biological process. Several researches are reported where one or more proteins were overexpressed with this aim, among which a study showing that overexpression of Rev3 led to increasing UV-induced mutagenesis [38]. In addition, gene overexpression is commonly used to rescue the phenotypic defects caused by mutations in another gene which is functionally associated. For example, in the case of MIP1, overexpression of RNR1, encoding the large subunit of the ribonucleotide reductase, lead to the discovery that increased levels of dNTP pools reduced the extended mutability caused by mutations in Mip1 [19,26,39]. This finding has had impact also in studies on human DNA polymerase gamma pathological mutations [26], where, starting from analysis in yeast, it has been shown that human DNA polymerase gamma harboring the pathological mutation H932Y has a ~200-fold reduced affinity to the incoming dNTPs. Here we found that increased expression of Pol zeta resulted in a significant reduction of extended mutability caused by mutations mapping in different domains of MIP1 and that overexpression of both Pol zeta and Rev1 led to a reduction in mtDNA point mutability.

**Materials and Methods**

**Yeast strains and media**

The yeast strains used in this work are DWM-5A (Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 mx4::HphM, DWM-4C (Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 mx4::HphM) [19], W303-1A CAN (Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 [40], BY4742 rev1Δ (Matα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ1, rev1Δ:KanR), BY4742 rev7Δ (Matα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ1 rev7::KanR) (Euroscarf collection), DWM-5A rev3Δ (Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 mx4::HphR), DWM-9A (Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 mx4::HphR). DWM-9A rev3Δ (Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 mx4::HphR rev7::KanR) [41], DWM-9A rev7Δ (Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 mx4::HphR rev7::KanR), DWM-9A rev3Δ (Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 mx4::HphR rev3::KanR).

Synthetic defined medium (SD) contained 6.9 g/l yeast nitrogen base without amino acids (Formedium), synthetic complete medium (SC) was SD supplemented with 1 g/l drop out mix [42]. YP medium contained 1% yeast extract (Formedium) and 2% peptone (Formedium), and YPA medium was SD supplemented with 40 mg/ml adenine base (Formedium). Carbon sources were added as indicated in the text. YPAG-Ery contained 1% yeast extract (Difco), 2% Bacto-peptone (Difco), 40 mg/l adenine base, 25 mM potassium phosphate pH 6.5, 3% ethanol, 3% glycerol and 3 g/l erythromycin (Sigma). CAN plates was SD medium supplemented with 0.006% L-canavanine sulfate (Sigma), amino acids and bases necessary to complement the auxotrophy and 2% glucose.

**Plasmids construction**

The DNA constructions produced in this work were obtained using plasmids pFL38, which is centromeric and brings the URA3 marker, pFL39 (centromeric, TRP1 marker) [43], YEpplac195 (multicopy, URA3 marker), YEpplac181 (multicopy, LEU2 marker) [44], pUG35 (centromeric, URA3 marker, for EGFP fusion) ([http://mips.helmholtz-muenchen.de/proj/yeast/info/tools/hagemann/gfp.html](http://mips.helmholtz-muenchen.de/proj/yeast/info/tools/hagemann/gfp.html)), pTL85 (multicopy, KanMX4 marker, for EGFP fusion) [41] and pAG32, an integrative plasmid used as template for gene disruption with the HphMX4 cassette [45].

Plasmids pFL38-MIP1 and pFL38 harboring wt or mutant mips alleles have been previously described [19], YEpplac195-REV3 was obtained by PCR-amplification of REV3, including the upstream and the downstream regions, from genomic DNA of strain DWM-5A with primers REV3-Fw (ggccggtaacctgataagacattcctggg) and REV3-Rv (ggccgaaccttcagagacattgcagagcct), digestion with KpnI and EcoRI of both the PCR fragment and YEpplac195 and ligation. YEpplac195-REV7 was obtained by PCR-amplification of REV7 from genomic DNA of strain DWM-5A with primers REV7-Fw (ggccggtaacctgataagacattcctggg) and REV7-Rv (ggccggtaaccttcagagacattgcagagcct), digestion with both the PCR fragment and YEpplac195 with SalI and KpnI and ligation. YEpplac195-REV3/ REV7 was obtained by subcloning REV7 from YEpplac195-REV7 to YEpplac195-REV3 digested with SalI and KpnI, YEpplac181-REV1 was obtained by PCR amplification of REV1 from genomic DNA of strain BY4742 with primers REV1-Fw (ggccggtaacctgataagacattgcagagcct) and REV1-Rv (ggccggtaaccttcagagacattgcagagcct), digestion of both the PCR fragment and YEpplac181 with SalI and KpnI.
Disruption of REV3, REV7 and REV1

REV3 was disrupted in strain DWM-5A by using the HphMX4 cassette amplified from plasmid pAG32 [34] by using primers DELREV3FW (gtataccgagcatcagacgccggtggaggt) and DELREV3RV (aagtaaatcgtctttgatttttgaatcttacattttgac) with the mutagenic primer REV3NONSRV (gaatccagcctgacgcgctgctgctggag) and with the mutagenic primer REV3NONSSRV, and with the primer REV3MUTRV (aagtaaatcgtctttgatttttgaatcttacattttgac) each of which contains a 5′ strech complementary to the REV3 NONSFW, R3GFPRVS primer. The final mutagenized product was obtained by using the overlapping PCR fragments as template and amplified with the primer REV3GFPFW and R3GFPRVS (gcgctgcaattgactttgactgacag) digested at the EcoRI site. All the cloned fragments were sequenced to check for the absence of mutations.

Disruption of REV3, REV7 and REV1

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Transformation of yeast strains

Yeast strains were transformed by lithium acetate methods [47]. We used the “Quick and easy method” (http://home.cc.umanitoba.ca/~gietz/Quick.html) in case of transformation with centromeric or episomal (multicopy) plasmids, and the “Best Method” (http://home.cc.umanitoba.ca/~gietz/method.html) in case of disruption cassettes.

Determination of mitochondrial and nuclear mutability

Petite frequency was determined as previously reported [48]. To determine the effect of antioxidant agents on the extended mtDNA mutability, wild-type and mutant strains, were grown on liquid SC medium supplemented with 40 μM dihydrolipoic acid (Sigma) or 10 μM MitoQ (a kind gift from Luigi Palmieri, University of Bari, Italy) for two 24-hour growth cycles. Dihydrolipoic acid was supplemented from a 40 mM stock solution in ethanol, while MitoQ was supplemented from a 5 mM stock solution in DMSO. Control experiment in which equal amounts of ethanol (0.1%) or DMSO (0.2%) were added to untreated cells was done in parallel. For each strain/condition at least three independent experiments were performed on three independent clones. Statistical analysis of petite frequency was performed by a two-tailed t-test.

