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# Mitochondrial DNA repair and damage tolerance

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## 1. ABSTRACT

The accurate maintenance of mitochondrial DNA (mtDNA) is required in order for eukaryotic cells to assemble a functional electron transport chain. This independently-maintained genome relies on nuclear-encoded proteins that are imported into the mitochondria to carry out replication and repair processes. Decades of research has made clear that mitochondria employ robust and varied mtDNA repair and damage tolerance mechanisms in order to ensure the proper maintenance of the mitochondrial genome. This review focuses on our current understanding of mtDNA repair and damage tolerance pathways including base excision repair, mismatch repair, homologous recombination, non-homologous end joining, translesion synthesis and mtDNA degradation in both yeast and mammalian systems.

## 2. INTRODUCTION

Mitochondria are essential organelles in eukaryotic cells, and the cellular energy produced by mitochondria is required for the survival of most eukaryotes. In addition to the obvious role in energy production, mitochondria perform a central role in amino acid, heme, lipid, and iron-sulfur cluster biosynthesis. These organelles contain an independently-maintained, multi-copy genome that must be correctly replicated and repaired for the proper function of the electron transport chain.

The size of the mitochondrial genome varies significantly between species. In human cells, it is a compact 16,500 bp, while in *Saccharomyces cerevisiae* it is much larger, ranging from 75,000 to 85,000 bp in size (1, 2). Unlike the nuclear genome, the mitochondrial genome is present in many copies per cell, and the copy number varies dependent on tissue type, and the energetic demands of the cell (3). Despite the significant difference in the size of these genomes in different species, the number and type of genes encoded is remarkably similar. The human and yeast mitochondrial genomes encode 13 and 8 polypeptides, respectively, in addition to the tRNAs and rRNAs required for their synthesis. The proteins encoded by the mitochondrial genome are some of the components of electron transport chain (ETC) complexes, therefore, the coordination of products of both the nuclear and mitochondrial genome are critical for the production of ATP, and thus for cellular function. Considering the requirement for the ETC in eukaryotic cells, proper replication and maintenance of the mitochondrial genome with high fidelity is essential to the survival of an organism.

Mitochondrial DNA (mtDNA) is packaged into nucleoprotein structures, called nucleoids, that are closely associated with the inner mitochondrial membrane (4-6). The most abundant yeast nucleoid protein is Abf2p, (TFAM, mammalian homolog) a high mobility group (HMG) protein family member that can facilitate the compaction of the mitochondrial genome by introducing sharp bends into the DNA (7). Abf2p binds mtDNA in a sequence-independent fashion and is present at high enough concentrations to coat the entire mitochondrial genome (8, 9). It is often stated in the literature that mtDNA has a greater susceptibility to damaging agents because it is “naked”. However, although the mitochondrial genome is not associated with histones, it has been well established that it is complexed with proteins that organize and stabilize the genome (reviewed in 10).

Mutations in mtDNA are directly linked to many heritable diseases, such as Kearns-Sayre Syndrome (KSS) and progressive external opthalmoplegia (PEO) (11). Additionally, an increase in mutational load has been detected in many neurological disorders such as Alzheimer's and Parkinson's diseases (12), but whether these mutations are causative or arise as a result of the disease pathology remains to be determined. The importance of a properly maintained mitochondrial genome was underscored in a mouse model that expressed proofreading deficient polymerase γ (POLG), the mitochondrial replicative polymerase. These mice have a shortened lifespan and an early onset of aging phenotypes (13). Taken together, it is clear that the accurate maintenance of the mitochondrial genome is vital for the health and survival of an organism.

MtDNA is exposed to the same chemical assaults as nuclear DNA, which generate various types of damage, including alkylation, abasic site formation, adduct formation, mismatched bases, single- and double-strand breaks, and oxidative lesions. Initially it was believed that DNA repair mechanisms might not be required in mitochondria, due to multi-copy nature of the genome. For many years, this led to models in which damaged mtDNA was simply degraded, rather than repaired. The mechanism by which these damaged genomes would be recognized and targeted for removal was not clear. In any case, there is now evidence for several DNA repair pathways in mitochondria, including base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ). This review will focus on our current mechanistic understanding of these repair pathways in both yeast and mammalian systems, where they have been the best studied.

Many of the mitochondrial repair pathways make use of proteins with dual nuclear and mitochondrial localization. As a result, many of the mitochondrial repair pathways are reminiscent of those found in the nucleus, however, the identified mitochondrial DNA repair pathways appear to operate with fewer proteins than the nuclear counterparts. While the known repair proteins are likely to maintain their recognized biochemical functions, such as DNA binding, it appears that at least some proteins may have been co-opted for use in mitochondrial specific pathways. These differences are perhaps not entirely surprising, as it is known that interactions with chromatin, and other nuclear-specific players impact nuclear repair. Distinct features of the mitochondrial milieu must impact repair pathways as well, so that simply extrapolating known nuclear repair pathways to the mitochondrial compartment will not allow us to generate a clear understanding of mtDNA metabolism.

This more limited repertoire of proteins used in mtDNA metabolism confounds our efforts to define specific repair mechanisms, in part, because there appears to be much overlap in the proteins utilized in various repair pathways and the replication machinery. For example, mtDNA polymerase, Pol γ is thought to perform both replicative and repair DNA synthesis, as additional processive polymerases have not been localized to mitochondria (reviewed in 14), and while multiple nuclear ligases are available in the nucleus, mitochondrial ligation is carried out by LIG3 in mammalian cells and Cdc9p in yeast (15, 16). In mitochondria, it is often difficult to elucidate whether a protein primarily functions in repair or replication. This is especially true of the putative HR proteins localized to mitochondria. For example, we know that stimulating damage can often lead to a transient increase in mtDNA copy number, and in mammalian cells this is dependent on the HR protein Rad51 (17).

Examination of mtDNA repair pathways has lagged significantly behind similar studies of nuclear repair. There are several factors that have contributed to this delay. Perhaps most significant was the erroneous assumption, made early on, that mtDNA was simply not repaired, and thus similar studies were not initiated. In addition, the yeast model system, which played a critical role in the identification and characterization of nuclear repair pathways has been less amenable to mitochondrial studies than nuclear studies until recently.

Dissection of nuclear repair pathways has relied on reporters of mutagenesis that read out different types of genomic changes. This is because different types of damage lead to different mutations. In addition, different lesions are recognized and repaired by different pathways, with some redundancy. In the mitochondria, however, all changes to mtDNA, including complete loss of the genome, lead to the same phenotypic output, which is the failure to grow on a non-fermentable carbon source, so the different mutations can't easily be distinguished.

