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Role of the DNA Repair Genes, *APN1* and *APN2*, in the Prevention of Mitochondrial Dysfunction in the Yeast *Saccharomyces cerevisiae*

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Abstract

Numerous pathologies and conditions such as cancer, neurodegenerative and cardiovascular diseases, and aging have been associated with mitochondrial dysfunction. This has driven the research field towards the development of therapeutic targets to cure or ameliorate these conditions. Mitochondria perform important functions for the organism, including cellular respiration and synthesis of ATP. A byproduct of cellular respiration are reactive species to oxygen (ROS), which in excess can lead to oxidative stress, a common denominator in the above-mentioned conditions. ROS can damage macromolecules such as the mitochondrial DNA (mtDNA). However, there is a mechanism that specifically repairs ROS-induced lesions, called based excision repair (BER). The goal of this study is to understand how mtDNA repair contributes to the prevention of excessive ROS production. Our hypothesis states that deficient mtDNA repair will lead to mitochondrial dysfunction in the form of increased ROS. *Saccharomyces cerevisiae* yeast strains harboring single and double mutations in the BER genes *APN1* and *APN2* were exposed to 0.3 mM and 1.0 mM H_2O_2 for 1 hour. Cells were then incubated with 2 mM MitoSox, a dye that emits fluorescence in the presences of mitochondrial ROS. MitoSox fluorescence was detected in a FACS instrument. We found that deletion of the *APN1* and *APN2* genes did not result in increased ROS formation in response to H_2O_2 damage. Even though these results do not support our hypothesis, we did observe that *APN1* was required for preventing ROS formation in the basal state. Thus, we conclude that mtDNA repair contributes to the prevention of mitochondrial dysfunction.

Chapter 1: Introduction

Origin

The mitochondrion is an organelle with many functions essential for cellular processes. There are several theories of how the organelle was acquired by the eukaryotic cells, one of them, the endosymbiotic theory, is based in the structural and functional resemblance of mitochondria to plastids, in the domain of protists. The endosymbiont hypothesis draws much of its contemporary support from the discovery of a unique genome in this organelle (Gray, M. 1999). There are two possible free-living progenitors, an *Archeabacteria* and a *Proteorbacteria*. It is hypothesized that either they merged and infected a eukaryotic cell or by a process of merging, an evolving eukaryotic cell without mitochondria was infected by a α-proteobacteria (Gray, M. 1982). This process can be observed in Figure 1.

The theory arose from the fact that although these organelles are not part of the cell endomembrane system, they contain their own membranous sheath. Furthermore, it has been recognized that the emergence of eukaryotic-specific traits probably required more metabolic energy per gene and the mitochondria could afford the amount of energy for such traits (Lane, N. 2014. Lane, N. 2010). These organelles tend to retain a miniaturized prokaryotic chromosome encoding 63 proteins or less in the case of mitochondria (Zimorski, V. 2014). For the mitochondria, the key point is that it contains its own genetic material, known as mitochondrial DNA (mtDNA).

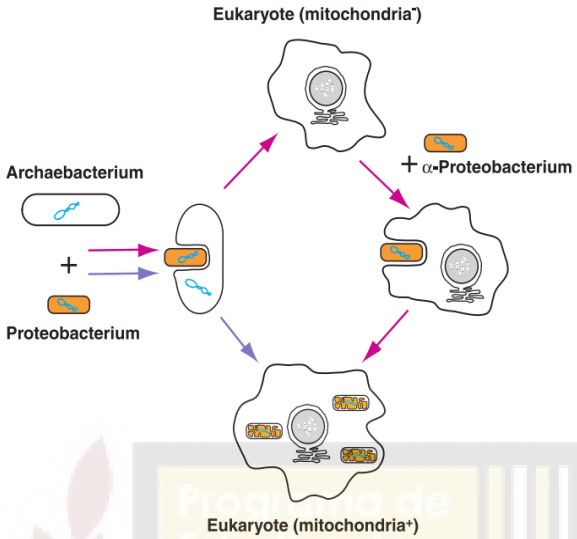


Figure 1. Endosymbiotic theory for the origin of mitochondria. Two alternative ways the mitochondria were acquired in eukaryotic cells. They arose from two microorganisms, both prokaryotic cells. Lavender arrows show the simultaneously fusion of Archeabacterium and the Proteobacterium creating the eukaryotic nucleus (gray) and mitochondrion (orange). Magenta arrows show a two-step fusion, first the Archeabacterium and Proteobacterium form an amitochondriate eukaryote cell followed by a second fusion of alfa Proteobacterium, acquiring the mitochondrion through endosymbiosis. Genomes, bacterial and mitochondrial, are blue (Gray, M. 1999). (Figure obtained from Gray, M. 1999)

The mitochondrial gene order has not been completely well conserved, instances of retention of what must have been the ancestral arrangement are still evident in other mtDNAs (Gray, M. 1999). Thus, the gene content among extant mtDNAs are best rationalized by assuming the occurrence of gene loss after divergence from the protomitochondrial genome (Gray, M. 1999). However, this can also be explained by a corollary to the endosymbiotic theory involving gene transfer to the nucleus, or endosymbiotic gene transfer (EGT). During the course of evolution, many genes were transferred from the mitochondria's chromosome to the host's nucleus. This process of organelle genome reduction has resulted in an expansion of the eukaryotic nuclear gene repertoire and in reductive genome evolution in the organelle of the mitochondria (Zimorski, V. 2014). The symbiosis process is depicted in Figure 2.