EryR mutant frequency was determined as previously reported [48]. Briefly, 15 independent colonies, isolated on SC medium supplemented with 2% glucose, were inoculated into 2 ml of SC liquid medium supplemented with 2% ethanol and grown to saturation (approximately 48 h). Cultures were then plated on YPAEG-Ery medium and dilutions were plated nonselectively on YM liquid medium supplemented with 3% glycerol (300–500 colonies) for a total cell count. After 7 days incubation, EryR mutant frequency was determined by the method of the median [49]. Each experiment was repeated at least three times on two independent clones for each strain. Statistical analysis of EryR mutant frequency was performed by a one-tailed t-test.

Nuclear point mutability was evaluated by measuring the mutation rate of CanR mutants in strain W303-1A CAN8, bearing the wt copy of GAL1, as previously reported [50], except that colonies were grown on SC medium supplemented with 2% glucose. Mutation rates were calculated using median values according to Lea and Coulson [49] by using the program FALCOR [51]. Rates are given as mean of four independent experiments in each of which 10 independent colonies for each clone were tested. Unpaired two-tailed t-test was used to evaluate the statistical significance.

In every test, frequencies or rates were considered significantly different when p<0.05.
shop, signals were quantified through Multianalyst software (Molecular Dynamics). To avoid saturation, signals were considered only if the signal deriving from the PCR amplification with 3-cycle less was 6–8-fold lower. Both in the case of Northern analysis and in the case of RT-PCR, relative levels of mRNA were quantified by comparison with a house-keeping gene signal (ACT1 or TAF10). All experiments were performed three times.

Fluorescence microscopy

Cells were grown in solid SC medium without methionine and supplemented with 2% glucose. After 24 hours, cells were resuspended in Tris–HCl 50 mM pH 7.5, incubated with 1 µg/ml DAPI (4',6-diamidino-2-phenylindole) for 15 min on ice and observed with a 100× magnification using DAPI or FITC filters. Pictures were taken with constant parameters (100 ms exposure for DAPI and 250–500 ms exposure for GFP with Nikon ACT-1 acquisition software) and processed to optimize contrast and brightness with Adobe Photoshop 7.0.

Results

Pol zeta overexpression rescues the mtDNA mutability induced by specific mip1 mutations

We have previously constructed a set of Saccharomyces cerevisiae mip1 mutant alleles, corresponding to POLG pathological mutations, and determined their effects on mtDNA extended and point mutability (Table 1) [19–21,23]. These mutant alleles localized in different Mip1 domains, displayed different in vivo and in vitro defects and behaved either as dominant or recessive (Table 1 and Figure 1). We analyzed the effect of overexpression and deletion of Pol zeta and Rev1 on the mtDNA mutability induced by these mip1 mutations, based on the recent finding that these polymerases were found in mitochondria, where Pol zeta and Mip1 belong to the same epistatic pathway [9].

REV3 and REV7 were cloned in the multicopy plasmid YEplac195 and introduced i) in the strain DWM-5A (mip1A) transformed with a number of recessive mip1 mutant alleles (haploid condition) and ii) in the strain W303-1B (MIP1) transformed with dominant mip1 mutant alleles (heteroallelic condition). Extended mtDNA mutability was first determined in these transformants by analyzing the frequency of spontaneous petite mutants.

In presence of overexpressed REV3, the level of petite mutants decreased; the rescue was mutation specific, being null for some mutations and significant for other ones (Figure 2). The strongest effect was observed for haploid strains bearing mutations G807R (50% reduction) and A692T (25% reduction). The REV7 overexpression, on the contrary, had slighter effects, which however were significant for mutations A692T and G807R (Figure 2A). In heteroallelic strains harboring wt MIP1 and dominant mip1F460S, mip1F460S or mip1Y537C mutant alleles (Figure 2B) no rescue was observed in the presence of either REV3 or REV7.

We also evaluated the combined effects of REV3 and REV7 overexpression by cloning the two genes together in the YEplac195 plasmid. Northern blot analysis indicated that REV3 and REV7 were expressed at high level (10–12 fold increase of mRNA levels) when cloned either alone or together, demonstrating that their concomitant presence in the plasmid did not affect the level of their expression (Figure 3A). Overexpression of whole Pol zeta had the same effect than overexpression of Rev3 (Figure 2), suggesting that Rev3 alone was responsible of the observed rescue or that the physiological levels of Rev7, which is more abundant than Rev3 [54], were sufficient to ensure the highest level of rescue. However, the slight decrease in petite frequency obtained overexpressing Rev7 alone suggests, as demonstrated below, that also Rev7 participates to the rescue and that the petite mutants’ reduction observed is most likely due to a slight increase of the whole Pol zeta.

Effect of REV3 deletion on extended and point mtDNA mutability

We next investigated the effect of REV3 deletion on mtDNA stability. No difference was observed between REV3 and rev3A regarding the frequency of petite mutants in strains harboring either wt MIP1 allele or mutant mip1 alleles, indicating that the REV3 deletion produced no effect on extended mutability (data not shown).

However, deletion of REV3 resulted in alteration of mtDNA point mutation, with 2.5 fold increase of EryR mutant frequency in the MIP1 wt strain, in agreement with results reported by Kalifa and Sia [10] (Table 2). In addition, strains carrying mip1F460S, mip1F460S and mip1F460S mutant alleles showed a further increase of EryR mutation (an increase of 3.9-, 3.3- and 5.1-fold, respectively) (Table 2), indicating that mutations in MIP1 and deletion of REV3 have a synergistic effect in rising point mutability. Furthermore, the ratios of EryR mutant frequencies of rev3A strains to REV3 wt strains parallelized the mutator phenotype of the mip1 mutant allele, being the lowest in mip1F460S strain showing a moderate increase of EryR frequency compared to wt MIP1 strain (approximately 3.4-fold), and being the highest in the strain mip1F460S showing an increase of 8.8 times the EryR frequency of MIP1 strain (Table 2). Therefore, Pol zeta is not physiologically involved in the maintenance of full mtDNA but is required for the fidelity of the mtDNA replication.