In addition, under normal laboratory conditions, budding yeast spontaneously lose mitochondrial function at high frequencies. Because non-respiring yeast cells give rise to smaller colonies on solid growth media, these have been termed *petites.* The vast majority of the spontaneously non-respiring colonies are termed cytoplasmic *petites*, in which the defect causing respiration loss is a mutation of the mitochondrial genome. When the mitochondrial genome in these cells are examined, they show large deletions of mtDNA, followed by an amplification of the sequence that remains. This type of *petite* genome is designated $\rho^-$, the wild-type genome is referred to as $\rho^+$, and cells lacking mtDNA entirely are $\rho^0$.

The mechanism by which the deletions in $\rho^-$ genomes arise is not known, however, these variants arise at frequencies many orders of magnitude greater than other types of mutations. Against this


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high background of spontaneous *petites,* one cannot score increases in the other types of mutations, such as base substitutions that result in non-respiratory phenotypes. In addition, depending on the sequences present in the $\rho^-$ genome, the mutant genome may have a replication advantage over the wild-type genome, or other $\rho^-$ genomes. Due to their preferential replication or inheritance in mating experiments, such $\rho^-$ genomes are known as "hypersuppressive" *petites* (reviewed in 18).

Prior to 2000, therefore, a researcher interested in studying mitochondrial mutagenesis in yeast would be able to measure the frequency of spontaneous *petites*, or a somewhat limited spectrum of point mutations via selection for resistance to mitochondrial ribosome-inhibiting antibiotics, such as erythromycin and spiramycin (19). In measuring point mutations, erythromycin appears to be the only drug that specifically selects for resistant mutations in the mitochondrial genome, without confounding nuclear mutations (19, 20). Because yeast are facultative anaerobes, this selection must be carried out on medium with a non-fermentable carbon source, as all yeast without active mitochondrial protein synthesis are resistant to the drug, limiting the conditions under which these experiments may be performed.

While the spectrum of gain-of-function mutations giving rise to $Ery^R$ is somewhat limited, these assays provided a more accurate estimation of point mutation rates overall than the sequencing methods available to researchers working at that time in vertebrate systems. Many of these methods required an amplification step prior to sequencing. Reported frequencies of mitochondrial mutations were extremely variable, and such disparities have been attributed to artifacts introduced during the amplification step, as methods requiring PCR amplification resulted in the highest frequencies (21-23). Methods that allow more accurate quantitation of random mutations and deletions at specific locations in the mitochondrial genome were subsequently developed (24, 25).

Yeast remain a powerful and attractive model system for studies of mtDNA repair, because of the relative ease with which genetic analysis of proteins that impact mitochondrial mutation rates can be performed. This is particularly true in view of new reporters that have become available in recent years, following the successful introduction of a selectable, recoded nuclear gene into the mitochondrial genome of yeast. Arg8p, acetylornithine aminotransferase, is encoded in the nuclear genome, translated on cytoplasmic ribosomes, and imported into the mitochondrial matrix, where it performs its catalytic function in the biosynthesis of arginine. Steele, *et al.* generated the synthetic *ARG8*$^m$ gene, to reflect the codon usage and bias of a mitochondrial gene, and introduced it into the mitochondrial genome by biolistic transformation, followed by recombination (26). Expression of *ARG8*$^m$ within the mitochondrial matrix successfully complements a nuclear *ARG8* deletion, and allows the yeast cells to grow on medium lacking arginine. Researchers in yeast now have a series of reporters available that utilize the *ARG8*$^m$ selectable marker, including reporters to measure frameshift mutations, microsatellite instability, spontaneous recombination at direct repeats, and double-strand break repair in mitochondria (27-32). These reporters have revealed proteins involved in mitochondrial genome mutagenesis and repair that do not significantly impact respiration loss or point mutation frequencies, and thus could not have been identified previously (27, 28, 31).

## 3. BASE EXCISION REPAIR

Base excision repair (BER) was the first identified repair pathway in mitochondria, in yeast and humans, and remains the best characterized (33-38). Mitochondrial BER has recently been extensively reviewed (39-43), therefore, we will only provide a brief summary here. The base excision repair pathways are primarily responsible for the removal of non-bulky base lesions that arise due to oxidation, alkylation, deamination, and methylation (44-46). This type of damage originates from both exogenous sources (radiation, environmental mutagens, chemotherapeutic drugs) and endogenous sources (reactive oxygen species ROS, spontaneous decay). The close proximity of mtDNA to the electron transport chain (ETC) may increase its susceptibility to ROS damage, and thus the need for an efficient BER pathway. The impact increased ROS exposure has on the mtDNA, in terms of frequency of base lesions, has been debated in the literature, but a general consensus is that mtDNA contains significantly higher proportion of oxidized bases when compared to the nucleus, ranging from 4X-40X more depending on the type of lesion (47-50).

BER is a highly conserved repair pathway, from bacteria to humans, consisting of four main steps: (1) base lesion recognition and removal by DNA glycosylases, resulting in an abasic (AP) site, (2) DNA end-processing, (3) gap-filling, and (4) ligation (Figure 1). There are two types of BER, short-patch (SP) or long-patch (LP), both of which occur in mitochondria. SP-BER is when a single nucleotide gap is formed, and repaired, while LP-BER generates a gap between 2-12 nucleotides and that is then subsequently filled and ligated restoring the DNA strand (44).

There are a plethora of base lesions that can occur including, but not limited to, oxidation, lipid peroxidation, deamination, alkylation, and spontaneous loss of the base (41, 43). The different types of lesions are recognized and removed by a specific enzyme or one of a group of DNA glycosylases. For example, 8–oxo-7,8-dihydroguanine (8-oxoG) is primarily removed by OGG1 (Ogg1p-*S. cerevisiae*) but can also be excised by NTH1


