Published in final edited form as:

DNA Repair (Amst). 2009 October 2; 8(10): 1242–1249. doi:10.1016/j.dnarep.2009.07.008.

Evidence for a Role of FEN1 in Maintaining Mitochondrial DNA Integrity

Lidza Kalifa¹, Gisela Beutner², Naina Phadnis¹, Shey-Shing Sheu², and Elaine A. Sia^{1,*}

¹ Department of Biology, University of Rochester, Rochester, NY 14627

² Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY 14642

Abstract

Although the nuclear processes responsible for genomic DNA replication and repair are well characterized, the pathways involved in mitochondrial DNA (mtDNA) replication and repair remain unclear. DNA repair has been identified as being particularly important within the mitochondrial compartment due to the organelle's high propensity to accumulate oxidative DNA damage. It has been postulated that continual accumulation of mtDNA damage and subsequent mutagenesis may function in cellular aging. Mitochondrial base excision repair (mtBER) plays a major role in combating mtDNA oxidative damage; however, the proteins involved in mtBER have yet to be fully characterized. It has been established that during nuclear long-patch (LP) BER, FEN1 is responsible for cleavage of 5' flap structures generated during DNA synthesis. Furthermore, removal of 5' flaps has been observed in mitochondrial extracts of mammalian cell lines; yet, the mitochondrial localization of FEN1 has not been clearly demonstrated. In this study, we analyzed the effects of deleting the yeast FEN1 homolog, *RAD27*, on mtDNA stability in *Saccharomyces cerevisiae*. Our findings demonstrate that Rad27p/FEN1 is localized in the mitochondrial compartment of both yeast and mice and that Rad27p has a significant role in maintaining mtDNA integrity.

Introduction

Mitochondrial DNA (mtDNA) incurs multiple forms of damage from both physiological and environmental sources. Since the presence of the mitochondrial genome is vital for eukaryotic viability, processes that maintain the integrity of mtDNA can likewise have global effects on cellular stability. The importance of mtDNA is underpinned by the identification of mtDNA mutations which result in a number of known genetic diseases. Additionally, the accumulation of mtDNA damage has been proposed to contribute to aging and age-related disorders, underscoring the importance of mtDNA maintenance for normal cellular function [1].

Progress in this field is revealing several pathways that are responsible for maintenance of the mitochondrial genome. Mitochondrial base excision repair (mtBER) and the proofreading 3'–5' exonuclease activity of the mitochondrial polymerase, Pol γ , are known to impact mtDNA maintenance [2–4]. In addition, recent work has begun to elucidate other possible mechanisms

Corresponding Author: Elaine A. Sia, University of Rochester, Rochester, NY 14627, Tel: 585-275-9275, Fax: 585-275-2070, esia@mail.rochester.edu.

Conflict of Interest Statement. The authors declare that there are no conflicts of interest.

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

for processing mtDNA lesions, including translesion DNA synthesis and double-strand break repair [5–9].

Of these multiple repair pathways, mtBER may be particularly significant. MtDNA is thought to be constantly bombarded with reactive oxygen species (ROS) due to its close proximity to the electron transport chain [10]. Studies have indicated that the mtBER pathway is largely responsible for repairing the oxidative damage to mtDNA [2,11,12]. Until recently, short-patch (SP) mtBER was the only accepted mtBER pathway, in which damaged nucleotides are replaced one at a time. There is now substantial evidence for long-patch (LP) BER in the mitochondrial compartment [13–15]; although the proteins involved in this pathway are disputed.

Analysis of yeast and mammalian nuclear BER indicates that the FEN1 flap endonuclease is responsible for cleavage of the 5' flap produced by LP BER [16]. The FEN1 family of proteins are evolutionarily conserved from archaeobacteria to humans and are involved in DNA replication and repair [17–19]. The *Saccharomyces cerevisiae* FEN1 ortholog, Rad27p, plays an integral role in cleavage of 5' DNA flaps created during Okazaki fragment processing [20–24], the processing of intermediates during nuclear LP BER [16,25], prevention of sequence duplications and repeat sequence expansions [17,25–30], as well as being implicated in double-strand break repair [18,27,31].

In contrast to the roles of FEN1 in nuclear DNA maintenance, the full extent of FEN1 utilization in the mitochondria is unclear. Recent studies demonstrated that a 5' flap removal activity exists in mitochondrial extracts of HeLa [13] and HCT116 [15] cell lines; however, these authors concluded that this activity was not due to FEN1. In contrast, another study identified FEN1 in mitochondrial extracts and concluded that LP mtBER is strongly dependent on FEN1 activity [14]. Although these studies provide clear evidence for LP mtBER, they differ in the enzyme proposed to cleave the 5' flaps. Here we use genetic and physiological analysis to examine the role of the yeast flap endonuclease Rad27p in mtDNA maintenance. We demonstrate, for the first time, that deletion of *RAD27* impacts the frequency of multiple types of mtDNA mutations, suggesting direct involvement in mtDNA repair. Additionally, we show evidence for Rad27p localization to yeast mitochondria through fractionation and fluorescence microscopy. Finally, we show evidence for mitochondrial localization of FEN1 in different types of mouse tissue, suggesting that its role in mtDNA maintenance is functionally conserved. These studies support a role for Rad27p/FEN1 in LP BER of mtDNA.

Results

Deletion of *RAD27* Results in Mitochondrial DNA Mutations

The mitochondrial genome encodes some of the components of the electron transport chain (ETC) as well as the tRNAs and rRNAs required for their translation. Specific point mutations, deletions, rearrangements, or complete loss of the mitochondrial genome may all impair the function of the ETC and lead to cellular respiration deficiency. To determine if deletion of *RAD27* caused mitochondrial genome instability, we measured the frequency of respiration loss, which is a functional correlate of major mtDNA defects. Since yeast are facultative anaerobes, we assess the respiration status of cells by their ability to grow on a non-fermentable carbon source. We found that the *rad27-Δ* strain showed no significant difference in respiration loss as compared to wild-type (2.5% and 3.4%, respectively; $P = 0.26$), indicating that deletion of *RAD27* does not affect general mtDNA maintenance. Since yeast cells contain approximately 50 copies of the mitochondrial genome per haploid cell [32] and it is unknown how many defective genomes need to accumulate in order to observe a measurable respiration loss, we proceeded to investigate specific types of mitochondrial mutations.