Effects of REV1 overexpression

Rev3 subunit of Pol zeta binds to Rev1, and the complex Pol zeta-Rev1 increases the efficiency of translesion synthesis by Pol zeta [8]. We tested whether overexpression of REV1, together with overexpression of Pol zeta, could further reduce the petite frequency due to mip1F460S, mip1F460S and mip1F460S mutant alleles. REV1 was cloned in the multicopy plasmid YEplac181 and introduced in strains overexpressing both REV3 and REV7 and carrying wt MIP1, mip1F460S, mip1F460S and mip1F460S mutant alleles. RT-PCR analysis confirmed that REV1 was 9–13-fold overexpressed in transformant cells (Figure 3B) and that REV3 and REV7 were overexpressed at the same levels (10–12 fold overexpression) as in strain not harboring YEplac181-REV1 (data not shown). Together with REV1, we overexpressed both REV3 and REV7, and not REV3 alone, because it is known that Rev1 can recruit Rev7 through a Rev3 independent Rev1-Rev7 binding thus depleting the levels of Rev7 [54]. REV1 overexpression did not alter the frequency of petite, both in presence and absence of overexpressed Pol zeta (Figure 4). This result indicates that either the physiological levels of REV1 are sufficient for the rescue by Pol zeta overexpressed or that Rev1 is not involved. Moreover, we observed that overexpression of both Pol zeta and Rev1, but not of either Pol zeta or Rev1 individually, resulted in a significant reduction of the EryR mutant frequency, in particular for the strain carrying the mutation G807R (Table 3). The results described here are symmetrical to those obtained previously by Kalifa and Sia [10], who showed an increase in EryR mutant frequency when REV1, REV3 or REV7 were disrupted.
## Table 1. POLG human pathological mutations, associated syndromes, *mip1* equivalent mutations analyzed in this study and phenotypes of *mip1* mutant strains.

| Human mutation | Pathology | Yeast mutation | Domain | In vivo phenotype: petite frequency | In vivo phenotype: Ery\(^b\) frequency (fold increase relative to wt) | Dominance/ recessivity | Polymerase activity (compared to wt) | Soluble protein levels (compared to wt) | Rescue by treatment with antioxidants |
|----------------|-----------|----------------|--------|------------------------------------|-------------------------------------------------|------------------------|------------------------------------|-------------------------------------|----------------------------------|
| G303R          | Alpers    | G259R          | Exo    | 80%                                | NT                                               | Recessive             | NT                                 | NT                                  | NT                               |
| S305R          | Alpers    | C261R          | Exo    | 85%                                | 6                                                 | Recessive             | Reduced                           | NT                                  | Slight                           |
| R574W          | PEO/myopathy/ dysphagia, Alpers | R467W  | Linker  | 60%                                | 4                                                 | Recessive             | As wt (Reduced processivity)      | As wt                               | No                               |
| A889T          | PEO       | A692T          | Pol    | 80%                                | 4                                                 | Slightly dominant\(^1\) | 30%                               | As wt                               | No                               |
| E895G          | Myspathic MDS | E698G  | Pol    | 100%                               | ND                                               | Dominant              | NT                                 | NT                                  | Strong                           |
| K947R          | PEO, ovarian failure | K749R  | Pol    | 100%                               | ND                                               | Dominant              | NT                                 | NT                                  | Strong                           |
| Y95SC          | PEO+/− Parkinsonism, Premature ovarian failure, Alzheimer’s disease | Y757C  | Pol    | 100%                               | ND                                               | Dominant              | <0.03%                            | NT                                  | Strong                           |
| G1051R         | arPEO, SANDO | G807R  | Pol    | 25%                                | 12                                               | Recessive             | 70–80%                            | 10–15%                             | No                               |
| E1143G         | Neutral polymorphism but may modulate disease mutations | E900G  | Pol    | 6%                                  | 2                                                 | Recessive             | 100–130%                          | 30–40%                             | NT                               |

NT: not tested.
ND: not detectable.

\(^1\)The mutation is slightly dominant in yeast, where the petite frequency in heterozygosis is approximately 1.5-fold compared to the wild type. In humans, the equivalent mutation has been associated either to sporadic PEO when it is in trans with mutation R579W, suggesting a recessive trait [80] and to PEO with tremor and peripheral neuropathy when it is in cis with E1143G SNP and in trans with wt POLG, suggesting a dominant trait [81].

doi:10.1371/journal.pone.0034322.t001
Figure 1. Linear representation of human Polg and yeast Mip1 with the mutations used in this study. NTD-Exo = N-terminal domain- 3’-5’ exonuclease domain. Pol = polymerase domain. doi:10.1371/journal.pone.0034322.g001

Figure 2. Petite frequency of mip1 strains overexpressing REV3, REV7, or both. Petite frequency was measured as reported in Materials and Methods on three independent clones for each strain. All values are means at least of three independent experiments. (A) Haploid mutant strains harboring mip1 recessive mutant alleles. (B) Heteroallelic strains harboring MIP1 wt allele and mip1 dominant mutant alleles. For each mip1 allele, * indicates that the petite frequency is significantly lower (p<0.05) than that of strain transformed with the empty plasmid (YEplac195). doi:10.1371/journal.pone.0034322.g002
Rescue of mtDNA Mutability by Rev3 Overexpression

We tested whether the rescue by Rev3 overexpression was dependent on the physiological levels of Rev7 and Rev1. The deletion of REV1 or REV7 had no effect on petite mutability (data not shown). However, the rescue of petite mutability in MIP1 mutants, exerted by overexpressed Rev3, occurred only when Rev7 was present (Table 4), indicating that the whole Pol zeta participated in the rescue. We furthermore observed that overexpression of REV7 in a rev3Δ strain did not reduce petite frequency both in wt MIP1 and in mip1 mutant strains (data not shown), corroborating this hypothesis. Unlike Rev7, Rev1 was dispensable (Table 4), thus supporting the hypothesis reported in the previous paragraph that Rev1 did not participate in the rescue of the extended mutability.

Combined action of REV3 overexpression and other chemical and genetic rescues

We have shown previously that a decrease of reactive oxygen species and/or an increase of the mitochondrial dNTP pool reduced the mtDNA extended mutagenesis induced by patholog- ical Pol gamma mutations in yeast [19]. Several mutations in MIP1, but not all, were rescued, albeit at different extent, by treatment with dihydrolipoic acid or with the mitochondrial specific ROS scavenger MitoQ [21]. To know whether this effect could be increased by REV3 overexpression the petite frequency was measured either in haploid (Figure 5A) or in heteroallelic strains (Figure 5B) treated with these molecules. We found that

there was a negative correlation between the rescue exerted by ROS scavengers and the rescue caused by REV3 overexpression (Figure 2 and Figure 5).

In fact, in haploid strains harboring A692T and G807R mutations, whose effect was reduced by overexpression of Rev3, no rescue was exerted by dihydrolipoic acid or MitoQ. In contrast, ROS scavengers strongly mitigated the effect of mip1 mutations E698G, K749R, Y757C, that are insensitive to Rev3 overexpression.

The only exception was mutation C261R, whose effect was slightly rescued both by treatment with ROS scavengers and by Rev3 overexpression (Fig. 2A and Fig. 5A). In this case, the additive effect of Pol zeta overexpression and ROS scavengers (data not shown) suggested that two different mechanisms were involved in the reduction of petite mutability observed.