Despite the above-mentioned reduction in the mitochondrial genome is highly conserved in its coding function (Gray, M. 1982). The mitochondrial encoded proteins are involved in a broad spectrum of pathways similar to their ancestrally prokaryotic biochemistry (Zimorski, V. 2014). The genes retained by the mitochondria encode proteins involved in the electron transport chain (ETC) of the bioenergetic organelle, and RNA components of the mitochondrial ribosome, also known as mitoribosomes, required for their protein synthesis and for tRNAs. It has been proposed that even within the ribosome, the same core of proteins has been retained independently by plastids and mitochondria, probably owing to constraints imposed by the process of ribosome assembly (Zimorski, V. 2014.Greber, B.J. 2016).

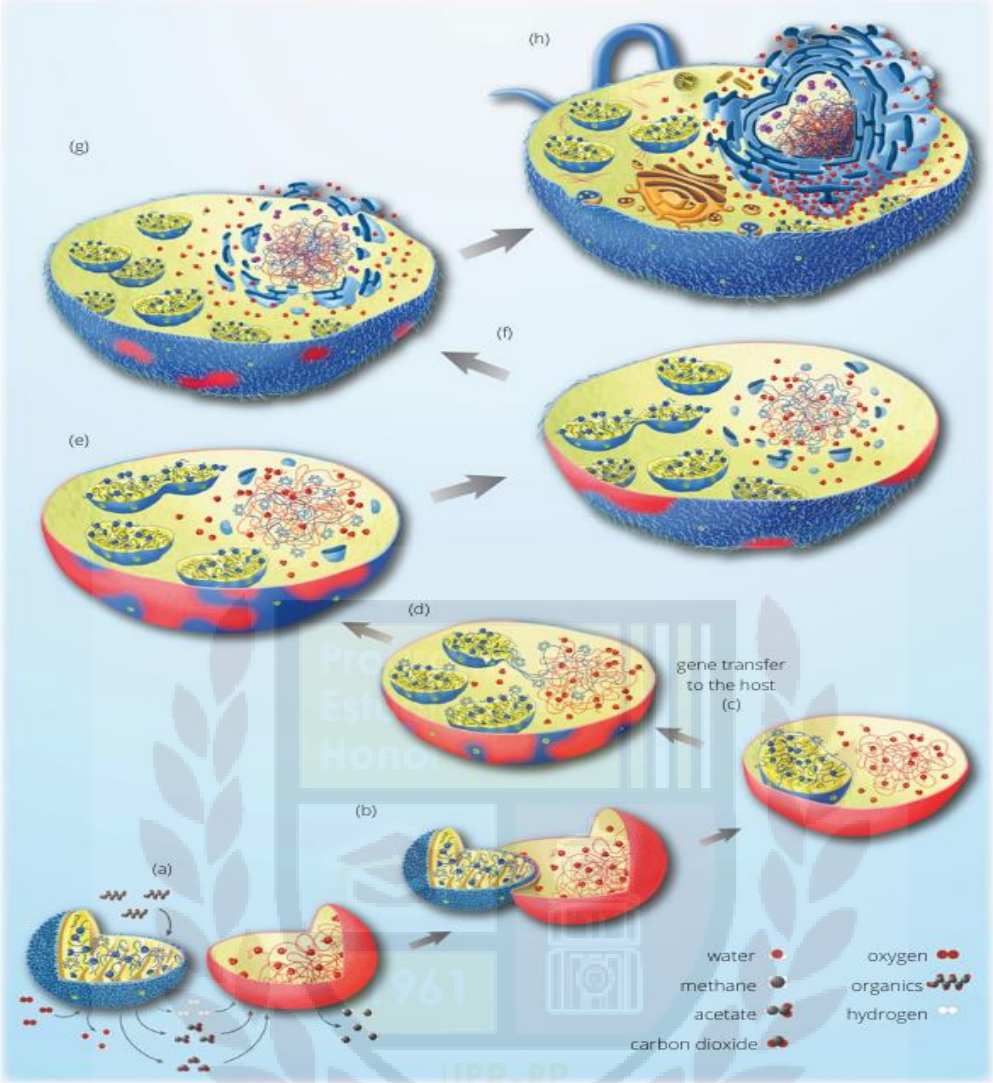


Figure 2. Transfer of mitochondrial genes into the nuclear genome. The model shows how mitochondria was incorporated in the host-eukaryotic cells and the gene transfer from the organelles to the nucleus. a) Anaerobic syntrophic host, a H_2 dependent archeon and the symbiont being a facultative anaerobe, respire in the presence of O_2 or in its absence produce fermentation. b) Anaerobic syntropy is thus the metabolic context of the host-symbiont association, leading the host to interact tightly with and adhere to their symbiont. c) The symbiosis is unstable thus the host needs to create importers to transfer for organics and gene information between the symbiont and host genome. d) The gene transfer has commenced. e) Molecular changes in the host genome begins, such as introns spreading in the host chromosomes. f) The newly acquired information in the genome begins to be transcribed and translated by the ribosomes. g) The host nucleus begins to compartmentalize. h) Eukaryotic cells evolve to what we know today (Martin, W. 2015). (Figure obtained from Martin, W. 2015).