It has been documented that deletion of *RAD27* results in 30 to 150-fold increases in the rate of nuclear point mutations [26,27,33,34]. To determine if deleting *RAD27* caused a similar change in the rate of mitochondrial point mutations, we performed fluctuation analysis to measure the rate of erythromycin resistance (E^R). Erythromycin is an antibiotic which targets the 21S rRNA in the mitochondrial compartment and specifically inhibits mitochondrial protein synthesis. As a result, E^R arises from specific mutations in the 21S rRNA locus in the mitochondrial genome and nuclear mutations conferring E^R have not been reported [6,35–38]. We found that a *rad27*- Δ strain displayed a rate of 84.8×10^{-7} mutations per cell division, which is a 10.5-fold increase in E^R as compared to the wild-type rate of 8.0×10^{-7} ($P < 0.03$).

Since deletion of *RAD27* results in a reduced growth rate [18,19], we decided to investigate whether additional mitochondrial point mutations accumulate over time. We observed that while the wild-type strain does not display an increase in mtDNA point mutations, the *rad27*- Δ strain showed significantly more mtDNA point mutations over time, suggesting that our original rate may be an underestimate of the mtDNA point mutations occurring in this strain (Figure 1A).

To determine if the E^R mutants that accumulate over time in the *rad27*- Δ strain are due to a slower growth rate or are arising later, we calculated the doubling times of E^R mutants that were obtained after 7 and 14 day incubations. The mutants obtained at 14 days were not visible at 7 days. We observed that the doubling times of the wild type and *rad27*- Δ E^R colonies that arose after 7 or 14 days differ significantly in that the *rad27*- Δ E^R strains have an increased doubling time relative to wild type ($P < 0.01$, in both cases). However, there is no significant difference in the growth rate between the *rad27*- Δ E^R mutants that arise after 7 or 14 days ($P = 0.40$) indicating that the E^R colonies that appear later in this strain are truly arising late and not simply slower growing.

Due to variability in yeast laboratory strains, we also measured the rate of nuclear mutations by canavanine resistance (Can^R), to confirm that our *rad27*- Δ strain shows rates similar to those reported previously [26,27,33,34]. We found that a *rad27*- Δ strain displayed a rate of 22.2×10^{-6} mutations per cell division which is a 103-fold increase in Can^R as compared to the wild-type rate of 21.6×10^{-8} ($P < 0.01$), consistent with previously published results [26, 27,33,34]. Analysis of nuclear point mutations after increased incubation times demonstrates that deletion of *RAD27* does not result in a significant increase in nuclear point mutations over time as it does for mitochondrial mutations (Figure 1B).

Nuclear phenotypes of *RAD27* mutants include an increase in nuclear recombination [18]. To determine the effects of *RAD27* deletion on recombination in both compartments, we constructed a strain harboring analogous direct-repeat reporters in the nucleus and the mitochondria (Figure 2A, B). The nuclear reporter consists of a *URA3* insertion into the *TRP1* gene such that 96 bp direct repeats of *TRP1* sequence flank *URA3*, resulting in a Ura^+/Trp^- phenotype. Recombination between these repeats eliminates *URA3* and restores functional *TRP1*, resulting in a Ura^-/Trp^+ phenotype. The mitochondrial direct-repeat reporter utilizes an *ARG8^m* insertion into the *COX2* gene, resulting in 96 bp of directly repeated *COX2* sequence as described previously [7]. Cells harboring both nuclear and mitochondrial intact reporters are $Ura^+/Arg^+/Trp^-$ and respiratory deficient. Maintenance of these reporters in the same strain allows for simultaneous measurement of both nuclear and mitochondrial direct-repeat mediated deletions (DRMD). Using this strain, we observed a 81.4-fold increase in nuclear DRMD and a 49.3-fold decrease in mitochondrial DRMD relative to wild type (Figure 2C). Both nuclear and mitochondrial DRMD rates in the *rad27*- Δ strain differ significantly from the wild-type rates ($P < 0.01$, in both cases).

RAD27 mutants cause an increase in nuclear repeat instability and repeat expansion [26–30]. To test the effects of the *rad27-Δ* allele on mitochondrial microsatellite instability, we used the mitochondrial reporters *arg8^m::(GT)₁₆₊₁* [39] and *arg8^m::(GT)₁₆₊₂* [6] to measure frameshifts within repetitive tracts (Figure 3A, B). These reporters contain a 32 bp dinucleotide repeat tract inserted into the *ARG8^m* gene, shifting the remaining sequence out of frame. Instability within this microsatellite tract that restores the *ARG8^m* reading frame, gives rise to Arg⁺ colonies. We observed that, in marked contrast to its nuclear phenotype, deletion of *RAD27* causes a significant 9.5-fold decrease ($P < 0.03$) and a 1.5-fold decrease ($P < 0.05$) in the median frequency of alterations in the *arg8^m::(GT)₁₆₊₁* and *arg8^m::(GT)₁₆₊₂* frameshift reporters, respectively (Figure 3C).

Rad27p Localizes to Yeast Mitochondria

We hypothesized that if Rad27p directly affects mtDNA stability, it would colocalize with mitochondrial structures. A previous large-scale screen in yeast found that Rad27p-GFP localizes to the nucleus [40]. However, this study also characterized Pif1p-GFP as a nuclear protein, although its dual localization to the mitochondria and nucleus has been confirmed [41,42]. To re-examine the localization of Rad27p, we constructed a C-terminal *RAD27*-GFP fusion integrated into the yeast genome, expressed from its own promoter. The expression of this construct allowed us to determine the cellular localization of Rad27p by fluorescence microscopy. Cells were grown aerobically in a non-fermentable carbon source and stained with DAPI, a fluorescent DNA binding dye, and Mitotracker, a dye that stains mitochondrial structures. The signal observed in the untagged *RAD27* strain demonstrates the level of background fluorescence produced by the yeast cells (Figure 4A). The images in Figure 4A *RAD27*-GFP are consistent with nuclear and mitochondrial staining. We show that Rad27p-GFP concentrates in the nucleus, however, it also colocalizes with the punctate staining of DAPI and Mitotracker, indicating that it also associates with mitochondria. To confirm that Rad27p-GFP is co-localizing to nuclear and mitochondrial structures, we analyzed GFP localization of a mitochondrial-specific protein Abf2p using a C-terminal *ABF2*-GFP fusion and nuclear GFP localization using a plasmid-based NLS-GFP fusion construct. The Abf2p-GFP displays punctate staining which colocalizes with mitochondrial structures based on DAPI and Mitotracker staining of live yeast cells. The nuclear control, NLS-GFP is small enough to diffuse through the nuclear pore channels and its retention in the nucleus is an active process, as a result some cytoplasmic fluorescence will be observed [43].