The second mechanism able to rescue the mip1 induced mtDNA extended mutability is the increase of the dNTP pools. In S. cerevisiae an increase of the dNTP pool was obtained either by overexpressing RNRI gene, which encodes the large subunit of the ribonucleotide reductase or by deleting SML1 gene, which encodes an inhibitor of the latter activity [55]. It has been shown that overexpression of RNRI or deletion of SML1, reduced the petite frequency in strains harboring specific MIP1 mutations [20,26,39,55].

To evaluate the relationship between the rescue exerted by REV3 overexpression and the rescue exerted by increasing the dNTP pools, we deleted SML1 in strains harboring mip1E698G, mip1K749R, and mip1Y757C mutant alleles, in presence of overexpressed REV3. We observed that, for every mutation studied, the

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Table 2. EryR mutant frequency in wt and rev3Δ strains transformed with wt or mutant mip1 alleles.

|            | EryR per 10^7 cells: wt strain | Fold increase mutant mip1/wt MIP1 | EryR per 10^7 cells: rev3Δ strain | Fold increase rev3Δ strain/wt strain |
|------------|-------------------------------|---------------------------------|-------------------------------|-----------------------------------|
| MIP1       | 1.5±0.3                   | 1.0                             | 3.7±0.6                      | 2.5                               |
| mip1E698G  | 5.6±0.8                   | 3.7*                            | 22.0±2.9                     | 3.9*                              |
| mip1K749R  | 5.1±0.6                   | 3.4*                            | 16.6±2.7                     | 3.3                               |
| mip1Y757C  | 13.2±1.9                  | 8.8*                            | 67.9±9.4                     | 5.1*                              |

EryR mutant frequency in wt and rev3Δ strains transformed with wt or mutant mip1 alleles was determined as reported in Materials and Methods. “Fold increase mutant mip1/wt MIP1” was obtained by dividing the EryR frequency related to each mip1 allele by the EryR frequency related to MIP1 wt allele. “Fold increase rev3Δ strain/wt strain” was obtained by dividing, for each mip1 allele, the EryR frequency of rev3Δ strain by that of wt strain. * indicates that the fold increase is significantly higher (p<0.05) than that of the MIP1 wt allele.

doi:10.1371/journal.pone.0034322.t002

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Figure 3. mRNA levels in overexpressing strains. A. Northern blot mRNA expression of REV3 and REV7 in strains transformed with YEplac195 empty plasmid (1st lane), YEplac195-REV3 (2nd lane), YEplac195-REV7 (3rd lane) and YEplac195-REV7/REV3 (4th lane). Numbers indicate the fold increase of REV3 and REV7 levels in overexpressing strains. mRNA of the ACT1 gene was used as a control. B. RT-PCR on REV1 in strains transformed with YEplac81-REV1 and YEplac181 empty plasmid. cDNA of the TAF10 gene was used as a control. Numbers over the gel indicate the number of PCR cycles, number under the gel indicate the fold increase of REV1 levels in overexpressing strains.

doi:10.1371/journal.pone.0034322.g003
two processes act synergistically (Figure 6). For example, in strain carrying \textit{mip1A692T} mutant allele the overexpression of \textit{REV3} reduced the petite frequency of approximately 1.3-fold, while deletion of \textit{SML1} reduced the petite frequency of 4.4 fold; together, the reduction was 9.1-fold. The synergistic effects were even stronger in the case of strain harboring \textit{mip1G807R} mutation (1.9-fold when \textit{REV3} was overexpressed, 4.1-fold when \textit{SML1} was deleted, 15.7-fold together). It is likely that the observed synergy is due to an increase of dNTP pools, which are the substrate for both Mip1 and Pol zeta.

### Construction of a mutant Rev3 which rescues mtDNA mutability without affecting nuclear mutability

It was previously shown that in UV-treated cells, enhanced expression of \textit{REV3} led to increase of nuclear point mutability, measured as increase in the rate of \textit{arg4–17} reversion. Pol zeta mutagenic function is dependent on Rev1 protein [38]. We measured nuclear mutability in cells transformed with \textit{REV3} and/or \textit{REV1} overexpressing plasmids to determine whether increased levels of Pol zeta/Rev1 exerted mutagenic effect as a consequence of a general mechanism associated to the translesion bypass. The overexpression of Rev3 or Rev1 caused an approximately two-fold increase of nuclear point mutability, measured as rate of Can\textsuperscript{R} mutants. When both Pol zeta and Rev1 were overexpressed, the rate of Can\textsuperscript{R} mutations increased approximately 3-fold (Figure 7). Thus, overexpression of Pol zeta is useful to reduce the mtDNA instability caused by specific mutations in \textit{MIP1} but is detrimental to spontaneous nuclear mutability.

A Rev3 mutant which could enter into mitochondria, but not in the nucleus is expected to reduce the mitochondrial mutability without increasing nuclear mutability. To this end, we thought of designing a \textit{REV3} gene whose product could not enter the nucleus. We searched inside Rev3 for the potential nuclear localization signal, by using the PredictNLS server [http://cubic.biocolumbia.edu/services/predictNLS/]. Rev3 protein is predicted to possess a unique bipartite nuclear localization signal encompassing amino acids 487–508 (Figure 8). Both upstream and downstream basic amino acid stretches composing the bipartite signal are critical for nuclear import [56].

To evaluate the localization of Rev3, we first cloned the whole coding sequence either in a centromeric plasmid or in a multicopy plasmid in frame with the EGFP gene. In both cases no GFP signal was detected in transformant cells, as it was found previously in high throughput analysis [57]. To overcome this problem, a shorter Rev3 fragment encompassing residues 1–534, which include the bipartite signal, was cloned in frame with EGFP. In this case, in transformant cells we observed a fluorescent signal corresponding to Rev3, localized in the nucleus. This was confirmed by DAPI staining of mitochondrial and nuclear DNA (Figure 6). Both in \textit{rho}\textsuperscript{+} strain, where DAPI stained both mtDNA and nuclear DNA, and in the \textit{rho}\textsuperscript{0}, where DAPI stained only nuclear DNA, the GFP signal co-localized with the nuclear DAPI signal.