Function

Mitochondria are energy-producing subcellular organelles, which perform cellular respiration, being the primary producers of ATP that our cells use for various biochemical processes to maintain their physiological roles, protein synthesis or the oxidative phosphorylation process (Kauppila, J. H. K. 2018 and Saki, M. 2017). Mitochondria also play a critical role in cell signaling, lipid oxidation, biosynthesis and assembly of protein aggregates (Saki, M. 2017). Moreover, this organelle is involved in calcium metabolism, contributes to the formation of reactive oxygen species (ROS) and initiates the process of apoptosis, being a homeostatic parameter acting as a signaling organelle for certain tissues (Moreira, O. 2017).

Mitochondrial biogenesis is characterized by their coordinated regulation between nuclear gene expression, protein translocation and the transcription of the mitochondrial genome (Carter, H.N. 2015). Its dynamism is regulated by several transcription factors, such as the peroxisome proliferator-activated receptor gamma co-activator 1-alfa (PGC-1α), and at the post-transcriptional level (Palikaras, K. 2014). Mitochondria also undergo other dynamic behaviors such as transport, directed movement within the cell, and degradation. All these processes are essential for the healthy functioning of the mitochondrial population (Mishra, P. 2016).

Structural mitochondrial DNA

The mtDNA is located in the mitochondrial matrix as a circular, double strand DNA molecule. It is free-mobile and lacks protection, like the nuclear envelop, and this makes the mtDNA subject to mutations a 100-times higher than the nuclear genome (Jackson,

M. 2019). Almost 93% of the mtDNA represents coding regions and is free from introns, histones, and epigenetic marks. Although the genetic role appears to be universally conserved, this genome exhibits remarkable variations in conformation, size, and actual gene content (Gray, M. 1999).

MtDNA encodes 37 genes of vital function for the mitochondria. Along with dozens of proteins encoded by the nuclear genome, they contribute to the formation and function of this organelle (Gray, M.W. 1982). Thirteen of the 37 mitochondrial genes encode subunits for the four respiratory complexes involved in oxidative phosphorylation (OXPHOS), situated in the inner mitochondrial membrane. The rest of the mitochondrial genes encode for 2 rRNAs and 22 tRNAs (Figure 3).

Heteroplasmy is another important feature of the mtDNA, meaning that not all copies of the mitochondrial genome within the cell are the same (Jackson, M. 2019). On average, there are about 1000 copies of mtDNA in each human cell, but only a subset of those molecules harbor sequence variation (Zekonyte, U. 2020). Hence, some mitochondria can contain mutations while others do not (Jackson, M. 2019). Mutations in the mtDNA have been implicated in neuromuscular and neurodegenerative disorders, as well as diabetes, cardiovascular disease, cancer, and aging (Zekonyte, U. 2020). Individuals carry low levels of mtDNA mutations without symptoms, because a certain threshold of mutations is needed to be reached for a biochemical phenotype to manifest. The symptoms of mitochondrial disease vary greatly because symptoms depend on the level of heteroplasmy in the affected tissue. Heteroplasmy can change within an individual over time and can cause late presentation or progression of disease over a lifetime.