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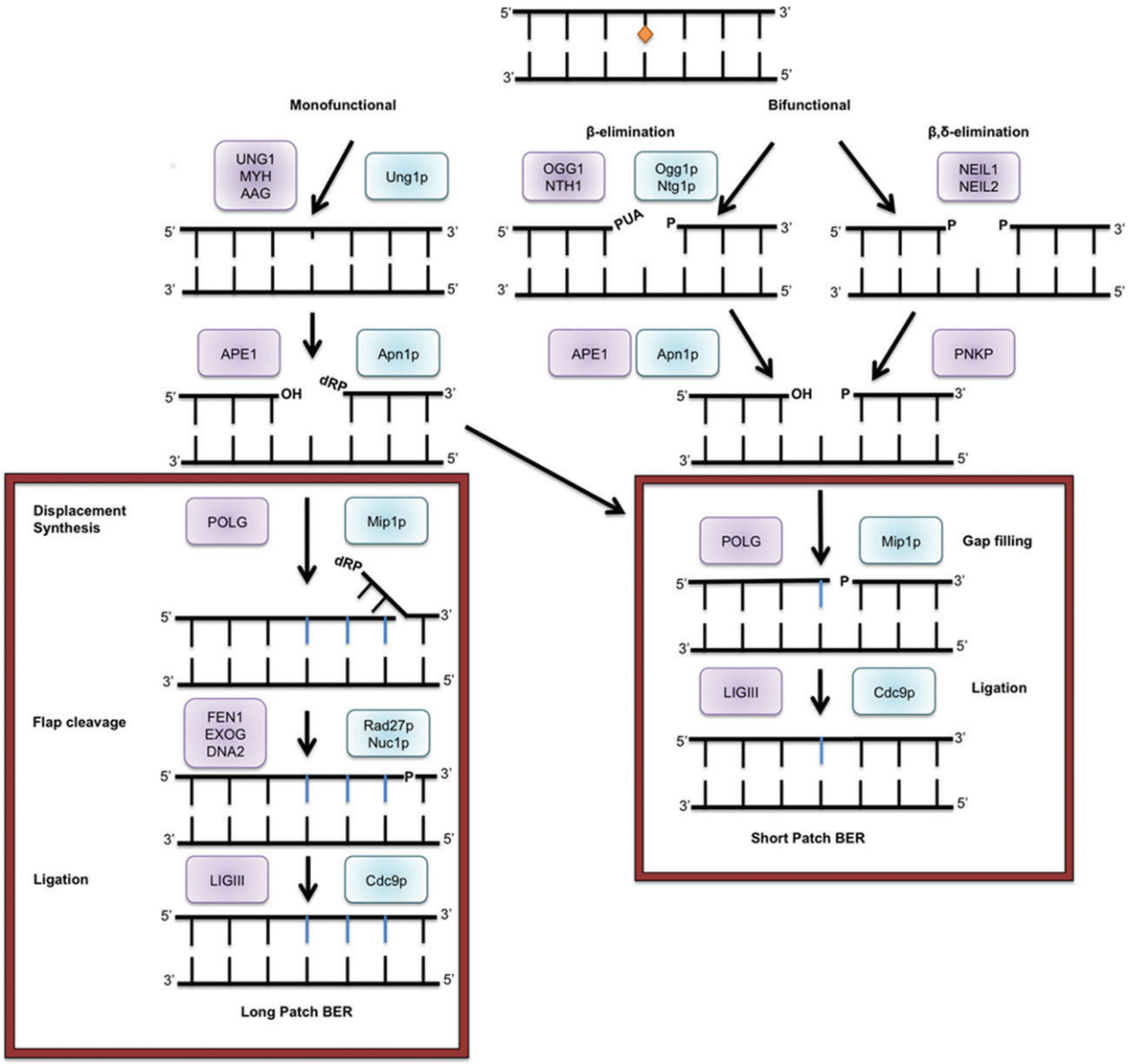


**Figure 1.** Mitochondrial BER. Mammalian and yeast homologs are indicated in purple and blue boxes, respectively. A base lesion (orange diamond) is recognized by either a monofunctional or bifunctional DNA glycosylase. Depending on the initial glycosylase the ends can be tailored by APE1 (Apn1p), PNKP, or POLG (Mip1p). POLG can then fill the remaining single nucleotide gap (SP-BER) or perform displacement synthesis (LP-BER). In LP-BER the resulting flap will be cleaved by an endonuclease. The remaining nick is sealed by LIGIII in both SP- and LP-BER.

(Ntg1p-*S. cerevisiae*). This redundancy in the recognition step allows for efficient repair of these common lesions in both the nucleus and mitochondria.

Currently there are seven glycosylases known to localize to mammalian mitochondria and three in yeast mitochondria (39, 41). These, and the other proteins discussed in this review, are summarized in Table 1. A thorough biochemical and structural review of these glycosylases can be found elsewhere (42). This comprehensive collection of BER glycosylases supports the hypothesis that efficient repair of the base lesions, at least in part, caused by ROS is vital for the maintenance of intact mtDNA and mitochondria.

There are two main classes of DNA N-glycosylases, monofunctional and bifunctional. Monofunctional DNA glycosylases are capable of cleaving the N-glycosidic bond, removing the damaged base and thus producing an AP site. AP endonuclease I (mammalian: APE1; *S. cerevisiae*: Apn1p) will then incise the DNA backbone 5' to the AP site, generating a 3' hydroxyl and 5' deoxyribosephosphate (5'dRP) flanking the single nucleotide gap. Bifunctional N-glycosylases can


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**Table 1.** Mitochondrial DNA repair, replication, and damage tolerance proteins

| Protein Name | Species | Mitochondrial localization confirmed | Known activity | Suggested Repair/ Tolerance pathway(s) |
|---|---|---|---|---|
| UNG1 | *H. sapiens* | Yes | Monofunctional uracil DNA glycosylase | BER |
| Ung1P | *S. cerevisiae* | Yes | | |
| MYH | *H. sapiens* | Yes | Monofunctional DNA glycosylase | BER |
| AAG | *H. sapiens* | Yes | Monofunctinoal alkyladenine DNA glycolsylase | BER |
| Mag1p | *S. cerevisiae* | No | | |
| NTH1 | *H. sapiens* | Yes | Bifunctional DNA glycosylase | BER |
| Ntg1p | *S. cerevisiae* | Yes | | |
| OGG1 | *H. sapiens* | Yes | Bifunctional 8-oxo guanine DNA glycosylase | BER |
| Ogg1p | *S. cerevisiae* | Yes | | |
| NEIL1 | *H. sapiens* | Yes | Endonuclease VIII-like bifunctional DNA glycosylase | BER |
| NEIL2 | *H. sapiens* | Yes | Endonuclease VIII-like bifunctional DNA glycosylase | BER |
| PNKP | *H. sapiens* | Yes | Polynucleotide kinase and 3' phosphatase | BER, NHEJ |
| APE1 | *H. sapiens* | Yes | AP endonuclease | BER |
| Apn1p | *S. cerevisiae* | Yes | | |
| POLG | *H. sapiens* | Yes | DNA polymerase | Replicative/repair polymerase |
| Mip1p | *S. cerevisiae* | Yes | | |
| FEN1 | *H. sapiens* | Yes | Flap endonuclease | LP-BER, HR, MMEJ |
| Rad27p | *S. cerevisiae* | Yes | | |
| EXOG | *H. sapiens* | Yes | Endo- and exonuclease | LP-BER, HR |
| Nuc1p | *S. cerevisiae* | Yes | | |
| DNA2 | *H. sapiens* | Yes | Flap endonuclease and ATP-dependent helicase | LP-BER, HR, |
| Dna2p | *S. cerevisiae* | No | | |
| LIGIII | *H. sapiens* | Yes | DNA Ligase | Replication, BER, HR, NHEJ, MMEJ, MMR |
| Cdc9p | *S. cerevisiae* | Yes | | |
| YB-1 | *H. sapiens* | Yes | Y-box binding protein | MMR, BER |
| Msh1p | *S. cerevisiae* | Yes | MutS homolog | HR, BER, MMR(?) |
| Mhr1p | *S. cerevisiae* | Yes | ATP independent strand exchange | HR, Replication |
| Cce1p | *S. cerevisiae* | Yes | cruciform cutting endonuclease | HR, Replication |
| Mgm101p | *S. cerevisiae* | Yes | Rad52-like protein | HR |
| RAD51 | *H. sapiens* | Yes | RecA homolog; strand exchange | HR |
| Rad51p | *S. cerevisiae* | Yes | | |
| RAD51C | *H. sapiens* | Yes | RAD51 paralog, Holliday junction resolution | HR |
| XRCC3 | *H. sapiens* | Yes | RAD51 paralog; strand exchange | HR |
| Rad59p | *S. cerevisiae* | Yes | Rad52 homolog | HR |
| MRE11 | *H. sapiens* | Yes | Binds dsDNA ends, part of MRN(X) complex | HR, NHEJ, MMEJ |
| Mre11p | *S. cerevisiae* | Yes | | |

(Contd...)