To obtain a version of Rev3 unable to migrate in the nucleus, we changed the 504–508 RKRKK downstream stretch to AAAAA (Figure 8). When Rev3 fragment encompassing amino acids 1–534 and harboring the 504–508 AAAAA stretch was fused

### Table 3. Ery\textsuperscript{R} mutant frequency in \textit{mip1} mutant strains overexpressing \textit{REV3}, \textit{REV7} and/or \textit{REV1}.

|            | Empty plasmid- empty plasmid | REV3/REV7- empty plasmid | Empty plasmid-REV1 | REV3/REV7-REV1 |
|------------|--------------------------------|--------------------------|---------------------|--------------|
| \textit{MIP1} | 1.4±0.2                        | 1.3±0.3                  | 1.6±0.3             | 1.0±0.3      |
| \textit{mip1C261R} | 4.9±0.7                        | 4.4±0.7                  | 4.2±0.4             | 1.9±0.2      |
| \textit{mip1A692T} | 4.3±0.5                        | 4.5±0.6                  | 4.7±0.6             | 2.3±0.3      |
| \textit{mip1G807R} | 16.7±2.1                       | 15.2±1.8                 | 16.4±3.7            | 6.5±1.1      |

Ery\textsuperscript{R} mutant frequency (per 10\textsuperscript{7} cells) was determined as reported in Materials and Methods. For each \textit{mip1} strain, *indicates that the Ery\textsuperscript{R} mutant frequency is significantly lower (p<0.05) than that of strain harboring both empty plasmids.

doi:10.1371/journal.pone.0034322.t003
with EGFP, the fluorescent signal corresponding to the fusion protein localized in the cytoplasm only (Figure 9). The AAAAA mutation was then introduced in the full REV3 gene, cloned in the multicopy plasmid YEplac195 and its effect was evaluated in strains harboring mip1A692T or mip1G807R. In the presence of overexpressed mutant Rev3, the mtDNA extended mutability decreased at the same extent as achieved in the presence of overexpressed wild type Rev3, indicating that the alteration of the nuclear signal did not affect the ability of Rev3 of operating in mitochondria (Table 5). If the mutated version of the enzyme did not enter the nucleus, then it was expected that its overexpression did not cause an increase in nuclear mutability. Indeed, in the presence of overexpressed mutant Rev3 the nuclear point mutability, measured as rate of CanR mutants accumulation, did not increase (Figure 7).

Discussion

In this study we showed that increased levels of Rev3, encoding the catalytic subunit of Pol zeta, reduces both the mtDNA extended mutability and the point mutability caused by specific mutations in Mip1, the S. cerevisiae mtDNA polymerase. The rescue of mtDNA extended mutability requires overexpression of the catalytic subunit Rev3, but not that of the accessory subunit encoded by REV7. However, a basal level of Rev7, which is known to be expressed more than Rev3 [54], is necessary, because in a rev7Δ strain the rescue does not occur (Figure 7).

Rev1, which interacts with Pol zeta for efficient bypass and extension past the DNA lesion, is not required to rescue mtDNA extended mutability, suggesting that Pol zeta can function independently of Rev1 in mitochondria. In contrast, Rev1 overexpression is necessary for the rescue of point mutability mediated by Rev3. These observations suggest that the rescues mediated by Rev3 overexpression are driven by two different mechanisms: i. the rescue of mtDNA extended mutability involving only Pol zeta and ii. the rescue of mtDNA point mutability for which both Pol zeta and Rev1 are required. These results are consistent with previous findings which indicate that the instability of mitochondrial DNA in general is caused by mechanisms different from those producing single point mutations [19].

Deletion of REV3 did not influence the mtDNA extended mutability (petite frequency), both in the wt strain and in mip1 mutant strains. In contrast, REV3 deletion caused an increase of point mutability. Therefore Pol zeta, in mitochondria, functions in a pathway less mutagenic than Mip1, supporting the hypothesis of Kalifa and Sia [10]. Moreover, strains carrying mip1 mutant alleles were themselves mutators, i.e. showed an EryR mutant frequency higher than that of the wt strain. The observation that the higher the mtDNA point mutability of the mutant strain, the higher the effect of REV3 deletion, indicates that mutator Mip1 isoforms are more sensitive to the absence of Rev3 than the wild type one and is coherent with that hypothesis.

### Table 4. Petite frequency in mip1 mutant strains deleted of REV1 or REV7 and overexpressing REV3 and REV7.

|     | Empty plasmid | REV3 | REV3/REV7 | Empty plasmid | REV3 | REV3/REV7 |
|-----|---------------|------|-----------|---------------|------|-----------|
| rev1Δ | 89.4          | 81.3 | 81.6      | 78.6          | 60.0 | 62.9      |
| rev7Δ | 91.5          | 89.4 | 82.7      | 79.2          | 80.2 | 57.3      |

Petite frequency was measured as reported in Figure 2. For each mip1 mutant rev1Δ or rev7Δ strain, *indicates that the frequency is significantly different (p<0.05) compared to that of strain transformed with the YEplac195 empty plasmid.

doi:10.1371/journal.pone.0034322.t004

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**Figure 5.** Petite frequency of mip1 strains treated with dihydrolipoic acid 40 μM (DHL) or 10 μM MitoQ. Petite frequency was measured as reported in Materials and Methods on three independent clones for each strain at least twice. (A) Haploid mutant strains harboring mip1 recessive mutant alleles. (B) Heteroallelic strains harboring MIP1 wt allele and mip1 dominant mutant alleles. For each mip1 strain, * indicates that the petite frequency is significantly lower (p<0.05) than that of non-treated (NT) strain.

doi:10.1371/journal.pone.0034322.g005
Until now, it has not been elucidated the physiological role of Pol zeta in mitochondria. Rev3, that is expressed at basal levels during the whole vegetative cell cycle and only moderately (~2-fold) overexpressed when cells are treated with UV, is expressed at levels comparable or higher (~18-fold) than those obtained in this study during the late phase of sporulation [58]. Interestingly, 

Figure 6. Petite frequency of \textit{mip1}^{\text{C261R}}, \textit{mip1}^{\text{G607R}} and \textit{mip1}^{\text{G807R}} strains disrupted in \textit{SML1} and overexpressing \textit{REV3}. In each graph the following petite frequencies are reported: \textit{mip1} strains transformed with YEplac195 empty plasmid (wt) or with YEplac195-\textit{REV3} (REV3); \textit{mip1 sml1} strains transformed with YEplac195 empty plasmid (\textit{sml1}); or with YEplac195-\textit{REV3} (\textit{sml1}/REV3 observed). In addition, the penultimate bar represents the petite frequency of \textit{sml1} strain transformed with YEplac195-\textit{REV3} that would be expected if the effects of the overexpression of \textit{REV3} and of the deletion of \textit{SML1} were additive (\textit{sml1}/REV3 expected). * indicates that the observed frequency is significantly lower than the expected one. NS: not significant. 
doi:10.1371/journal.pone.0034322.g006

Figure 7. Rate of Can\textsuperscript{R} mutants. Rate of Can\textsuperscript{R} mutants (A) in wt strains overexpressing \textit{REV3}, \textit{REV7} and/or \textit{REV1} or (B) wt \textit{REV3} and mutant \textit{REV3}, encoding for the Rev3 protein harboring the 504–508 AAAAA stretch. Rate of Can\textsuperscript{R} mutants was measured as indicated in Materials and Methods on three independent clones. If no stated differently, * indicates that the rate is significantly different from that of strain transformed with the empty plasmid(s). NS: not significant. 
doi:10.1371/journal.pone.0034322.g007
maintenance of a full non-mutated mtDNA genome is fundamental in meiosis, since respiratory-deficient diploid strains are unable to sporulate or show increased levels of dead spores [59,60]. A possible interpretation is that increased levels of Pol zeta could have importance in the maintenance of wt mtDNA during sporulation.