To support this finding, we next purified yeast mitochondria, in cells expressing a C-terminal *RAD27*-HA fusion expressed from the endogenous *RAD27* locus. Equal amounts of protein for whole cell extracts, crude mitochondria, and pure mitochondria were analyzed by western blot using anti-HA antibody (Figure 4B). We observed an enrichment of Rad27p in both crude and pure mitochondrial fractions relative to the whole cell extract. Antibodies against Por1p, an outer mitochondrial membrane (OMM) protein, and Cox3p, an integral inner mitochondrial membrane (IMM) protein, confirm that we are concentrating mitochondria in our purification. Probing with antibody against histone H4 verifies that the mitochondrial fractions are devoid of significant nuclear contamination. The enrichment indicates that the fraction of Rad27p in the mitochondria is greater than the fraction of Rad27p present in the whole cell. To confirm that Rad27p resides within the mitochondrial compartment, we performed a protease protection assay (Figure 4C). Using our crude mitochondrial fraction, we found that Rad27p is protected from proteinase K digestion. This protection is lost when mitochondria are lysed prior to proteinase K treatment, confirming localization of Rad27p within the mitochondrial compartment.

FEN1 Co-fractionates with Mouse Mitochondria

To determine if mitochondrial localization of FEN1 is conserved in mice, we isolated mitochondria from mouse brain, heart, and kidney (Figure 5A). We find that FEN1 is present in mitochondrial fractions of all these mouse tissues. To determine if FEN1 is present within the mitochondrial compartment, we treated mouse brain mitochondria with 10 µg/mL proteinase K for up to 30 minutes (Figure 5B). We determined that FEN1 remains present in the mitochondrial fraction even after protease digestion. The detection of the OMM protein VDAC is significantly reduced in the proteinase K treated samples, however the detection of Cytochrome C remained unchanged, suggesting that our proteinase K treatment removed proteins which were bound or loosely associated with the OMM, but did not disturb the integrity of the inner membrane. Therefore, this result is indicative of FEN1 localization within the mitochondrial compartment of mouse brain tissue.

Discussion

FEN1 activity is critical for the normal maintenance of nuclear DNA. This enzyme has demonstrated roles in genome replication, DNA repair, and recombination. A role for FEN1 in mtDNA maintenance has only recently been suggested [14], however, the mitochondrial subcellular localization of FEN1 has been debated [13–15]. Here we corroborate the findings of Liu *et al.* 2008 [14] with our genetic analysis of *RAD27* deletion strains, and validate the localization of FEN1 in yeast and mice. We have positively identified FEN1 in mitochondrial fractions of yeast and mice and found that it colocalizes with yeast mitochondrial structures by fluorescence microscopy.

Our comprehensive genetic analysis of *RAD27* deletion strains supports a mitochondrial role for Rad27p, and shows that this protein may perform different functions in the nuclear and mitochondrial compartments. While previous studies of *RAD27* mutant strains demonstrate increases in various types of nuclear mutations including recombination, point mutations, reversions, and frameshifts [17,18,25–30,33,34]; our study reveals increases in mitochondrial point mutation accumulation but decreases in mitochondrial DRMD and mitochondrial microsatellite instability.

Unlike nuclear DNA, mtDNA in yeast is dispensable under laboratory growth conditions. As a result, mtDNA can be lost when cells are provided with a fermentable carbon source such as dextrose, resulting in non-respiring cells called *petites*. If Rad27p plays a critical role in mtDNA replication, we would expect loss of the mitochondrial genome at a high frequency under non-selective conditions in a *RAD27* mutant. Since the *rad27-Δ* strain does not show an increase in the frequency of *petites*, we conclude that Rad27p does not play a significant role in mtDNA replication, or that there is redundancy in its function in mtDNA replication in yeast. However, our genetic data do suggest a role for Rad27p in mtDNA repair, consistent with the *in vitro* evidence implicating FEN1 in mitochondrial LP BER [14].

We observe a significant increase in mitochondrial mutations resulting in erythromycin resistance. While our increase in mtDNA point mutations seems to be at odds with the observation that deletion of *RAD27* does not increase respiration loss, it is important to note that respiration loss primarily results from large-scale deletions and rearrangements of the mitochondrial genome and is measured as percent of total cells. In contrast, erythromycin resistance results specifically from point mutations, and is measured on a scale of 10^{-7} cells. Therefore these events are mechanistically distinct, and even dramatic increases in mtDNA point mutations may not induce significant increases in respiration loss; furthermore, not all mtDNA point mutations give rise to respiratory deficient cells.

Because the *rad27-Δ* strains have reduced growth rates relative to the wild-type strains, we tested whether the number of E^R colonies continued to increase after increased incubation times up to 21 days. We found that deletion of *RAD27* causes a significant increase in mtDNA point mutations that continue to accumulate over time, and this phenomenon is not evident for nuclear point mutations. These experiments are performed on solid medium, so the additional mutants that arise are independently-derived. In addition, because nuclear mutations do not increase during these extended incubations, the E^R mutants cannot arise as an indirect consequence of nuclear mutations. How these mutations continue to accumulate is not clear, however, mtDNA replication is not tied to cell cycle regulation [44] and mtDNA replication has been demonstrated in post-mitotic eukaryotic cells [45]. In the *rad27-Δ* yeast strain there may be increased turnover of mtDNA even in cells that are no longer dividing, resulting from a failure to complete BER.

We have developed a powerful reporter system that allows us to measure the rates of nuclear and mitochondrial DRMD within the same cells. Using these reporters, we show that deletion of *RAD27* results in a nuclear hyper-recombination phenotype, and simultaneously causes a reduction in mitochondrial DRMD. This suggests that the mechanism of nuclear and mitochondrial DRMD may differ significantly. In addition, Rad27p acts to promote mitochondrial DRMD, although this effect may result from direct action of the enzyme on recombination intermediates, indirect effect of interaction with mitochondrial recombinational repair proteins, or competition for mtDNA substrates.

Nuclear repeat tract expansion in *RAD27* mutants is attributed to the role of Rad27p in Okazaki fragment maturation, and it is thought that the expansion occurs on the lagging strand during DNA replication [46]. Using our mitochondrial microsatellite reporter system, we observed a decrease in microsatellite instability in *RAD27* deficient strains. However, the mechanism by which Rad27p may affect these types of mutations in the nucleus and mitochondria is likely to differ due to the different modes of DNA replication in these compartments, or because Rad27p may not play a critical role in mtDNA replication.

We have employed several different techniques to investigate the mitochondrial subcellular localization of Rad27p/FEN1. In yeast, we found that Rad27p is localized to mitochondria using western blotting and fluorescence microscopy. Furthermore, we have observed FEN1 in mitochondria purified from mouse brain, heart, and kidney tissue, and we show that in yeast and mouse brain mitochondria FEN1 is protected from protease digestion indicative of its localization within the mitochondrial compartment. These data support our hypothesis that Rad27p impacts mtDNA mutation frequencies via direct interaction with mitochondrial substrates.