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**Table 1.** *Continued...*

| Protein Name | Species | Mitochondrial localization confirmed | Known activity | Suggested Repair/ Tolerance pathway(s) |
|---|---|---|---|---|
| RAD50 | *H. sapiens* | No | Binds dsDNA ends, part of MRN(X) complex | HR, NHEJ, MMEJ |
| Rad50p | *S. cerevisiae* | Yes | | |
| NBS1 | *H. sapiens* | No | Binds dsDNA ends, part of MRN(X) complex | HR, NHEJ, MMEJ |
| Xrs2p | *S. cerevisiae* | No | | |
| KU70 | *H. sapiens* | No | Binds dsDNA ends, forms heterodimer with Ku80 | NHEJ |
| Ku70p | *S. cerevisiae* | No | | |
| KU80 | *H. sapiens* | Yes | Binds dsDNA ends, forms heterodimer with Ku70 | NHEJ |
| Ku80p | *S. cerevisiae* | No | | |
| REV3 | *H. sapiens* | Yes | Catalytic subunit of pol zeta | TLS |
| Rev3p | *S. cerevisiae* | Yes | | |
| Primpol | *H. Sapiens* | Yes | DNA polymerase and DNA primase | TLS |
| BER : Base excision repair; HR: Homologous recombination; LP-BER: Long patch base excistion repair; MMEJ: Microhomology mediated nonhomologous end joining; MMR: Mismatch repair; NHEJ: Nonhomologous end joining; TLS: Translesion synthesis | | | | |

cleave the N-glycosidic bond and nick the DNA strand 3' of the lesion. This intrinsic lyase activity can process the lesion by one of two mechanisms. First, β-elimination will leave a 3' phospho-α,β-unsaturated aldehyde (3'PUA) and a 5' phosphate on either side of the gap. APE1 (Apn1p) can remove the replication blocking 3'PUA and convert it to a 3'OH with its phosphodiesterase activity. Second, a β-δ elimination reaction will cleave the DNA backbone resulting in 3'-phospate and 5'-phosphate (51). The 3'-phosphate is processed, generating a 3'-OH, potentially by polynucleotide kinase 3' phosphatase (PNKP), which has been recently localized to the mitochondria (52-54). These nicked repair intermediates are then processed via one of two pathways.

In short patch BER, polymerase γ (mammalian: Pol γ; *S. cerevisiae*: Mip1p) will convert the 5'dRP residues to a 5' phosphate and fill the single nucleotide gap (55). The mitochondrial ligase (mammalian: LigIII; *S. cerevisiae*: Cdc9p) then seals the nick (56, 57). Alternatively, in long patch BER, the 3' end at the nick is extended by Pol γ, which displaces a 5' flap containing the dRP, which is subsequently cleaved. The 5' flap is potentially cleaved by FEN1 (*S. cerevisiae*: Rad27p), DNA2 (*S. cerevisiae*: Dna2p, mitochondrial localization not demonstrated), EXOG (*S. cerevisiae*: Nuc1p) or a combination of these enzymes (27, 58-61). Once the flap has been removed LigIII (Cdc9p) will seal the remaining nick.

In humans, there are 3 monofunctional DNA glycosylases known to localize to mitochondria, uracil-DNA glycosylase 1 (UNG1), *Escherichia coli* MutY homolog (MYH) and N-methylpurine DNA glycosylase (AAG/MPG). In *S. cerevisiae* mitochondria, the only monofunctional glycosylase is Ung1p. Given the conservation of mitochondrial BER among diverse species, and the high levels of oxidative damage predicted, one might expect loss of BER proteins to result in significant cellular defects. However, analysis of mutants lacking specific BER enzymes typically results in subtle, and sometimes contradictory phenotypes. In some cases, redundancy in the repair network to remove the most frequent base lesions may obscure phenotypes in single mutants. In addition, the glycosylases in this pathway can convert potentially miscoding errors, for example, uracil in DNA, into potentially cytotoxic lesions, like abasic sites or single-strand breaks. Several studies have indicated that imbalanced expression of factors in the BER pathway can be mutagenic when the generation of intermediates outpaces later processing. These observations suggest that a careful balance must be struck between factors in BER that allows repair of lesions without generating mutations (62-65). The reported mitochondrial phenotypes of glycosylase mutants in yeast and mice are described more fully below.

Human UNG1 is encoded by the UNG locus but uses an alternative promoter as well as alternative splicing to generate the mitochondrial form of UNG1, which contains a N-terminal mitochondrial targeting sequence (MTS) (66). *In vitro* assays indicate that purified human Ung1 recognizes and removes misincorporated uracil and 5-fluorouracil (67, 68). UNG1 knockout mice exhibit increased levels of incorporated uracil as expected, but overall resulted in no major pathologies in young mice (69). In contrast, older mice exhibited a reduction in lifespan and had a predisposition for B-cell lymphomas (70). There were no mitochondrial-specific phenotypes reported. In yeast, both the deletion and


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overexpression of Ung1p led to an increase in *petite* colonies suggesting that an optimal balance is needed in this repair pathway to maintain a stable mitochondrial genome (71). The mitochondrial point mutation frequency appears to be unaffected in the *ung1*-Δ strain (71, 72).

Unlike other glycosylases, MYH will remove the undamaged base in a pair instead of the damaged base. MYH excises adenine when it is paired with 8-oxoG, guanine or cytosine (42, 67, 73-75). The nuclear and mitochondrial isoforms of MYH are the result of alternative splicing, with the mitochondrial isoform containing a 14-amino acid N-terminal MTS (42, 74, 75). Double knockout mice lacking both MYH and OGG1 did not exhibit any detectable increase in mtDNA mutations, instability or incidence of cancer (76).