Regarding extended mutability, some MIP1 mutations equivalent to human pathological mutations were rescued by overexpression of Rev3 while others were not, Y757C, E698G and K749R, dominant mutations located in the polymerase domain, together with the recessive mutation in the exomuclease domain G259R, belong to this second group. These mutations are rescued by treatment with antioxidant molecules such as dihydrolipoic acid and MitoQ. Biochemical properties are available only for human Y955C mutation, equivalent to yeast Y757C, that strongly reduces the polymerase activity (<0.03% compared to the wild type), the processivity and the nucleotide binding affinity, but does not affect the DNA binding affinity [61,62]. It was previously reported also that human Pol gamma harboring the Y955C mutation increases the in vitro incorporation of 8-oxo-dGTP, the most abundant oxidized nucleotide in mitochondria, and the translesion synthesis opposite to an 8-oxo-dG residue [63]. The last observation

Figure 8. Position and sequence of the bipartite nuclear localization sequence. The stretch RKRRKK was mutagenized to AAAAA to obtain a mutant Rev3 fragment fused with EGFP for protein localization and a mutant Rev3 isoform for analysis of mtDNA mutability and nuclear point mutability.

doi:10.1371/journal.pone.0034322.g008

Table 5. Petite frequency in mip1 strains overexpressing REV3 or the mutagenized form of REV3.

|                        | Empty plasmid | REV3 | Mutant REV3 |
|------------------------|---------------|------|-------------|
| mip1Δ             | 2.4±0.3       | 2.0±0.2 | 2.2±0.2 | |
| mip1Δ   | 80.4±4.3      | 60.2±4.1 | 63.2±3.6 | |
| mip1Δ   | 23.5±2.4      | 12.6±1.4 | 11.6±1.7 | |

Petite frequency was measured as reported in Figure 2. For each mip1 strain, *indicates that the frequency is significantly different (p<0.05) compared to that of strain transformed with the YEplac195 empty plasmid. The differences between strains overexpressing wt REV3 or mutant REV3 are not significant.

doi:10.1371/journal.pone.0034322.t005

Figure 9. Localization of Rev3. Localization of Rev3 and mutagenized Rev3 fused with EGFP in (A) a rho+ strain and in (B) a rho0 strain. Images have been acquired and adjusted as reported in Materials and Methods. In rho+ strain DAPI stains both the nuclear DNA (bigger spot) and the mtDNA (small spots in the cell periphery). In rho0 strain, which is depleted of mtDNA, DAPI stains the nuclear DNA only.

doi:10.1371/journal.pone.0034322.g009
suggestions that, although most of the mtDNA extended mutability by Y757C mutation is caused by stalled replication due to strongly reduced polymerase activity [61,62,64,65], the mtDNA damage can be partially induced also by the accumulation of oxidized bases. In addition, for heteroallelic strains containing dominant mutations, it is possible that replication stalling by mutant Mip1 itself causes oxidative damage on mtDNA, which can partially block replication by wt Mip1. Independently of the mechanisms, it is known that there is a correlation between mtDNA extended mutability and oxidative stress: petite frequency is higher in strains with a reduced antioxidant potential [66], in strains treated with hydrogen peroxide [67] and in strains deleted both in MSH1 and in OGG1, encoding the mitochondrial glycosylase/hyrase that specifically excises 8-oxo-dG residues in mtDNA [68].

Differently from Y757C, E698G, K749R and G259R, mutations C261R, R467W, A692T and G807R are rescued by Rev3 overexpression. These mutations are known to reduce the polymerase activity and/or the processivity of Mip1 [20,27]. Yeast C261, equivalent to human S305, is part of a loop-helix orienter module (amino acids 304–316) that creates steric constraints leading to a destabilization of the template strand and a modification in the arrangement of the primer strand [27]: this subdomain in the 3′–5′ exonuclease plays a crucial role in the DNA polymerase function with no major effect on the exonuclytic activity. yG807, equivalent to hG1051, is part of a partitioning loop (amino acids 1050–1095) [12] that modulates the partitioning of the primer strand between the polymerase and the exonuclease active sites by forming stable contacts with correctly base-paired primer-template DNA and destabilizing primer-template DNA that contains mismairs [69]. This subdomain is adjacent to the orienter module, whose role is to position correctly the partitioning loop. Mutations in the orienter loop and in the partitioning loop both result in reduced DNA-binding affinity, reduced polymerase activity, increased point mutability and extended mutability [20,26,27]. yA692T, equivalent to hA889, is part of a β-sheet which surrounds the catalytic residues in the palm [12]. Mutations in this β-sheet are predicted to reduce polymerase activity [69], and this effect was observed for A692T mutation in yeast [26]. yR467, equivalent to hR574, is part of a subdomain responsible for the intrinsic processivity of Pol gamma [12]. As a matter of fact, mutation yR467W results in a decreased processivity [27].

We showed that these recessive mutations with reduced polymerase activity or processivity are insensitive to antioxidants treatment. Thus, we speculate that different Pol gamma mutations exert their negative effect according to two distinct ways. The first one, rescued by the treatment with antioxidants, can be related to the increased incorporation of 8-oxo-dG: in presence of antioxidant molecules the concentration of oxidized nucleotides and, as a consequence, their incorporation in the mtDNA is reduced. The inability of Pol zeta to rescue these Mip1 mutations may depend partially on the low efficiency (approximately 12%) of Pol zeta to incorporate nucleosides opposite to 8-oxo-dG [70,71]. The second way, rescued by Rev3 overexpression, could be related to reduced polymerase activity, processivity and/or TLS synthesis of mutated mtDNA polymerase as in the case of C261R, R467W, A692T and G807R mutations. Two modes of action may account for ability of Rev3 to rescue this kind of mlp1 mutations. i) Pol zeta could participate directly to the replication of undamaged mtDNA at the replicative fork partially playing the role of Mip1, as it was recently demonstrated in the case of defective nuclear replisome due to mutations in the replicative polymerases [72]. Interestingly, the rescue is observed for mutations which cause reduced DNA binding affinity (C261R) or for which reduced DNA binding affinity is predicted (R467W and G807R) [27,69]. ii) Alternately, Pol zeta could partially substitute mutant Mip1 defective of TLS, either during the incorporation opposite to a lesion or the extension from mismatched terminally nucleotides. Reports of translesion synthesis by Pol gamma are limited. In vitro studies showed that Pol gamma from higher eukaryotes is an enzyme with a low TLS activity: it can bypass 8-oxo-dG incorporating dCMP (~73%) or dAMP (~27%), but stalls the majority of the time opposite abasic sites and opposite adducts containing benzo[α]pyrene and benzo[c]phenanthrene [73,74]. Moreover, it has been recently reported that human Pol gamma barely bypasses cyclobutane T-T dimers incorporating dAMP (~80%) or dCTP (~20%), whereas it extends very inefficiently past the dimer [75].