For some time, nuclear and mitochondrial BER have been known to share protein components. Most mammalian mitochondrial N-glycosylases are products of alternative splicing of the same transcript that encodes the nuclear enzymes [47–50]. Similarly, the yeast mitochondrial N-glycosylase proteins Ogg1p, Ung1p, and Ntg1p are also isoforms of nuclear proteins [51–53]. The identification of a role for Rad27p in mitochondrial LP BER is perhaps more significant, however, given its important role in other pathways of nuclear DNA maintenance, and has raised the possibility of its action in similar mitochondrial pathways. Our results are consistent with those of Liu *et al.* [14], and suggest that the endo/exonuclease activity of Rad27p may be important in mitochondrial LP BER, however, further studies need to be conducted to determine if Rad27p performs additional functions.

Material and Methods

Growth Media and Strains

All growth media used in this study were previously described [6]. All *S. cerevisiae* strains used in this study (Table 1) are isogenic with DFS188 (*MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG*; ρ^+), a derivative of D273-10B, except EAS736 and EAS738, which are derived from DFS160 (*MATaade2-101 leu2 Δ ura3-52 arg8- Δ ::URA3 kar1-1*) [54]. The *rad27- Δ* strain was constructed by one-step gene transplacement of the wild-type gene with the *kanMX* marker using standard methods [55]. The *RAD27*-citrine-3HA and *RAD27*-yEGFP gene fusions were constructed by one-step gene insertion. Primers containing 40 bp 5' of the *RAD27* stop codon and 40 bp 3' of the stop codon were used to amplify the citrine-3HA sequence from plasmid pKT220 and the yEGFP from plasmid pKT209 of the Sheff and Thorn plasmid collection [56]. The *ABF2*-GFP gene fusion was constructed by amplifying the GFP-*HIS3MX6* sequence of *ABF2* from the UCSF yeast GFP clone collection (clone ID YMR072W) from Invitrogen [40] and integrating it into DFS188 using one-step gene insertion. The NLS-GFP plasmid as described in [43] was transformed into DFS188 and selected for on synthetic medium lacking leucine.

The DRMD reporter (LKY196) was constructed by cloning 80 bp 5' of the start to the first 99 bp of the *TRP1* gene followed by 231 bp 5' of the start to 86 bp 3' of the stop of the *URA3* gene followed by 4 bp 5' to 532 bp 5' of the start of *TRP1* into pBlueScript II SK⁺. This plasmid was subsequently digested with *Bam*HI and *Hind*III to isolate the reporter construct. The fragment was transformed into EAS748 by standard methods and transformants were selected on medium lacking uracil and arginine. The LKY196 (DFS188 REP96::*URA3::trp1, mit⁻ REP96::ARG8^m::cox2*) strain was confirmed by standard Southern blot analysis using a 179 bp *TRP1* fragment which includes 80 bp 5' of the start to the first 99 bp of the *TRP1* gene. The wild-type *RAD27* gene was subsequently deleted in this strain as described above.

To introduce the mitochondrial frameshift reporter constructs by cytoduction, strains were first made ρ^0 by treatment with ethidium bromide as described previously [57]. These strains were then mated to the DFS160 reporter constructs EAS736 (*arg8^m::(GT)₁₆+2*) or EAS738 (*arg8^m::(GT)₁₆+1*). Haploid cytoductants with the DFS188 nuclear background and the mitochondrial reporter were selected on synthetic glycerol medium lacking adenine and were screened for a Lys⁻ phenotype on synthetic dextrose lacking lysine.

Measuring Mitochondrial and Nuclear Mutations

Determination of the frequency of respiration loss and mitochondrial frameshifts in the *arg8^m::(GT)₁₆+1* and *arg8^m::(GT)₁₆+2* microsatellite tracts were determined as previously described [58,59]. To determine the rate of mtDNA point mutations, independent colonies were isolated on YPD, inoculated into YPD liquid medium, and grown to saturation at 30 °C. Appropriate dilutions were plated on YG and YG + 4 g/L erythromycin. Plates were incubated at 30 °C for 7 days. The rate of erythromycin resistance (E^R) was determined using the method of the median [60]. The average frequency of E^R over time was performed as above, except total colony forming units on the YG + erythromycin plates were determined after 7, 10, 14, 17, and 21 days. The growth rate of E^R mutants was determined by growing independent E^R colonies in YG + 4 g/L erythromycin and measuring the optical density over time. Doubling time was calculated using the log phase growth of the culture and performed using at least two independent E^R colonies. The rates of nuclear point mutation accumulation were determined as previously described [61]. The average frequency of Can^R over time was performed as above, except total colony forming units on the SD-Arg + 60 μ g/mL canavanine plates were determined after 3, 6, and 9 days.

The rate of nuclear and mitochondrial repeat-mediated deletions was determined in strains containing both reporters. Individual colonies were isolated on YPD, resuspended in water, and appropriate dilutions were plated on SD-Trp for nuclear recombinants, YG for mitochondrial recombinants, and YPD for total viable cells. All plates were incubated at 30°C for 3 days, then the rate of nuclear and mitochondrial recombination was determined using the method of the median [60].

For each experiment, 10–20 independent cultures were used and each experiment was performed 2 or more times. All statistical analyses were performed using InStat 3 for Macintosh (GraphPad Software, Inc., San Diego, CA). Unpaired t-tests were used to calculate a two-tailed *P* value in the comparison of average or median rates and frequencies.

Microscopy

Yeast strains were grown overnight at 30°C then diluted and grown to early log phase. Strains were grown in synthetic complete medium containing 2% glycerol, except LKY527 which was grown in synthetic medium lacking leucine containing 2% glycerol. Cells were first supplemented with 100 pM Mitotracker (Molecular Probes, Eugene, OR) and incubated for 30 min. at 30°C and secondly with 10 nM DAPI and incubated for an additional 5 min. Selective mtDNA staining with DAPI is achieved by the short incubation time in live cells. Cells were harvested and washed twice with 1X PBS. Fluorescence microscopy was performed on a Zeiss Axioplan 2 microscope. The exposure settings for DIC 0.006 sec with 4% gain and 32% offset, DAPI 0.050 sec 68% gain and 0% offset, GFP 0.300 sec with 30% gain and 0% offset, and Mitotracker Red 0.500 sec with 30% gain and 0% offset.