AAG recognizes and excises a variety of alkylated bases in both single and double-stranded DNA (77). Recently, mitochondrial localization of AAG has been confirmed, where it was shown to interact with the mitochondrial single-stranded binding protein (mtSSB) (78). This interaction with mtSSB inhibited AAG's ability to excise alkylated bases on ssDNA, thus preventing the formation of ssDNA breaks (78). Mitochondrial mutagenesis in mammals lacking AAG has not yet been assessed.

In yeast and mammalian mitochondria, there are two and four bifunctional glycosylases, respectively. The two mammalian specific mitochondrial bifunctional glycosylases are Endonuclease VIII-like glycosylase 1 and 2 (NEIL1 and NEIL2) (54, 79). These glycosylases facilitate the repair of several types of base lesions including various oxidized pyrimidines, ring-opened formamidopyrimidines (FaPy) lesions, and thymine glycol (51, 80). Unlike other DNA glycosylase mutants, NEIL1 knockout mice exhibit a strong a mitochondrial phenotype, with an increase in steady state damage and mtDNA deletions, and these mice display symptoms of metabolic syndrome (81).

The 8-oxoguanine DNA glycosylase (mammalian: OGG1; *S. cerevisiae*: Ogg1p) recognizes 8-oxoG and FaPy lesions. In humans, alternative splicing generates a mitochondrial-specific isoform of OGG1, while in yeast there is a N-terminal MTS (34, 82). Interestingly, OGG1 knockout mice do not exhibit any identifiable mitochondrial mutator phenotype but overexpression of the mitochondrial isoform of OGG1, in a mouse model of mammary tumorigenesis, resulted in a decrease in tumor size and reduction in metastasis (83-85).

The loss of Ogg1p in yeast causes an increase in mitochondrial point mutations. Consistent with the prediction that both Ogg1p and Ntg1p can recognize some of the same substrates, a number of studies consistently show a reduction in point mutants in *ntg1*-Δ *ogg1*-Δ double mutants (34, 86, 87). However, this result suggests that although Ogg1p and Ntg1p compete for the same lesion, the Ntg1p-dependent pathway is more mutagenic (39).

NTH1 (*S. cerevisiae*: Ntg1p), a homolog of *Escherichia coli* endonuclease III, repairs oxidized pyrimidines (5-hydroxycytosine and 5-hydroxyuracil), thymine glycol, 8-oxoG, and FaPy lesions. Human NTH1 predominantly localizes to the nucleus while mouse NTH1 predominantly localizes to the mitochondria. Deletion of the nuclear localization signal in the human NTH1 results in mitochondrial localization, suggesting that a cryptic mitochondrial targeting sequence may exist (88). Although NTH1 knockout mice display no obvious phenotypes (89), mitochondria from mouse liver extracts from NTH1 knockout lines lack the ability to incise thymine glycol containing substrates (90).

Yeast Ntg1p contains an N-terminal mitochondrial targeting sequence, and has been shown to localize to both the nucleus and mitochondria (38, 91). Loss of Ntg1p has been reported to result in a range of phenotypes, from no effect on mtDNA point mutants, to subtle increases, and finally decreases in point mutations, frameshifts, spontaneous direct repeat mediated deletions, and mtDNA copy number (34, 63, 92, 93). This reason for this variation is still unclear, but it has been proposed that yeast strain-specific differences in gene expression, or in experimental design may play a role (63).

Despite the fact that BER is the best characterized pathway in mitochondria, in both yeast and mammals, significant questions remain. For example, while it is known that a number of BER components are shared between the nucleus and mitochondria in both systems, the signals and regulatory factors involved in sorting the proteins into the appropriate cellular compartment, under the appropriate conditions are not well understood. The dynamic localization of yeast Ntg1p is perhaps the best characterized. A single isoform contains an N-terminal mitochondrial targeting sequence, as well as nuclear localization signals that must be regulated to target the protein to either compartment (38, 91). Griffiths, *et al*. have demonstrated that localization of Ntg1p changes in response to damage concentrated in the different organelles. Exposure of yeast cells to $H_2O_2$ results in enrichment of Ntg1p-GFP fusion in the nucleus. Treatment of cells with antimycin, in addition to $H_2O_2$, results in increased mitochondrial oxidative stress. Following this treatment, increased mitochondrial reactive oxygen species (ROS) and GFP fluorescence were observed, indicating that the increased mitochondrial damage triggered a relocalization of the glycosylases enzyme to mitochondria. This localization was dependent on the presence of mtDNA, suggesting that there is a mechanism for sensing the damaged genomes, and not simply elevated ROS (94). A clear understanding of the signals required for the cell to recognize damage in


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the different subcellular compartments and respond to by sorting Ntg1p to the appropriate compartment will require additional studies, however, sumoylated Ntg1p is found in the nucleus, suggesting that post-translational modification of the protein may be important (94, 95).

## 4. MISMATCH REPAIR

Our current understanding of mismatch repair (MMR) remains limited compared to our knowledge of mitochondrial BER. Mason *et al*. first reported MMR activity in rat liver mitochondrial extracts (96). In contrast to BER, in which shared nuclear and mitochondrial isoforms of proteins are prevalent, vertebrate mitochondrial MMR is not dependent on known nuclear MMR proteins. Instead, mitochondrial MMR in vertebrates is dependent on the Y box binding protein, YB-1 (97). YB-1 is multifunctional protein, implicated in a wide variety of nuclear processes ranging from transcriptional regulation to mRNA splicing (98-101). YB-1 had previously been shown to preferentially separate DNA duplexes containing mismatches or cisplatin induced crosslinks (102). The depletion of YB-1 led to the decreased ability of mitochondrial extracts to perform MMR *in vitro,* and the cells displayed an increase in mtDNA mutagenesis (97). These observations support the model that YB-1 functions in a mitochondrial MMR pathway that appears to be unique from the MSH2-dependent MMR that occurs in the nucleus.

In yeast, MMR activity has not been clearly demonstrated despite the localization of the bacterial MutS homolog, Msh1p, to mitochondria (103, 104). Purified Msh1p binds DNA mismatches *in vitro,* and early models placed Msh1 in a mitochondrial mismatch repair pathway (105). However, the catastrophic loss of mtDNA integrity in an *msh1*-Δ strain was not entirely consistent with a primary function in MMR. Pol γ proofreading mutants show mutation rates orders of magnitude above wild-type rates, yet retain the ability to maintain mtDNA (106). In addition, substitutions in Msh1p residues, conserved among the MutS homologs required for MMR, do not display phenotypes analogous to the nuclear counterparts (107, 108). Genetic studies suggest a more complicated view, in which Msh1p is involved in multiple pathways of mtDNA maintenance (87, 103, 105, 107, 108). The MutS homologs in the nucleus of eukaryotes are involved in a diverse set of DNA transactions including, mismatch repair, oxidative damage repair, mitotic and meiotic recombination (109-115). It is perhaps not surprising then, that *in vivo* evidence supports a role of Msh1p in mitochondrial BER and recombination in yeast (87, 93, 107, 108, 116). Whether Msh1p participates in additional pathways required for the maintenance or repair of the mitochondrial genome remains to be determined. Interestingly, YB-1 also has been shown to function in BER by stimulating the glycosylase activity of NEIL2 and NTHL1 in nuclear BER (117, 118). Due to the limited, albeit increasing, repertoire of repair proteins in the mitochondria compared to the nucleus it is not surprising that multifunctional proteins, such as YB-1 and Msh1p, have been co-opted to perform in multiple, and perhaps novel, mitochondrial repair pathways.