These observations suggest that DNA polymerase gamma can promote mutagenesis during TLS.

It is worthwhile mentioning that the Rev3 rescue was observed only in the absence of wt Mip1. It could be possible that in heteroallelic strains wt Mip1 out-competes even a 10-fold overexpression of Pol zeta for the stalled replication that is caused by mutant Mip1. As a consequence, putative beneficial effects of overexpression of Pol zeta in heteroallelic strain could be hidden by the presence of wt Mip1.

It has been demonstrated previously that mtDNA instability caused by specific mutations in MIP1 is reduced by increasing the dNTPs in the cell, either by overexpressing the RNR1 gene, which encodes the large subunit of the ribonucleotide reductase, or by deleting the SML1 gene, which encodes an inhibitor of the latter activity [55]. Increased concentration of dNTPs is beneficial since the basal levels of mitochondrial dNTP pools are a limiting factor to maintain mtDNA copy number in yeast [76,77].

Here we describe that overexpression of Rev3 and increased activity of ribonucleotide reductase have a synergistic effect on reducing the frequency of petites. The synergistic effect is probably due to the fact that the activity of Pol zeta but also of Mip1 have improved with the increase of the dNTP pools, which are the substrate of both DNA polymerases. In addition, the REV3 overexpressing mld1A strains harboring mlp1 mutations A692T and G807R reached petite frequency values which are slightly higher or similar, respectively, to those of wt strain, thus indicating that through genetic modifications it is possible to erase completely the detrimental effects of such mlp1 mutations on extended mutability.

It is worth mentioning that the human Pol zeta protein does not localize to the mitochondria [9]. From the data here described, we suggest that in mammalian cells carrying mutant Pol gamma a beneficial effect could be obtained by overexpressing the yeast Rev3 mutant isoform unable to enter the nucleus but capable of rescuing mtDNA mutability, or as an alternative a mitochondrial localizing version of human Rev3. It has been reported that expression of a yeast mitochondrial repair/recombination enzyme, Cce1, the cruciform-cutting endonuclease, in human cells harboring partially duplicated mtDNA induced the appearance of wt mtDNA, thus demonstrating that expression of a yeast protein could be beneficial for mtDNA stability in mammalian cells [70]. Such a genetic rescue could be associated to a treatment with dNTPs, which seems to be beneficial for the detrimental effects caused by specific mutations in human POLG, the processivity defect of human DNA polymerase gamma harboring Y955C mutation was overcome by increasing the dATP or dTTP concentration during in vitro synthesis of mtDNA fragments [65] and the treatment with dAMP and dGMP leads to a slight increase in the levels of mtDNA in myoblasts deriving from an Alpers’ patient harboring mutations A467T and K1191N in POLG [79].
Acknowledgments

We are deeply grateful to Antonietta Cirasolo and Roberto Silva for their skilful technical assistance, and to Antoni Barrientos for the gift of strain W305-1A CAN6.

References

1. Lawrence CW (2004) Cellular functions of DNA polymerase zeta and Rev1 protein. Adv Protein Chem 69: 167–203.
2. Nelson JR, Lawrence CW, Hinkle DC (1996) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science 272: 1646–1649.
3. Morrison A, Christensen RB, Alley J, Beck AK, Bernstein EG, et al. (1989) REV3, a Saccharomyces cerevisiae gene whose function is required for induced mutagenesis, is predicted to encode a non-essential DNA polymerase. J Bacteriol 171: 3639–5667.
4. Lawrence CW, Hinkle DC (1996) DNA polymerase zeta and the control of DNA damage induced mutagenesis in eukaryotes, in: Lindahl T, ed. Cancer Surveys: Genetic Stability in Cancer, vol. 28. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. pp 31–.
5. Lawrence CW, Gibble PEM, Muraute RS, Wang X-D, Li Z, et al. (2000) Roles of DNA polymerase zeta and Rev1 protein in eukaryotic mutagenesis and translesion replication, in: Proceedings of the Cold Spring Harbor Symposia on Quantitative Biology, vol. 65. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. pp 61–69.
6. Johnson RE, Washington MT, Haracska L, Prakash S, Prakash L (2000) Mutations in mismatch extension and for extension opposite from DNA lesions. Mol Cell Biol 20: 6555–5663.
7. Johnson RE, Washington MT, Haracska L, Prakash S, Prakash L (2000) Mutations in mismatch extension and for extension opposite from DNA lesions. Mol Cell Biol 20: 6555–5663.
8. Johnson RE, Washington MT, Haracska L, Prakash S, Prakash L (2000) Mutations in mismatch extension and for extension opposite from DNA lesions. Mol Cell Biol 20: 6555–5663.
9. Johnson RE, Washington MT, Haracska L, Prakash S, Prakash L (2000) Mutations in mismatch extension and for extension opposite from DNA lesions. Mol Cell Biol 20: 6555–5663.
50. Huang ME, Rio AG, Galibert MD, Galibert F (2002) Pol32, a subunit of *Saccharomyces cerevisiae* DNA polymerase delta, suppresses genomic deletions and is involved in the mutagenic bypass pathway. Genetics 160: 1409–1422.

51. Hall BM, Ma C, Liang P, Singh KK (2009) Fluctuation Analysis Calculator (FALCOR): a web tool for the determination of mutation rate using Luria-Delbrück fluctuation analysis. Bioinformatics 25: 1564–1565.

52. Aussel FM, Brent R, Kingston RE, Moore DD, Seidman JG, et al. (1994) Current Protocols in Molecular Biology. New York: Wiley.

53. Sherman F, Fink GR, Hicks JB (1986) Laboratory Course Manual for Methods in Yeast Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

54. Acharaya N, Haraczka L, Johnson RE, Unk I, Prakash S, et al. (2003) Complex formation of yeast Rev1 and Rev7 proteins: a novel role for the polymerase-associated domain. Mol Cell Biol 23: 9743–9744.