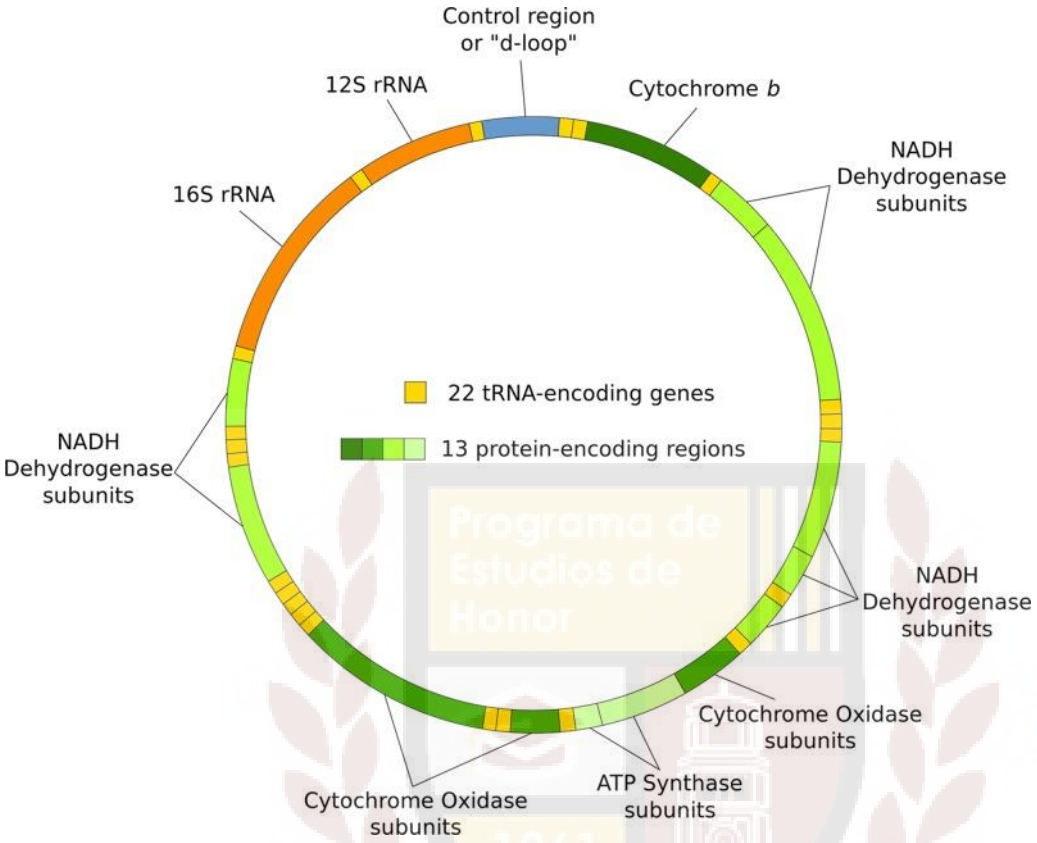


Figure 3. The mitochondrial genome. Circular and double stranded DNA that codes 37 genes involved in the respiratory complexes in oxidative phosphorylation (OXPHOS), tRNA and rRNA (Jackson, M. 2018). (Figure obtained from Wikimedia Commons: Public Domain).

Another unique feature of the mtDNA is that it is inherited maternally; in other words, a female will pass her mitochondria to all her children while a male will not pass on any of their mitochondria. This is an important phenomenon, especially when considering the inheritance of the mitochondrial mutations from a carrier mother. Pathogenic mutations in mtDNA can have very serious consequences and lead to diseases with onset in the neonatal period or adult life that are pleiotropic with muscular, metabolic, and/or neurological symptoms (Kauppila, J.H.K. 2018).

Despite the essential coding contribution of mtDNA to the cell, most of the genetic information for the mitochondrial biogenesis and function resides in the nuclear genome, determined by the import of nuclear DNA-specific proteins and in some cases small RNAs, especially tRNAs into the mitochondria (Gray, M.W. 1999). As we can see, the majority of the components of the respiratory complexes are encoded by the nuclear genome, synthesized in the cytosol and translocated inside the mitochondria to form the different components of the ETC (Kauppila, J. H. K. 2018).

Oxidative phosphorylation

Oxidative phosphorylation (OXPHOS) is the process by which energy from nutrients is transformed in the form of ATP, therefore it is the responsible for the production of most cellular energy. ATP synthesis occurs because the action of the electron transport chain (ETC) which utilizes several protein complexes that move electrons along the ETC while it moves protons from the mitochondrial matrix to the intermembrane mitochondrial space, generating an electrochemical gradient. The final

electron acceptor is oxygen, which then is converted into water. This electrochemical gradient is used to add a phosphate to ADP thus generating ATP (Zhao, R. 2019).

The ETC is composed of transmembrane protein complexes I, II, III and IV as well as the freely mobile electron transporters ubiquinone and cytochrome c. They exist in the folded inner membrane (IMM) called cristae (Figure 4) (Zhao, R. 2019). Although the majority of the complex components are encoded by nuclear genes, they are assembled inside the mitochondria together with the product of the mitochondrial-encoded genes.

Complex I is also known as NADH-ubiquinone oxidoreductase and it is the largest multisubunit enzyme complex in the ETC in charge for transfering electrons from to ubiquinone via iron-sulfur (FeS) clusters arranged from low to high potential, and ubiquinone (CoQ) is reduced to ubiquinol (QH_2). The transfer of a pair of electrons from NADH to CoQ in this complex induces the pumping of four protons (H^+) from the matrix into the intermembrane space (Zhao, R. 2019).

Complex II namely succinate dehydrogenase, is a component of the Krebs cycle as well as the ETC. Therefore, it serves as a link between metabolism and OXPHOS. Complex II catalyzes the oxidation of succinate to fumarate and it is another entry point for electrons from succinate to CoQ, similar to Complex I via a cluster of FeS. FAD is reduced to $FADH_2$ after receiving electrons from succinate and then transfered to the FeS cluster, then CoQ is reduced to QH_2 after obtaining the electron from the FeS cluster. This complex is the only one from the ETC that does not pump protons (Zhao, R. 2019).

Complex III is also known as the cytochrome c complex or CoQ-cytochrome c reductase. This complex transfers electrons carried by QH_2 to cytochrome c. The electron transfer process for this complex is accomplished by the Q-cycle. QH_2 is oxidized to ubisemiquinone (QH^-) after transferring an electron to the FeS cluster and concurrently two protons are released into the intermembrane space (IMS) from the matrix. Then a series of transfer of the electron occurs from different cytochromes and CoQ. When the second molecule of QH_2 is oxidized, it displaces the other two protons (Zhao, R. 2019).