Isolation of Mitochondria

Whole cell extracts from wild-type and *RAD27*-citrate-3HA yeast strains were prepared by cell disruption with glass beads as described previously [62]. To obtain crude and pure mitochondrial fractions, cells were grown in synthetic glycerol complete medium in a Microferm fermentor to OD₆₀₀ ~ 1.5. The 11 L of cells were concentrated and mitochondria were isolated by differential centrifugation and further purified on a sucrose gradient as described by Meisinger *et al.* [63]. 100 µg of total protein for whole cell extracts, crude, and pure mitochondrial fractions were analyzed by western blot analysis. Yeast mitochondrial protease protection assay was performed as described in [64] using 1 mg crude mitochondrial extract. 20 µg or 100 µg of sample was subjected to western blot analysis.

Intact functional mitochondria were isolated from the brain, heart, and kidneys of 6 week old male mice. Isolation of mitochondria from the brain and heart was performed by methods described by Rehncrona *et al.* 1979 [65] and from the kidneys by Pallotti and Lenaz 2001 [66]. 50 µg of total protein from each sample was subjected to western blot analysis. Freshly isolated brain mitochondria were treated with 10 µg/mL proteinase K for up to 30 minutes. After the indicated time, a 10-fold excess of phenylmethanesulfonyl fluoride (PMSF) was added to each sample. For the 0 time point, Proteinase K was added to this sample just prior to the addition of the PMSF. The samples were centrifuged for 5 minutes at 13,000 g to separate the mitochondrial sediment from the digested proteins. 30 µg of total protein from each sample was subjected to western blot analysis.

Samples were subjected to electrophoresis on 10% or 12% SDS-PAGE and transferred to nitrocellulose membranes. Antibodies used for detection are as follows: 1:2500 dilution of anti-HA mouse HRP-conjugated primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:1500 dilution of anti-Por1p mouse monoclonal antibody (Molecular Probes, Eugene, OR), 1:500 dilution of anti-Cox3p mouse monoclonal antibody (Molecular Probes, Eugene, OR), 1:2500 to 1:5000 dilution of anti-FEN1 rabbit polyclonal antibody (Abcam, Cambridge, MA),

1:200 dilution of anti-VDAC rabbit polyclonal antibody (Abcam, Cambridge, MA), 1:2000 dilution of anti-VDAC mouse monoclonal antibody (Calbiochem, Gibbstown, NJ), 1:2000 dilution of anti-Cytochrome C mouse monoclonal antibody (BD Bioscience, San Jose, CA or Abcam, Cambridge, MA), 1:500 dilution of anti-histone H4 rabbit polyclonal antibody (Abcam, Cambridge, MA), 1:10,000 dilution of anti-mouse HRP-conjugated secondary antibody (Abcam, Cambridge, MA), and 1:2000 dilution of anti-rabbit HRP-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA).

Acknowledgments

Our research was supported by US National Science Foundation grant MCB0543084 (E.A.S.), US National Institutes of Health grants HL-33333 (S-S.S.) and 1 F31 GM078700 (L.K.). We are grateful to Dr. Robert Maul and Dr. Shona Mookerjee for critical reading of the manuscript and helpful comments and Dr. Thomas D. Fox for the gift of the anti-Cit1p antibody.

References

- Wallace DC. Mitochondrial Diseases in Man and Mouse. *Science* 1999;283:1482–1488. [PubMed: 10066162]
- Bohr VA. Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. *Free Radic Biol Med* 2002;32:804–812. [PubMed: 11978482]
- Hu JP, Vanderstraeten S, Foury F. Isolation and characterization of ten mutator alleles of the mitochondrial DNA polymerase-encoding MIP1 gene from *Saccharomyces cerevisiae*. *Gene* 1995;160:105–110. [PubMed: 7628702]
- Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly-Y M, Gidlof S, Oldfors A, Wibom R, Tornell J, Jacobs HT, Larsson N-G. Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 2004;429:417–423. [PubMed: 15164064]
- Zhang H, Chatterjee A, Singh KK. *Saccharomyces cerevisiae* Polymerase Zeta Functions in Mitochondria. *Genetics* 2006;172:2683–2688. [PubMed: 16452144]
- Kalifa L, Sia EA. Analysis of Rev1p and Pol zeta in mitochondrial mutagenesis suggests an alternative pathway of damage tolerance. *DNA Repair (Amst)* 2007;6:1732–1739. [PubMed: 17689152]
- Phadnis N, Sia RA, Sia EA. Analysis of Repeat-Mediated Deletions in the Mitochondrial Genome of *Saccharomyces cerevisiae*. *Genetics* 2005;71:1549–1559. [PubMed: 16157666]
- Morel F, Renoux M, Lachaume P, Alziari S. Bleomycin-induced double-strand breaks in mitochondrial DNA of *Drosophila* cells are repaired. *Mutat Res*. 2007
- Pinz KG, Shibutani S, Bogenhagen DF. Action of mitochondrial DNA polymerase gamma at sites of base loss or oxidative damage. *J Biol Chem* 1995;270:9202–9206. [PubMed: 7721837]
- Stuart JA, Brown MF. Mitochondrial DNA maintenance and bioenergetics. *Biochim Biophys Acta* 2006;1757:79–89. [PubMed: 16473322]
- Croteau DL, Stierum RH, Bohr VA. Mitochondrial DNA repair pathways. *Mutat Res* 1999;434:137–148. [PubMed: 10486588]
- Bohr VA, Stevnsner T, de Souza-Pinto NC. Mitochondrial DNA repair of oxidative damage in mammalian cells. *Gene* 2002;286:127–134. [PubMed: 11943468]
- Akbari M, Visnes T, Krokan HE, Otterlei M. Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis. *DNA Repair (Amst)* 2008;7:605–616. [PubMed: 18295553]
- Liu P, Qian L, Sung JS, de Souza-Pinto NC, Zheng L, Bogenhagen DF, Bohr VA, Wilson DM 3rd, Shen B, Demple B. Removal of Oxidative DNA Damage via FEN1-Dependent Long-Patch Base Excision Repair in Human Cell Mitochondria. *Mol Cell Biol*. 2008
- Szczesny B, Tann AW, Longley MJ, Copeland WC, Mitra S. Long patch base excision repair in mammalian mitochondrial genomes. *J Biol Chem* 2008;283:26349–26356. [PubMed: 18635552]
- Wu X, Wang Z. Relationships between yeast Rad27 and Apn1 in response to apurinic/apyrimidinic (AP) sites in DNA. *Nucleic Acids Res* 1999;27:956–962. [PubMed: 9927726]