55. Zhao X, Muller EG, Rothstein R (1998) A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects G1/S progression. Mol Cell 2: 329–340.

56. Dingwall C, Robbins J, Dilworth SM, Roberts B, Richardson WD (1988) The DNA polymerase. Mol Cell Biol 8: 15225–15228.

57. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, et al. (2003) Global analysis of protein localization in budding yeast. Nature 425: 686–691.

58. Singhal RK, Hinkle DC, Lawrence CW (1992) The REV3 gene of *Saccharomyces cerevisiae* is transcriptionally regulated more like a repair gene than one encoding a DNA polymerase. Mol Gen Genet 236: 17–24.

59. Kientz MT, Tingle MA, Halvorson HO (1974) Sporulation of *Saccharomyces cerevisiae* in the absence of a functional mitochondrial genome. J Bacteriol 117: 80–88.

60. Codona AC, Gaseurt-Ramirez JM, Benitez T (1995) Factors which affect the frequency of sporulation and tetrad formation in *Saccharomyces cerevisiae* baker's yeasts. Appl Environ Microbiol 61: 630–638.

61. Ponnamarev MV, Longley MJ, Nguyen D, Kunkel TA, Copeland WC (2002) Active site mutation in DNA polymerase gamma associated with progressive external ophthalmoplegia causes error-prone DNA synthesis. J Biol Chem 77: 1435–1439.

62. Northam MR, Robinson HA, Kuchenova OV, Scherbakova PV (2010) Participation of DNA polymerase zeta in replication of undamaged DNA in *Saccharomyces cerevisiae*. Genetics 184: 27–42.

63. Kishimura H, Takahiro S, Okamoto K, Ishikawa T, Inoue K (2004) Nucleotide incorporation by human DNA polymerase zeta opposite benz[a]pyrene and benz[a]phenanthrene diolepoxide adducts of deoxyguanosine and deoxyadenosine. Nucleic Acids Res 32: 397–405.

64. Taylor SD, Zhang H, Eaton JS, Redheffer MS, Lebedeva MA, et al. (2005) The conserved Mecl/Rad53 nuclear checkpoint pathway regulates mitochondrial DNA copy number in *Saccharomyces cerevisiae*. Mol Cell Biol 15: 3010–3018.

65. Lebedeva MA, Shadel GS (2007) Cell cycle- and ribonucleotide reductase-driven changes in mitochondrial DNA copy number influence mtDNA Inheritance without compromising mitochondrial gene expression. Cell Cycle 6: 2048–2057.

66. Semlodi H, Di Re M, Bokori-Brown M, Holt IJ (2007) The yeast Holliday junction resolution, CCR4, can restore wild-type mitochondrial DNA to human cells carrying rearranged mitochondrial DNA. Hum Mol Genet 16: 2306–2319.

67. Gibson BR, Prescott KA, Smart KA (2008) Pol6 mutation in aged and oxidatively stressed ale and lager brewing yeast. Lett Appl Microbiol 46: 636–642.

68. Kiani A, Dzierzbicki P, Rogowska AT, Mâle E, Fikar M, et al. (2009) Msh1p counteracts oxidative lesion-induced instability of mtDNA and stimulates mitochondrial recombination in *Saccharomyces cerevisiae*. DNA Repair (Amst) 8: 318–329.

69. Eroo L, Farnum GA, Palin E, Suomalainen A, Kaguni LS (2011) Clustering of Alpers disease mutations and catalytic defects in biochemical variants reveal new features of molecular mechanism of the human mitochondrial replicase, Pol γ. Nucleic Acids Res 39: 9072–9084.

70. de Padula M, Sležák G, Auffert van Der Kemp P, Boiteux S (2004) The post-replication repair RAD13 and RAD6 genes are involved in the prevention of spontaneous mutations caused by 7,8-dihydro-8-oxoguanine and 8-oxo-7,8-dihydroguanine. Mol Cell Biol 24: 1455–1459.

71. Kienen MT, Tingle MA, Halvorson HO (1974) Sporulation of *Saccharomyces cerevisiae* in the absence of a functional mitochondrial genome. J Bacteriol 117: 80–88.

72. Northam MR, Robinson HA, Kuchenova OV, Scherbakova PV (2010) Participation of DNA polymerase zeta in replication of undamaged DNA in *Saccharomyces cerevisiae*. Genetics 184: 27–42.

73. Pinc KG, Shihabutani S, Bogdenhausen DF (1995) Action of mitochondrial DNA polymerase gamma at sites of base loss or oxidative damage. J Biol Chem 270: 9202–9206.

74. Graziewicz MA, Sayer JM, Jerina DM, Copeland WC (2004) Nucleotide incorporation by human DNA polymerase gamma opposite benz[a]pyrene and benz[a]phenanthrene diolepoxide adducts of deoxyguanosine and deoxyadenosine. Nucleic Acids Res 32: 397–405.

75. Kasiviswanathan R, Gustafson MA, Copeland WC, Meyer JN (2011) Human mitochondrial DNA polymerase γ exhibits potential for bypass and mutagenesis at UV-induced cyclobutane thymine dimers. J Biol Chem 10: 10.1074/jbc.M111.306852. jbc.M111.306852.

76. Taylor SD, Zhang H, Eaton JS, Redheffer MS, Lebedeva MA, et al. (2005) The conserved Mecl/Rad53 nuclear checkpoint pathway regulates mitochondrial DNA copy number in *Saccharomyces cerevisiae*. Mol Cell Biol 15: 3010–3018.

77. Lebedeva MA, Shadel GS (2007) Cell cycle- and ribonucleotide reductase-driven changes in mtDNA copy number influence mtDNA Inheritance without compromising mitochondrial gene expression. Cell Cycle 6: 2048–2057.

78. Semlodi H, Di Re M, Bokori-Brown M, Holt IJ (2007) The yeast Holliday junction resolution, CCR4, can restore wild-type mitochondrial DNA to human cells carrying rearranged mitochondrial DNA. Hum Mol Genet 16: 2306–2319.

79. Bahl S, Ahita A, Holinska-Feder E, Möller-Ziemann S, Koehler U, et al. (2009) In situ supplementation with dAMP/dGMP leads to partial restoration of mtDNA levels in mitochondrial deletion syndromes. Hum Mol Genet 18: 1590–1599.

80. Filosto M, Mancuso M, Nishigaki Y, Pancrudo J, Harayi V, et al. (2003) Clinical and genetic heterogeneity in progressive external ophthalmoplegia due to mutations in polymerase gamma. Arch Neurol 60: 1279–1284.

81. Hisama FM, Mancuso M, Filosto M, DiMauro S (2002) Progressive external ophthalmoplegia: a new family with tremor and peripheral neuropathy. Am J Med Genet A 135: 217–219.