Complex IV is also called cytochrome c oxidase. This complex transfer electron from cytochrome c to the terminal electron acceptor oxygen (O_2) to generate water (H_2O). Cytochrome c is a mobile electron carrier loosely connected to the outer surface of the IMM and allows the interaction with complex III and to accept electrons. The reduced cytochrome c moves along the membrane and interact with complex IV, simultaneously transferring electron that ends up forming H_2O and pumping four H^+ into the IMS (Zhao, R. 2019).

Complex V is the ATP synthase. The ETC transfer two electrons and forms H_2O, which is accompanied by the pumping of four, four and two protons from the matrix to the IMS from complex I, III and IV, respectively. Then the protons pass from the IMS to the matrix which transfer stored energy created by the proton's electrochemical gradient, so ADP can be phosphorylated to form ATP (Zhao, R. 2019).

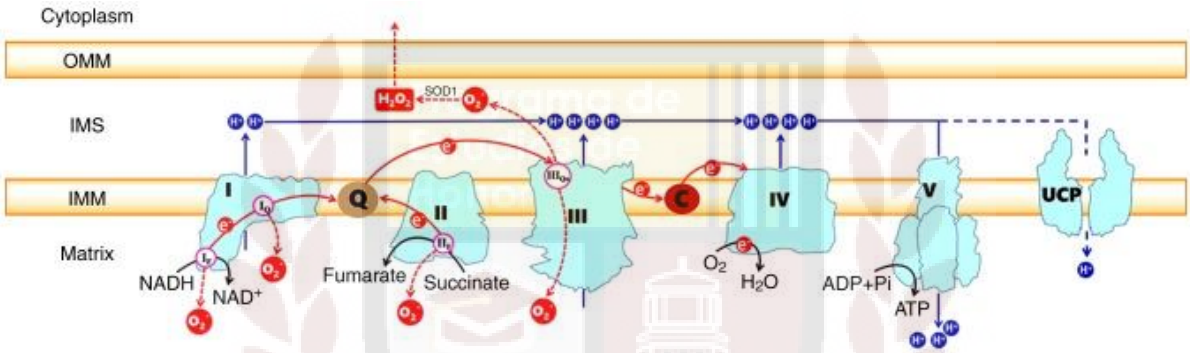


Figure 4. Model of the electron transport chain. Model of the ECT and ATP synthase and how energy is produced. UCP, uncoupling protein is not part of the ETC (Zhao, R. 2019). (Figure obtained from Zhao, R. 2019).

Reactive Oxygen Species

The ETC reduces the reactivity of the electrons, but sometimes 0.2-2% of these electrons escape, reacting with oxygen resulting in very reactive intermediates known as reactive oxygen species (ROS) (Zhao, R. 2019). These ROS are associated with substrate oxidation, identified in ETC. By reacting with the DNA, proteins and lipid, high levels of ROS lead to inhibited cell growth, and impaired biomass and lipid yield (Zhang, S. 2020). Therefore, the mtDNA has been thought to be target of oxidative damage because the OXPHOS system produces superoxide (O_2^-) and/or hydrogen peroxide (H_2O_2) as a natural side product of cellular respiration (Kauppila, J.H.K. 2018). In addition, the mtDNA repair systems are more error prone than nuclear DNA repair systems because of the limited repertoire of DNA repair mechanisms in mitochondria (Jackson, M. 2018)

ROS are oxygen-derived molecules with oxidizing properties, or molecules which rapidly convert to radicals (Xu, N. 2020). They generally comprise both free radical forms such as the superoxide radical ($\cdot O_2$-) and the hydroxyl radical (-OH), and non-radical forms such as H_2O_2 and singlet oxygen ($^1O_2$) (Zhang, S. 2020). Superoxide holds a prominent position in this context, as it initiates a cascade of reactions generating ROS. Such reactive derivates are generated by peroxide radical metabolism in mitochondria and specific oxidase, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthene oxidase (XO), and arachidonic acid (AA)-metabolizing enzymes (Xu, N. 2020). Similarly to what happens with mitochondrial complex I and complex III, oxygen binds to an electron to form a peroxide radical ($O_2^{\cdot -}$), which is converted to H_2O_2 by the, enzyme, superoxide dismutase (SOD) (Zhang,S.2020). The balance between the

antioxidant systems and ROS play an important role in regulating redox homeostasis. The antioxidants include enzymatic antioxidants, such as SOD, catalase, glutathione, peroxidase, glutathione S-transferase; nonenzymatic antioxidants, such as glutathione, vitamin A, vitamin C, and vitamin E (Xu, N. 2020).

ROS have important physiological roles in the cells, such as second messengers that mediate different intracellular pathways, upregulation of cytoprotective autophagy, regulation of synaptic plasticity, necessary for long-term potentiation (Zhao, R. 2019). ROS also have an important role in the establishment of neuronal polarity and regulate cytoskeletal organization and dynamics by regulating intracellular Ca^{2+} release. Besides, during hypoxia ROS stabilizes hypoxia-inducible factors in the cytosol and triggers cellular adaptation (Ajith, T.A. 2017).

Oxidative lesions in mtDNA

Mitochondria are a main source of cellular ROS. The amount of ROS generated as a result of a stimulus determines whether ROS play beneficial or harmful roles, which means different physiological or pathological pathways, are activated (Zhao, R. 2019). When the ROS concentration surpass the natural buffer of the mitochondria, they cause oxidative stress (Saki, M. 2017). Thus, oxidative stress occurs when there is an imbalance between the pro-oxidant molecules and the antioxidant defense mechanisms. Oxidative stress caused by increased mitochondrial ROS production is considered a form of mitochondrial dysfunction.