17. Liu Y, Kao HI, Bambara RA. Flap endonuclease 1: a central component of DNA metabolism. *Annu Rev Biochem* 2004;73:589–615. [PubMed: 15189154]
18. Sommers CH, Miller EJ, Dujon B, Prakash S, Prakash L. Conditional lethality of null mutations in RTH1 that encodes the yeast counterpart of a mammalian 5'- to 3'-exonuclease required for lagging strand DNA synthesis in reconstituted systems. *J Biol Chem* 1995;270:4193–4196. [PubMed: 7876174]
19. Reagan MS, Pittenger C, Siede W, Friedberg EC. Characterization of a mutant strain of *Saccharomyces cerevisiae* with a deletion of the *RAD27* gene, a structural homolog of the *RAD2* nucleotide excision repair gene. *J Bacteriol* 1995;177:364–371. [PubMed: 7814325]
20. Zhu FX, Biswas EE, Biswas SB. Purification and characterization of the DNA polymerase alpha associated exonuclease: the RTH1 gene product. *Biochemistry* 1997;36:5947–5954. [PubMed: 9166764]
21. Turchi JJ, Huang L, Murante RS, Kim Y, Bambara RA. Enzymatic completion of mammalian lagging-strand DNA replication. *Proc Natl Acad Sci U S A* 1994;91:9803–9807. [PubMed: 7524089]
22. Waga S, Bauer G, Stillman B. Reconstitution of complete SV40 DNA replication with purified replication factors. *J Biol Chem* 1994;269:10923–10934. [PubMed: 8144677]
23. Ishimi Y, Claude A, Bullock P, Hurwitz J. Complete enzymatic synthesis of DNA containing the SV40 origin of replication. *J Biol Chem* 1988;263:19723–19733. [PubMed: 2848839]
24. Ayyagari R, Gomes XV, Gordenin DA, Burgers PM. Okazaki fragment maturation in yeast. I. Distribution of functions between FEN1 AND DNA2. *J Biol Chem* 2003;278:1618–1625. [PubMed: 12424238]
25. Rossi ML, Bambara RA. Reconstituted Okazaki fragment processing indicates two pathways of primer removal. *J Biol Chem* 2006;281:26051–26061. [PubMed: 16837458]
26. Johnson RE, Kovvali GK, Prakash L, Prakash S. Requirement of the yeast RTH1 5' to 3' exonuclease for the stability of simple repetitive DNA. *Science* 1995;269:238–240. [PubMed: 7618086]
27. Tishkoff DX, Filosi N, Gaida GM, Kolodner RD. A novel mutation avoidance mechanism dependent on *S. cerevisiae* *RAD27* is distinct from DNA mismatch repair. *Cell* 1997;88:253–263. [PubMed: 9008166]
28. Kokoska RJ, Stefanovic L, Tran HT, Resnick MA, Gordenin DA, Petes TD. Destabilization of yeast micro- and minisatellite DNA sequences by mutations affecting a nuclease involved in Okazaki fragment processing (rad27) and DNA polymerase delta (pol3-t). *Mol Cell Biol* 1998;18:2779–2788. [PubMed: 9566897]
29. Callahan JL, Andrews KJ, Zakian VA, Freudenreich CH. Mutations in yeast replication proteins that increase CAG/CTG expansions also increase repeat fragility. *Mol Cell Biol* 2003;23:7849–7860. [PubMed: 14560028]
30. Yang J, Freudenreich CH. Haploinsufficiency of yeast FEN1 causes instability of expanded CAG/CTG tracts in a length-dependent manner. *Gene* 2007;393:110–115. [PubMed: 17383831]
31. Wu X, Wilson TE, Lieber MR. A role for FEN-1 in nonhomologous DNA end joining: the order of strand annealing and nucleolytic processing events. *Proc Natl Acad Sci U S A* 1999;96:1303–1308. [PubMed: 9990019]
32. Ejchart A, Putrament A. Mitochondrial mutagenesis in *Saccharomyces cerevisiae*. I. Ultraviolet radiation. *Mutation Research* 1979;60:173–180. [PubMed: 379626]
33. Greene AL, Snipe JR, Gordenin DA, Resnick MA. Functional analysis of human FEN1 in *Saccharomyces cerevisiae* and its role in genome stability. *Hum Mol Genet* 1999;8:2263–2273. [PubMed: 10545607]
34. Xie Y, Liu Y, Argueso JL, Henricksen LA, Kao HI, Bambara RA, Alani E. Identification of rad27 mutations that confer differential defects in mutation avoidance, repeat tract instability, and flap cleavage. *Mol Cell Biol* 2001;21:4889–4899. [PubMed: 11438646]
35. Sor F, Fukuhara H. Identification of two erythromycin resistance mutations in the mitochondrial gene coding for the large ribosomal RNA in yeast. *Nucleic Acids Research* 1982;10:6571–6577. [PubMed: 6757866]
36. Sor F, Fukuhara H. Erythromycin and spiramycin resistance mutations of yeast mitochondria: nature of the *rib2* locus in the large ribosomal RNA gene. *Nucleic Acids Research* 1984;12:8313–8318. [PubMed: 6095195]