## 5. HOMOLOGOUS RECOMBINATION, NON-HOMOLOGOUS END JOINING AND DOUBLE-STRAND BREAK REPAIR

Homologous recombination (HR) activity has been well documented in *S. cerevisiae* mitochondria. HR in yeast is particularly easy to detect due to the biparental inheritance of mtDNA in this organism. As such, recombination between differentially marked mitochondrial genomes can be easily measured. The frequency of recombination is quite high in yeast mitochondria, as genetic markers on yeast mtDNA separated by 1kb or more behave as unlinked genes in crosses (18).

Due to the inheritance of only maternal mtDNA in mammalian systems, HR is more difficult to detect. It has been demonstrated that human mitochondrial protein extracts are capable of catalyzing the HR of plasmid DNA substrates *in vitro* (119). The *in vivo* relevance was demonstrated in patients that inherited both paternal and maternal mtDNA, in which recombinant mtDNA molecules were readily detected (120, 121). Despite this support for HR pathways in both mammalian and yeast mitochondria, our knowledge of the proteins involved and the molecular mechanisms that underlie these events is lacking in comparison to the nuclear models. Below we will begin with an overview proteins implicated in mitochondrial HR in yeast and/or mammals and then explore DSB repair specifically.

Mhr1p was originally identified as a yeast mitochondrial protein that is required for gene conversion events, but has little effect on crossing over in mtDNA (122). The *mhr1-1* mutants also exhibited a temperature sensitive loss of mtDNA (122). Subsequent studies revealed that Mhr1p promotes homologous pairing of DNA in an ATP independent manner (123). In mammalian cells, mitochondrial genomes are generally monomeric circular structures. However, in yeast, the mtDNA is found predominantly as long linear concatemers, with circular monomers being found primarily in the budding daughter cell (123). This led to the proposition of a rolling circle mechanism of replication and inheritance of the mitochondrial DNA that is dependent on Mhr1p in *S. cerevisiae* (reviewed in 124). In this recombination initiated rolling circle replication model, Mhr1p promotes the homologous base pairing of a 3' single-stranded tail with an intact circular double-stranded mtDNA molecule. This leads to the formation of a concatemeric DNA that is then transmitted into the daughter cell during cell division. The amount of concatemeric DNA increases


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when *MHR1* is overexpressed and is reduced in cells expressing the *mhr1-1* mutant allele, further supporting the role of Mhr1p in the generation of these concatemers of mtDNA (123). Furthermore, recent studies on hypersuppressive $\rho^-$ genomes demonstrate that ROS-induced Ntg1-dependent DSBs can stimulate mtDNA replication that is dependent on Mhr1p. The observation that cells stressed with ROS exhibit an increase in mtDNA copy number is consistent with this model. However, it is important to keep in mind that the mechanisms of replication and inheritance of $\rho^-$ genomes, and especially hypersuppressive $\rho^-$ genomes are likely to be different than $\rho^+$ genomes, as the protein requirements are not the same. For example, replication of the wild-type mitochondrial genome requires the mitochondrial RNA polymerase, Rpo41p (125). Fangman, *et al.* demonstrated that two *petite* genomes, one of them hypersuppressive, could be maintained independently of Rpo41p (126). All $\rho^-$ *petite* genomes do not share the same requirements, however, as Mgm101p is required for the maintenance of $\rho^+$ and non-hypersuppressive $\rho^-$ genomes, while hypersuppressive *petites* are maintained in its absence (127). In addition, recombination between $\rho^+$ genomes is either unaffected, or only slightly affected in *cce1-Δ* and *mhr1-1* backgrounds, while recombination between $\rho^-$ genomes or $\rho^+$ and $\rho^-$ genomes is significantly altered (122, 128).

Cce1p is a mitochondrially-localized protein with cruciform cutting activity *in vitro.* This protein is associated with the inner membrane, and is proposed to resolve Holliday junctions that result from recombination events (129, 130). The loss of *CCE1* has surprisingly little effect on mitochondrial genome stability, however, as *CCE1* deletion does not result in a significant decrease in the mtDNA recombination rates, or a substantial increase in the generation of $\rho^-$ genomes. Loss of Cce1p results in aggregation of mtDNA molecules, linked by recombination junctions, that are organized into fewer nucleoids (108).

In wild-type yeast cells, during amino acid starvation, the mtDNA is divided into a large number of nucleoids. This parsing of mtDNA is dependent on Cce1p, thus suggesting that the resolution of recombination intermediates is required to disperse the mtDNA into new nucleoids. According to the mtDNA replication model proposed by Shibata and Ling, Cce1p also functions in a crossing over recombination pathway that generates concatamer intermediates independently from the Mhr1p dependent rolling circle replication and transmission model (124). In the absence of functional Mhr1p and Cce1p, yeast cells are devoid of mtDNA, possibly due to the cell's inability form concatemers, and thus the prevention of mtDNA transmission from the mother cell to the budding daughter cell. It is remains unknown what initiating events or lesions generate the Holliday junctions that Cce1p may act upon.

Studies in yeast, using a reporter strain that allowed for the measurement of direct repeat mediated deletions (DRMD), have permitted a more direct interrogation of the possible HR mechanisms at work in mitochondria in mitotic cells. In addition, these reporters aid in the identification of the proteins involved (29). Initial studies concluded that in this context Cce1p had no effect on the generation of spontaneous deletions (29). This further supports a model in which there are several HR-like pathways that appear to be at least partially redundant.