ROS can damage macromolecules and mitochondrial DNA (mtDNA) is an important target within mitochondria. A large amount of ROS cause lipid peroxidation, DNA damage, protein oxidation, irreversible impairment of mitochondria, insufficient ATP

generation and, eventually cell death (Zhao, R. 2019). ROS act through oxidative modification of numerous types of proteins, particularly receptors, kinases, phosphatases, caspases, ion channels and transcription factors (Zhao, R. 2019).

MtDNA is particularly susceptible to ROS-induced damage because of its proximity to the ETC. . The radical is able to react with any cellular macromolecule, including mtDNA inducing a large spectrum of DNA lesions (Kauppila, J.H.K. 2018). These DNA lesions, if not repaired, can lead to point mutations, rearrangements, and deletions (Wei, Y. 2002, Griffiths, L. 2009). In cell culture, exposure to extracellular H_2O_2 or rotenone has been reported to lead to mtDNA damage (Kauppila, J.H.K. 2018). The accumulation of mutations can result in mitochondrial dysfunction, thus affecting processes such as aging, apoptosis and different pathologies. MtDNA mutations have been implicated in many pathologies such as muscle contraction dysfunction, neurodegenerative diseases, cancer and aging. In addition, mitochondrial dysfunction, and oxidative stress trigger signals, such as elevated Ca^{2+} and NAD^{+} levels, that then orchestrate a nuclear gene expression response to mitigate the defect. Signaling between the two organelles, the nucleus, and mitochondria, is essential for deciding cellular fate (Saki, M. 2017). Figure 5 summarizes the crosstalk between the nucleus and mitochondria.

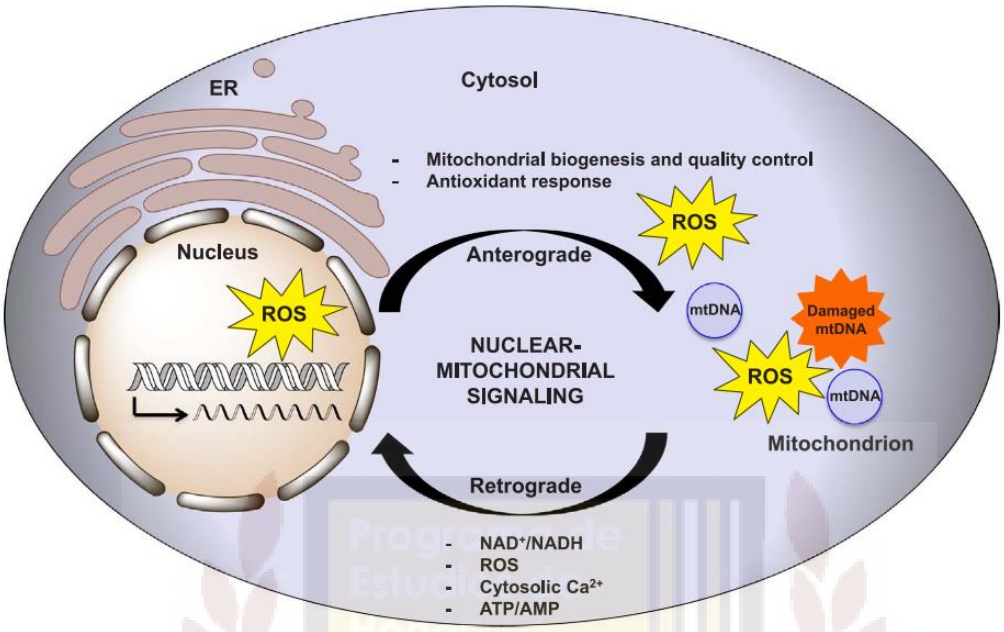


Figure 5. Crosstalk between nucleus and mitochondria. ROS damage signals a crosstalk between these two organelles via changes in levels of NADH, Ca^{2+} and ATP to try to fix the lesion or signal apoptosis (Figure obtained from Saki, M. 2017)

Base Excision Repair

Base excision repair (BER) is a DNA repair mechanism which has been extensively characterized in the nucleus. Its main function is to repair damage induced by ROS and other chemical agents such as alkylation agents. BER has been also shown to be present in mitochondria. It is a tightly coordinated process that is carried out in a stepwise matter and includes: recognition and excision of the damaged DNA base, removal of the resulting abasic site, end processing, gap filling and ligation (Saki, M. 2017). BER occur via the sequential actions of DNA glycosylases, endonucleases, DNA polymerases and other associated proteins. Class II endonucleases act on apurinic/apyrimidinic sites (AP sites) which are intermediates in BER.