37. Cui Z, Mason TL. A single nucleotide substitution at the *rib2* locus of the yeast mitochondrial gene for 21S rRNA confers resistance to erythromycin and cold-sensitive ribosome assembly. *Current Genetics* 1989;16:273–279. [PubMed: 2697468]
38. Vanderstraeten S, Van den Brule S, Hu J, Foury F. The Role of 3'-5' Exonucleolytic Proofreading and Mismatch Repair in Yeast Mitochondrial DNA Error Avoidance. *Journal of Biological Chemistry* 1998;273:23690–23697. [PubMed: 9726974]
39. Mookerjee SA, Lyon HD, Sia EA. Analysis of the functional domains of the mismatch repair homologue Msh1p and its role in mitochondrial genome maintenance. *Curr Genet* 2005;47:84–99. [PubMed: 15611870]
40. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK. Global analysis of protein localization in budding yeast. *Nature* 2003;425:686–691. [PubMed: 14562095]
41. Lahaye A, Stahl H, Thines-Sempoux D, Foury F. PIF1: a DNA helicase in yeast mitochondria. *EMBO J* 1991;10:997–1007. [PubMed: 1849081]
42. Zhou J, Monson EK, Teng SC, Schulz VP, Zakian VA. Pif1p helicase, a catalytic inhibitor of telomerase in yeast. *Science* 2000;289:771–774. [PubMed: 10926538]
43. Shulga N, Roberts P, Gu Z, Spitz L, Tabb MM, Nomura M, Goldfarb DS. In vivo nuclear transport kinetics in *Saccharomyces cerevisiae*: a role for heat shock protein 70 during targeting and translocation. *J Cell Biol* 1996;135:329–339. [PubMed: 8896592]
44. Newlon CS, Fangman WL. Mitochondrial DNA synthesis in cell cycle mutants of *Saccharomyces cerevisiae*. *Cell* 1975;5:423–428. [PubMed: 1098780]
45. Kai Y, Takamatsu C, Tokuda K, Okamoto M, Irita K, Takahashi S. Rapid and random turnover of mitochondrial DNA in rat hepatocytes of primary culture. *Mitochondrion* 2006;6:299–304. [PubMed: 17098481]
46. Schweitzer JK, Livingston DM. Expansions of CAG repeat tracts are frequent in a yeast mutant defective in Okazaki fragment maturation. *Hum Mol Genet* 1998;7:69–74. [PubMed: 9384605]
47. Nakabeppu Y. Regulation of intracellular localization of human MTH1, OGG1, and MYH proteins for repair of oxidative DNA damage. *Prog Nucleic Acid Res Mol Biol* 2001;68:75–94. [PubMed: 11554314]
48. Nilsen H, Otterlei M, Haug T, Solum K, Nagelhus TA, Skorpen F, Krokan HE. Nuclear and mitochondrial uracil-DNA glycosylases are generated by alternative splicing and transcription from different positions in the UNG gene. *Nucleic Acids Res* 1997;25:750–755. [PubMed: 9016624]
49. Otterlei M, Haug T, Nagelhus TA, Slupphaug G, Lindmo T, Krokan HE. Nuclear and mitochondrial splice forms of human uracil-DNA glycosylase contain a complex nuclear localisation signal and a strong classical mitochondrial localisation signal, respectively. *Nucleic Acids Res* 1998;26:4611–4617. [PubMed: 9753728]
50. Slupphaug G, Markussen FH, Olsen LC, Aasland R, Aarsaether N, Bakke O, Krokan HE, Helland DE. Nuclear and mitochondrial forms of human uracil-DNA glycosylase are encoded by the same gene. *Nucleic Acids Res* 1993;21:2579–2584. [PubMed: 8332455]
51. Chatterjee A, Singh KK. Uracil-DNA glycosylase-deficient yeast exhibit a mitochondrial mutator phenotype. *Nucleic Acids Res* 2001;29:4935–4940. [PubMed: 11812822]
52. Singh KK, Sigala B, Sikder HA, Schwimmer C. Inactivation of *Saccharomyces cerevisiae* *OGG1* DNA repair gene leads to an increased frequency of mitochondrial mutants. *Nucleic Acids Res* 2001;29:1381–1388. [PubMed: 11239005]
53. You HJ, Swanson RL, Harrington C, Corbett AH, Jinks-Robertson S, Senturker S, Wallace SS, Boiteux S, Dizdaroglu M, Doetsch PW. *Saccharomyces cerevisiae* Ntg1p and Ntg2p: broad specificity N-glycosylases for the repair of oxidative DNA damage in the nucleus and mitochondria. *Biochemistry* 1999;38:11298–11306. [PubMed: 10471279]
54. Steele DF, Butler CA, Fox TD. Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. *Proc Natl Acad Sci U S A* 1996;93:5253–5257. [PubMed: 8643562]
55. *Methods in yeast genetics*. Cold Spring harbor Laboratory Press; Cold Spring Harbor: 1998.
56. Sheff MA, Thorn KS. Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast* 2004;21:661–670. [PubMed: 15197731]

57. Fox, TD.; Folley, LS.; Mulero, JJ.; McMullin, TW.; Thorsness, PE.; Hedin, LO.; Costanzo, MC. Analysis and Manipulation of Yeast Mitochondrial Genes. In: Guthrie, C.; Fink, GR., editors. *Methods in Enzymology*. Academic Press, Inc; San Diego: 1991. p. 149-165.
58. Phadnis N, Sia EA. Role of the Putative Structural Protein Sed1p in Mitochondrial Genome Maintenance. *J Mol Biol* 2004;342:1115–1129. [PubMed: 15351639]
59. Sia EA, Butler CA, Dominska M, Greenwell P, Fox TD, Petes TD. Analysis of microsatellite mutations in the mitochondrial DNA of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 2000;97:250–255. [PubMed: 10618404]
60. Lea DE, Coulson CA. The distribution of the number of mutants in bacterial populations. *J Genet* 1949;49:264–284.
61. Phadnis N, Mehta R, Meednu N, Sia EA. Ntg1p, the base excision repair protein, generates mutagenic intermediates in yeast mitochondrial DNA. *DNA Repair* 2006;5:829–839. [PubMed: 16730479]
62. Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. *Current Protocols in Molecular Biology*. Wiley; New York: 1994.
63. Meisinger C, Sommer T, Pfanner N. Purification of *Saccharomyces cerevisiae* Mitochondria Devoid of Microsomal and Cytosolic Contaminations. *Anal Biochem* 2000;287:339–342. [PubMed: 11112284]
64. He S, Fox TD. Membrane translocation of mitochondrially coded Cox2p: distinct requirements for export of N and C termini and dependence on the conserved protein Oxa1p. *Mol Biol Cell* 1997;8:1449–1460. [PubMed: 9285818]
65. Rehncrona S, Mela L, Siesjo BK. Recovery of brain mitochondrial function in the rat after complete and incomplete cerebral ischemia. *Stroke* 1979;10:437–446. [PubMed: 505482]
66. Pallotti, F.; Lenaz, G. Isolation and Subfractionation of Mitochondria from Animal Cells and Tissue Culture Lines. In: Pon, LA.; Schon, EA., editors. *Methods in Cell Biology*. Academic Press; San Diego: 2001.
67. Bonnefoy N, Fox TD. In vivo analysis of mutated initiation codons in the mitochondrial *COX2* gene of *Saccharomyces cerevisiae* fused to the reporter gene *ARG8^m* reveals lack of downstream reinitiation. *Mol Gen Genet* 2000;262:1036–1046. [PubMed: 10660064]
68. Mookerjee SA, Sia EA. Overlapping contributions of Msh1p and putative recombination proteins Cce1p, Din7p, and Mhr1p in large-scale recombination and genome sorting events in the mitochondrial genome of *Saccharomyces cerevisiae*. *Mutat Res* 2006;595:91–106. [PubMed: 16337661]

Figure 1.

Accumulation of DNA point mutations over time measured by erythromycin resistance (ER^R) for mtDNA and canavanine resistance (Can^R) for nuclear DNA. (A) The average median frequency of ER^R per 10^7 cells is plotted over time in days. Each point represents the average of 2–7 independent experiments with 5–10 cultures per experiment except for the 17 and 21 day time points of $rad27-\Delta$ in which the data points are represented by one experiment as all other experiments had too many colonies to accurately count. Error bars indicate the SEM. (B) The average median frequency of Can^R per 10^8 cells is plotted over time in days. The experiment was performed twice with 10–20 independent cultures. A representative experiment is shown. The wild-type strain is indicated by $\circ$ and the $rad27-\Delta$ strain is indicated by Δ .