Additional studies using the DRMD reporter system concluded that the generation of spontaneous DRMDs was synergistically dependent on the non-homologous end joining (NHEJ) complexes, Mre11p, Rad50p and Xrs2p (MRX), and Ku70p, and Ku80p (Ku). This result suggests that end joining at spontaneously occurring double strand breaks may play a role in generating deletions (28). *In vitro* studies with mammalian mitochondrial extracts have demonstrated the capacity to catalyze DNA end-joining activities (131), but proteins responsible for this activity remain unknown. The repair of induced mitochondrial DSBs in mice did result in one detectable deletion event that occurred via NHEJ (132). This implies that in mammalian mitochondria, while NHEJ is possible, it may not be the predominant DSB repair mechanism. Nuclear NHEJ candidates have been localized to the mitochondria in both yeast (Rad50p and Mre11p) (133) and mammalian cells (Ku80 isoform, MRE11) where they may function in a mitochondrial NHEJ pathway. (134, 135)

Several other nuclear repair proteins have been shown to have dual mitochondrial localization as well. The RecA homolog, Rad51, which is important for nuclear homologous recombination (HR), has been localized to the mitochondria in both mammals and yeast where it was shown to bind directly to mtDNA (31, 136). In mammals, the recruitment to mitochondria of Rad51 and its paralogs, Rad51C and Xrcc3, is stimulated after the cells have been exposed to oxidative stress (136). The specific enrichment of Rad51 after oxidative stress is dependent on active mtDNA replication (17). Previously it had been observed that both mammalian and yeast mtDNA copy number is temporarily elevated in response to oxidative stress (137, 138). In mammalian cells it was shown that stress induced increases in mtDNA copy number are dependent on Rad51 (136). One could postulate that that this temporary increase in copy number is due to a mechanism similar to the ROS-induced *MHR1*-dependent replication model proposed above in yeast.

Recent studies in yeast clearly demonstrated a role for the HR proteins Rad51p, Rad52p, and Rad59p in the generation of both spontaneous deletions and those initiated by an inducible double-strand break (31).


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Interestingly Rad51p promotes the generation DRMDs in the mitochondria, whereas in the nucleus Rad51p inhibits the generation of similar deletion products (31). This suggests that there are unique mitochondrial HR pathways that have yet to be characterized and that these pathways may utilize nuclear HR proteins in novel ways.

Double-strand breaks (DSBs) are extremely problematic DNA lesions if not efficiently repaired. DSBs can occur spontaneously due to ROS-induced damage, replication stalling, or radiation. While it is not always clear what initiates spontaneous recombination events, they can be stimulated by DSBs. There are two primary DSB repair pathways in the nucleus, HR and NHEJ (reviewed in 139, 140, 141). HR is typically considered error-free repair due to the use of homologous sequences as template for the repair the DSB. However, there are sub-pathways of HR, such as single-strand annealing (SSA), that are quite mutagenic. SSA occurs when a DSB forms between repetitive elements. After 5' end resection has occurred, these repetitive elements will anneal. The annealing of the repetitive elements with 3' non-homologous single stranded tails, generates flap structures that can be cleaved by endonucleases and ligated to generate a deletion that has lost of one of the repeats and the intervening sequences (142, 143).

NHEJ requires minimal end processing, followed by ligation of the processed ends. The end processing often will lead to small insertions or deletions at the site of the DSB thus making NHEJ more mutagenic than classical HR. There is also a sub pathway of NHEJ, microhomology-mediated end joining (MMEJ) that utilizes microhomologies that are revealed during end processing to facilitate the ligation of the broken ends (144, 145). This typically leads to larger deletions than classical NHEJ, but smaller deletions than SSA.

This type of mitochondrial mutation is important, because mtDNA deletions are a commonly associated with aging, cancer, Kearns-Sayre syndrome, myopathies, progressive external opthalmoplegia, diabetes and deafness (146, 147). The majority of mtDNA deletions (85%) are flanked by short direct repeats (148-150). It is plausible then that these deletions are generated due to mutagenic DSB repair pathways such as SSA, NHEJ, and MMEJ.

The study of DSB repair in mitochondria is an emerging area. Initial studies constitutively expressed mitochondrially targeted *Pst*I (mito*Pst*I) in mice (132). These mice exhibited myopathy associated with mtDNA depletion and had mitochondrial deletions that closely resembled those in humans. The depletion of mtDNA suggested that the mouse mitochondrial DSB repair network was incapable of handling the continuous cleavage of the mtDNA, which led to degradation of a significant fraction of the population of mitochondrial genomes. This degradation could be due to a regulated pathway, or simply result from the generation of a large proportion of unstable linear mtDNA fragments (151, 152).

Later studies utilized a neuronal specific tetracycline inducible mito*Pst*I system, to study the effects of more transient DSBs on mouse mtDNA (153). These mice did not exhibit any mtDNA depletion after transient expression of mito*Pst*I suggesting that if mitochondrial DSB levels are below a threshold, the cell can effectively repair them (153). In mice that had undergone DSB induction the authors were able to detect mtDNA with deletions that were not present in control samples. Analysis of the deletion products, from this study and others, indicate that error prone pathways, likely HR and NHEJ, can generate mtDNA deletions after DSB induction (153, 154).

Recent *in vitro* studies demonstrated significant MMEJ activity in rat mitochondrial protein extracts (155). The MMEJ efficiency increased as homology length increased. *In vitro* MMEJ activity was dependent on known nuclear MMEJ proteins, CtIP, FEN1, MRE11, Ligase III, and PARP1, as the efficiency of MMEJ reactions was impaired in extracts immunodepleted for these proteins (155). This points to MMEJ as yet another mutagenic repair pathway that can lead to the generation of mtDNA deletions. Reminiscent of yeast *petite* genomes (18), these deleted genomes were present in much higher proportions in older mice relative to younger mice suggesting that the smaller genomes may have a replication or inheritance advantage (153). This phenomenon may explain why aging individuals typically have an accumulation of deleted mtDNA molecules (156).

In yeast, we have developed an inducible DSB system that generates a single mitochondrial DSB within the *ARG8*$^{m}$ reporter gene. Utilizing this system we were also able to detect deletion products after the induction of the DSB (28, 31), suggesting conserved mutagenic DSB repair pathways between yeast and mammalian systems. In addition to the deletion products, reciprocal products that are only generated during classical HR were also detected (28, 29). This would suggest that there at minimum two pathways acting at mitochondrial DSBs, those that generate reciprocal products (HR) and those that do not (SSA or MMEJ). Mutant analyses using this system have shown that the yeast mitochondrial DSB-induced deletions are partially dependent on Rad51p, Rad59p and the Ku and MRX complexes (28, 31). The contribution of Rad51p to the generation of deletions in this context is in contrast to the nuclear SSA model as previously stated (157), since nuclear SSA is a Rad51p-independent process.

## 6. LESION BYPASS

The conversion of DNA lesions to mutations is an active process in the cell. As described for DSBs, error prone repair pathways may generate mutations, however,


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mutations may also be introduced during DNA replication. Bulky lesions block the progress of most replicative polymerases, as the stringent substrate requirements of the active sites, and the presence of exonuclease proofreading domains inhibit progress across from the damaged region (158-160). Specialized polymerases that have the ability to bypass lesions have been identified in diverse organisms from bacteria to humans. These translesion polymerases can allow replication to continue, albeit at the cost of decreased fidelity. These error-prone enzymes clearly play an important role in the replication of the genome after damage, as the majority of mutations induced by UV-light in the nucleus of yeast, for example, are dependent on the presence of the error-prone polymerase, Pol zeta comprised of Rev3p and Rev7p (161-163). While these polymerases do not play a role in the repair of DNA, they would be more accurately considered a pathway of damage tolerance, they play an important role in the cellular response to lesions.