The first step is carried out by DNA glycosylases, specialized enzymes, that recognizes the damaged base and catalyze the cleavage resulting in an AP site. Bifunctional DNA glycosylases are involved in the removal of oxidized DNA bases and nick the DNA backbone 3' to the lesion. DNA polymerase in the mitochondrion is responsible of the gap filling synthesis step in the mitochondria. The final ligation step in the mitochondria, is carried out by DNA ligase III. Given that BER is the major repair pathway in the mitochondria, diseases states associated with mutations and deletions in the BER machinery could result in aging-related neurodegenerative disorders, ataxia, diabetes, and cardiomyopathy (Saki, M. 2017). It has been shown that a decreased mitochondrial BER in combination with increased mitochondrial ROS production can induce more mtDNA point mutations (Kauppila, J.H.K. 2018).

Apn1 and Apn2 are major multifunctional nucleases involved in the repair of oxidatively damaged DNA, that possess endonuclease and 3' to 5' -exonuclease activities (Chalissery, J. 2017). Two AP endonucleases have been identified in the yeast *Saccharomyces cerevisiae*, *APN1* and *APN2* (Johnson,R.E. 1998). Yeast strains carrying a null mutation in the *APN1* gene (*apn1*Δ) are sensitive to oxidative agents such as hydrogen peroxide (H_2O_2). A yeast strain carrying a double mutation (*apn1*Δ*apn2*Δ) is extremely sensitive to H_2O_2 indicating that both AP endonucleases are required for proper cell function. AP endonucleases have been conserved throughout evolution and they are present in humans. Their role is highlighted by the fact that mutations in AP endonucleases genes have been identified in many cancer types.

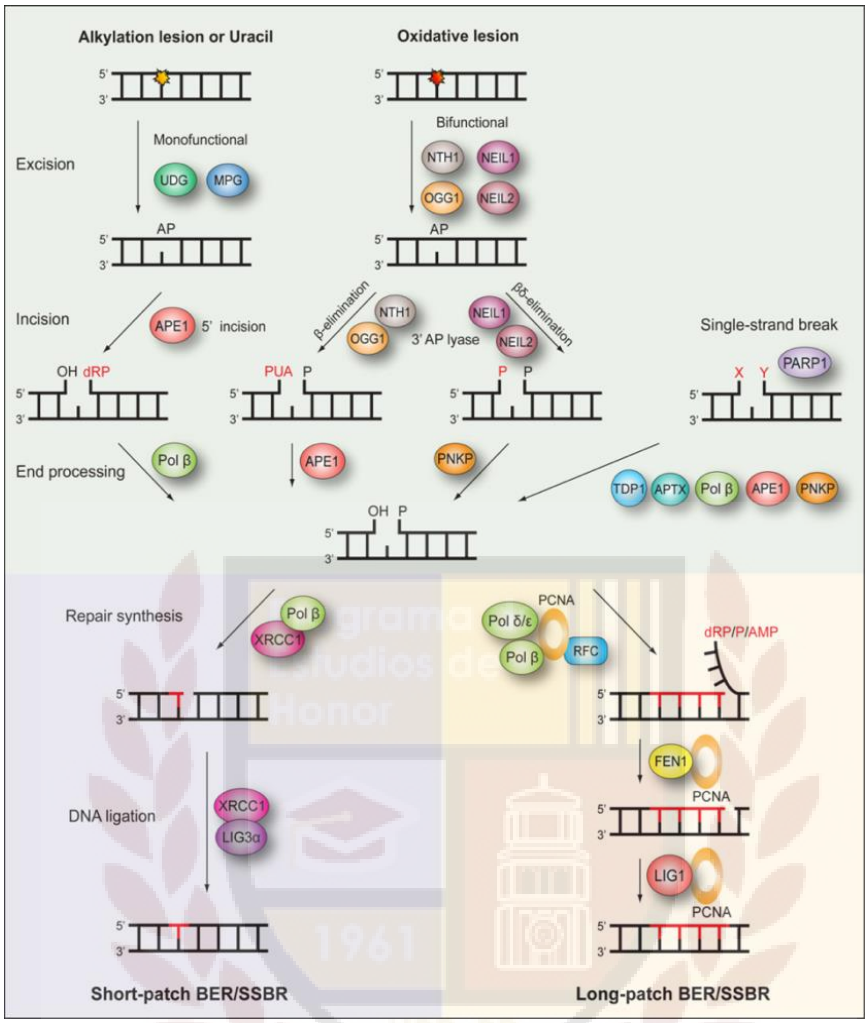


Figure 6. BER oxidative lesion and repair. MtDNA lesions can be cause alkylation or oxidative. ROS damage the mtDNA and several steps and proteins involved in the BER mechanism attempt to correct such lesion. If not corrected, it becomes a mutation either the survival cell or it may induce apoptosis. BER involves multiple enzymes to carry out the repair of DNA containing damaged bases or AP sites. It begins with an excision of the damage base by DNA glycosylase. An AP site is generated due to the cleavage of the N-glycosidic bond of the damage base. Then it is followed by AP-endonuclease that cleaves the AP site to generate 3' OH terminus at the damage site, Then the DNA polymerase fill in the single nucleotide gap generated due to lesion base removal. As the final step, the nick and sealing is done by a DNA ligase. The short-patch BER pathway leads to a repair tract of a single nucleotide. Alternatively, the long-patch BER pathway produces a repair tract of at least two nucleotides (Hedge, M. 2008). (Figure obtained from Jeppensen, E. 2011)

A main question in the literature is understating how mtDNA damage affects the function of mitochondria. Mitochondrial dysfunction in the form of increased ROS have been linked to different pathologies such as Huntington's disease, Parkinson's disease, multiple sclerosis, cancer, diabetes and cardiovascular dysfunction (Thakur, S. 2014). Thus, targeting mitochondrial dysfunction has the potential to ameliorate or prevent the progression of many diseases. Mitochondria is also an important target not only because of its role in cellular bioenergetics, but also because of its role in maintaining nuclear genome stability (Kaniak-Golik, A. 2015).