Figure 2.

Nuclear and mitochondrial direct-repeat mediated deletion (DRMD) reporters. (A) The nuclear DRMD reporter consists of the *URA3* gene inserted 99 bp into the *TRP1* gene followed by the entire *TRP1* gene lacking the ATG. (B) The mitochondrial DRMD reporter consists of the *ARG8^m* gene inserted 99 bp into the *COX2* gene followed by the entire *COX2* gene lacking the ATG as described previously [7]. The solid boxes represent the 96 bp of directly repeated sequence. (C) Average rates of nuclear and mitochondrial DRMD for wild type and *RAD27* deficient yeast are plotted on a log scale. Error bars indicate the SEM.

Figure 3.

Reporter constructs to measure mitochondrial microsatellite instability (A) *arg8^m::(GT)₁₆+1* [39] and (B) *arg8^m::(GT)₁₆+2* [6]. *ARG8^m* is a derivative of *ARG8* modified for the codon preference of the mitochondria. This gene has been inserted in the mitochondrial genome 5' of the *COX2* gene and is being expressed using the transcriptional and translational signals of *COX2*. The dinucleotide (GT)₁₆ sequence has been inserted 9 bp into *ARG8^m* and are 32 bp in each reporter. The flanking sequences have been altered to generate a +1 and +2 frameshift. (C) The effect of mitochondrial microsatellite instability in *RAD27* deficient strains as compared to wild type. The average median frequency of frameshifts per 10⁷ cells is plotted. Error bars indicate the SEM.

Figure 4.

Rad27p localizes to yeast mitochondria *in vivo*. (A) Fluorescence microscopy of wild type, *RAD27*-GFP, *ABF2*-GFP, and NLS-GFP yeast grown in synthetic media containing 2% glycerol (Sgly) to select for respiring cells. Yeast were washed with 1X PBS and stained with DAPI and Mitotracker, and viewed with DIC, DAPI, Texas Red, and GFP filters. (B) Mitochondrial fractionation of wild-type and *RAD27*-HA yeast strains grown in Sgly Complete. Lane: 1 – wild-type whole cell extracts; 2 – *RAD27*-HA whole cell extracts; 3 – wild-type crude mitochondrial fraction; 4 – *RAD27*-HA crude mitochondrial fraction; 5 – wild-type pure mitochondrial fraction; 6 – *RAD27*-HA pure mitochondrial fraction. Blots were probed with anti-Por1p and anti-Cox3p as a control for mitochondrial enrichment, anti-HA for Rad27p, and anti-histone H4 for a nuclear control. (C) Protease digestion of *RAD27*-HA crude mitochondria to determine submitochondrial localization. Lane: 1 – mock treated mitochondria; 2 – proteinase K treated mitochondria; 3 – mock treated mitoplasts; 4 – proteinase K treated mitoplasts; 5 – proteinase K treated ruptured mitochondria. The blots were probed with anti-HA for Rad27p, anti-Cox2p, anti-Cit1p, and anti-Por1p. All blots represent 20 µg sample per lane except for the anti-HA blot which contains 100 µg sample per lane.

Figure 5.

FEN1 localizes to mouse mitochondria. (A) Mouse mitochondria were isolated from brain, heart, and kidney and subjected to SDS-PAGE. For each tissue type, lane 1 contains nuclear material and unbroken cells, lane 2 contains cytoplasmic material, and lane 3 represents purified mitochondria. The blot was probed with anti-VDAC for mitochondrial content, anti-histone H4 for nuclear content, and anti-FEN1. (B) Proteinase K digestion of mouse brain mitochondria. Lane 1 – whole tissue homogenate; 2 – cytosol; 3 – purified mitochondria; 4–8 – mitochondria treated with proteinase K for indicated time in minutes. Blots were probed with anti-FEN1, anti-VDAC for detection of an OMM protein, anti-Cytochrome C for detection of release of mitochondrial intermembrane space protein, and anti-histone H4 as a control for nuclear contamination.

Table 1

Strains used in this study

Strain	Relevant Nuclear Genotype	Relevant Mitochondrial Genotype	Reference
DFS188	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG</i>	ρ^+	[59]
DFS160	<i>MATa ade2-101 leu2Δ ura3-52 arg8-Δ::URA3 kar1-1</i>	ρ^0	[54]
TF236	<i>MATa ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2 cox3::arg8m-1</i>	ρ^+	[67]
NYP181	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG rad27-Δ::kanMX</i>	ρ^+	This study
NPY054	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG ARG8</i>	ρ^+	[58]
LKY215	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG ARG8 rad27-Δ::kanMX</i>	ρ^+	This study
EAS748	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG</i>	mit- <i>REP96::ARG8^m::cox2</i>	[7]
LKY196	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG REP96::URA3::trp1</i>	mit- <i>REP96::ARG8^m::cox2</i>	This study
LKY205	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG REP96::URA3::trp1 rad27-Δ::kanMX</i>	mit- <i>REP96::ARG8^m::cox2</i>	This study
EAS738	<i>MATa ade2-101 leu2Δ ura3-52 arg8-Δ::URA3 kar1-1</i>	$\rho^+ \text{arg8}^m::(\text{GT})_{16+1}$	[68]
EAS744	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG</i>	$\rho^+ \text{arg8}^m::(\text{GT})_{16+1}$	[68]
LKY226	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG rad27-Δ::kanMX</i>	$\rho^+ \text{arg8}^m::(\text{GT})_{16+1}$	This study
EAS736	<i>MATa ade2-101 leu2Δ ura3-52 arg8-Δ::URA3 kar1-1</i>	$\rho^+ \text{arg8}^m::(\text{GT})_{16+2}$	[6]
EAS741	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG</i>	$\rho^+ \text{arg8}^m::(\text{GT})_{16+2}$	[6]
LKY224	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG rad27-Δ::kanMX</i>	$\rho^+ \text{arg8}^m::(\text{GT})_{16+2}$	This study
LKY217	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG RAD27-yECitrine-3HA-KAN^r</i>	ρ^+	This study
LKY426	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG RAD27-yEGFP-CaURA3</i>	ρ^+	This study
LKY049	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG GFP-ABF2-HIS3MX6</i>	ρ^+	This study
LKY527	<i>MATa ura3-52 leu2-3, 112 lys2 his3 arg8::hisG + pGAD-NLS-GFP</i>	ρ^+	This study