Early experiments with yeast indicated that $Ery^R$ point mutations could be efficiently induced by exposure of cells to UV light (164), suggesting the presence of translesion synthesis in this organelle. Subsequent studies revealed that UV-induced cytoplasmic *petites*, both spontaneous and induced frameshifts, and microsatellite instability were dependent on Pol zeta (20, 165). Surprisingly, however, loss of Pol zeta resulted in increased point mutations as measured by $Ery^R$, suggesting that there is alternative pathway of error-prone damage tolerance in yeast mitochondria (20). Baruffini, *et al.* confirmed this observation, and further demonstrated that overexpression of a mitochondrial-specific mutant of *REV3*, with its protein partners, can suppress the increased mutagenesis of Pol γ mutator alleles (166). This finding may implicate Pol γ or at least some of the pathogenic mutant forms of the enzyme, in the bypass of DNA lesions.

REV3, the catalytic subunit of Pol zeta has recently been localized to mitochondria in mammalian cells (167), and mutations, both deletions and base substitutions, have been shown to accumulate in human skin, in association with exposure to UV light, suggestive of error-prone bypass (168-170). *In vitro* experiments with purified human Pol γ indicate that this enzyme bypasses thymidine dimers with dramatically reduced efficiency relative to undamaged templates (171). In contrast, purified Pol γ can perform error-prone synthesis across from acrolein derived DNA adducts at relatively high efficiency. Acrolein is one of the products of lipid peroxidation, and a common environmental toxin. As such, the error-prone bypass of these adducts by Pol γ has been proposed to be a significant source of damage-induced mutations in mammalian cells (172).

The recent identification of PrimPol, an enzyme active in both the nucleus and mitochondria, has begun to change the view of Pol γ as the "jack of all trades" in mtDNA synthesis in vertebrates. As its name suggests, the enzyme has DNA primase activity as well as DNA-dependent DNA polymerase activity. Loss of PrimPol results in defects in mitochondrial synthesis (173). In addition, the purified enzyme is capable of synthesizing on templates with DNA lesions, including 8-oxo-G, pyrimidine photoproducts, and abasic sites, suggesting that, in mammals, this polymerase may be the source of significant mitochondrial lesion bypass (173, 174).

## 7. DEGRADATION

Bypass of pyrimidine dimers allows replication to proceed, but does not remove the lesion from the template strand. In the nucleus, damage of this type is removed by the nucleotide excision repair pathway. Early experiments failed to reveal evidence of mitochondrial DNA repair of pyrimidine dimers (175, 176). This led to the proposition that mtDNA was not repaired and instead the compromised genomes were simply degraded. Further support for this hypothesis was garnered when it was then determined that the mitochondrially-localized endonuclease G preferentially cleaved DNA opposite SSBs generated after oxidative damage (177). Recent studies have provided more direct evidence that selective mtDNA degradation may function as a quality control measure in the mitochondria that removes highly mutagenized genomes from the replication cycle (132, 154, 178-186).

Treating both yeast and mammalian cells with ethanol led to an increase in oxidative stress to the cells. After ethanol treatment, these cells experienced a decrease in mtDNA copy number, followed by a rebound to wild-type copy number levels after the stress was removed (181-183). A similar phenomenon was observed in a rat model for cerebral ischemia/reperfusion where a transient loss in mtDNA was followed by a full recovery of copy number to wild-type levels within 24 hours (180). In order to more directly measure the effect of oxidative damage on mtDNA copy number, Shokolenko *et al*. induced oxidative stress by inhibiting complex I of the ETC (185). This treatment leads to an increase in superoxides in the matrix. After stimulation of mitochondrial ROS, they were able to detect an increase in SSBs and abasic sites as well as a decrease in mtDNA copy number. This loss of mtDNA was exacerbated when the BER repair pathway was inhibited, suggesting that there is a competition between repair and degradation of the mtDNA under oxidative stress (185).

Recently it has also been shown that inducing chronic mitochondrial DSBs, by expressing various mitochondrially-targeted restriction enzymes in mammalian cells, will lead to mtDNA depletion (132, 153, 154, 179). Yeast mitochondria appear to tolerate the induction of exogenous DSBs


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better, as there is no detectable loss of mtDNA after DSB induction (31, 187). This may result from an increased capacity for DSB repair in yeast, as HR may be more robust (18), or it may simply be that the experimental system allows for the generation of more limited, and effectively repaired, DSBs.

Species-specific differences in DSB repair could also be due to the differences in the physical organization of yeast mtDNA and mammalian mtDNA. Mammalian mtDNA is estimated to be present at 1.45 copies per nucleoid, while yeast nucleoids can contain up to 10 copies (6, 188, 189). In order for mammalian mtDNA to find a suitable partner for HR, it may need to interact with mtDNA from a separate nucleoid, which does not appear to occur frequently in mammalian cells (190, 191). It is worth noting such interactions have not been tested in cells that have undergone DSB induction, or other genotoxic stress. We currently have little understanding of nucleoid dynamics under stressed conditions. While this evidence clearly demonstrates that under extreme stress, cells struggle to maintain their mtDNA, it remains unclear when this loss of DNA is simply a result of the mitochondrial genome becoming extensively fragmented due to the mitochondrial repair networks being overwhelmed, or if there is an active pathway in place that recognizes highly damaged mtDNA and targets it for degradation. This can be clarified by identifying the protein(s) involved in sensing the damaged genomes and the endo/exonuclease(s) required for degradation, as well as garnering a better understanding of nucleoid/mtDNA dynamics under stress.

## 8. CONCLUDING REMARKS

Eukaryotic cells clearly have a diverse suite of mtDNA repair and tolerance pathways that act in concert to maintain an error free mitochondrial genome. Developing additional, novel mtDNA reporters in yeast will continue to provide us with necessary tools to identify new proteins involved in repair. In addition, the powerful haploid genetics in yeast will allow us to better understand redundancy in overlapping repair pathways. While we continue to identify more DNA repair proteins that localize to both the nucleus and mitochondria, we are also identifying mitochondrial-specific players. A clear understanding of the integration of these factors will require studies to elucidate the signals that recognize mtDNA damage and modulate the dynamic localization of proteins shared between nuclei and mitochondria.

It is important to recognize that mitochondria themselves are dynamic structures, undergoing fission and fusion, and changes to distribution throughout the cell in response to environmental and metabolic signals. In this context, mitochondria often serve as the integration point for cellular inputs. In future studies, it will be vital to begin to integrate mtDNA metabolism and inheritance with these morphological changes.

## 9. ACKNOWLEGEMENTS

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Repair, MMR, Homologous Recombination, HR, Non-homologous End Joining, NHEJ, mtDNA Degradation, Translesion Synthesis, TLS, Microhomology-mediated End Joining, MMEJ, Yeast

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