Hypothesis and Specific Aim

The purpose of the research is to analyze the effects of ROS-induced damage on mitochondrial function. Our hypothesis states that deficient mtDNA repair will lead to mitochondrial dysfunction in the form of increased ROS. To test this hypothesis, we used yeast strains harboring mutations in mtDNA repair genes to determine the effect of these mutations in ROS production. Our hypothesis states that mtDNA repair deficient strains will generate more ROS than wild type (WT) cells after an insult with H_2O_2. To test this hypothesis, we established the following specific aim: to determine if yeast strains harboring mutations in the DNA repair *APN1* and *APN2* genes exhibit increased ROS production after an oxidative insult with H_2O_2.

Chapter 2: Material and Methods

Experimental Design

To test our hypothesis, *Saccharomyces cerevisiae*, commonly known as Baker's yeast, was used as experimental model. This organism is a unicellular eukaryotic model, easy to handle as compared to more complex organisms such as human beings.

Wild type and isogenic strains harboring mutations in DNA repair genes were used. These yeast strains were exposed to an oxidizing agent and then the production of ROS was determined using MitoSox, a molecule that when oxidized, emits fluorescence.

Definition of Variables

- Independent variable: Concentration of Hydrogen Peroxide
- Dependent Variable: Production of ROS
- Control: Wild Type *Saccharomyces cerevisiae*
- Experimental: *Saccharomyces cerevisiae* with mutations in the *APN*1 and *APN*2 genes.

Yeast strains, media and H_2O_2 treatment

The organism model used in this research is the yeast *Saccharomyces cerevisiae*. The strain used wereEMY74.7 (MATa his3-Δ1 leu2-3, -112 trp1Δ ura3-52), and its isogenic derivatives harboring single and double mutations in the *APN1* (*apn1Δ*) and *APN2* (*apn2Δ*) genes. A total of four strains were studied: WT, *apn1Δ,apn2Δ and apn1Δapn2Δ.*

Cells were cultured overnight in YPD medium (1% yeast extract, 2% peptone and 2% dextrose; Fisher Chemicals). The next day cell density was determined using a hemocytometer and diluted to 1 x 10^7cells/ml. After 2 hours cells were treated by adding H_2O_2at 2 different concentrations (0.3 mM or 1.0 mM). Cells were treated for 1 hour at 30ºC with vigorous aeration. At the end of the treatment period cells were centrifuged for 3 minutes at 2,500 rpm and washed with H_2O. Cells were then resuspended in 1 mL of H_2O before proceeding to MitoSox incubation.

Determination of the generation of mitochondrial superoxide anions in the mitochondria

ROS will be measured using MitoSox Red (Thermo Fisher Scientific). ROS levels will be measured in wild type and mutant yeast strains after treatment with H_2O_2 as described above. Cells will be washed, centrifuged, and resuspended in 1 mL of PBS. MitoSox Red was added to a final concentration of 2 mM, followed by an incubation of 20 minutes at 30ºC in the dark. After incubation with the dye, the cells were centrifuged, washed, and resuspended in 1 mL of PBS. The fluorescence was detected using a Flow Cytometry instrument located in the laboratory of Dr. Yamil Gerena at the University of Puerto Rico Medical Sciences Campus. All the flow cytometric analyses were carried out using a FACSCelesta cytometer (BD Biosciences, CA). FACSDIVAsoftware (BD Biosciences, CA) was used for data acquisition. Cells were gated in forward/side scatter dot plots. ROS emissions were measured in the FL2 (585 nm band-pass filter) channel. Data on scatter parameters and histograms were acquired in log mode. Ten thousand events were

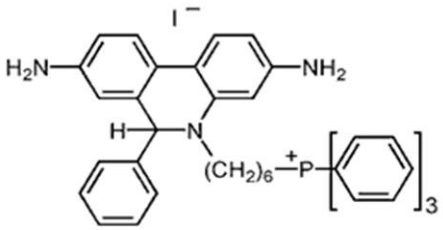
evaluated for each sample, and the median peak channel obtained from the histograms was used to determine the levels of ROS.

Ethical considerations

The ethical considerations that will be involved in this investigation to be carried out are the honesty in the handling of the information, in the collection of data and in the analysis of it. Likewise, compliance with the standards established by the Institutional Biosafety Committee of the Medical Sciences Campus of the University of Puerto Rico.

Statistical Analysis

Outputs from the FACS instrument were transferred to Excel and then statistical analyses were performed using PRISM 7. One-way analysis of variance (ANOVA) was used to demonstrate the effect of H_2O_2 on each of the strains. The means and standard errors were represented graphically. P values below 0.05 ($p < 0.05$) were considered statistically significant.



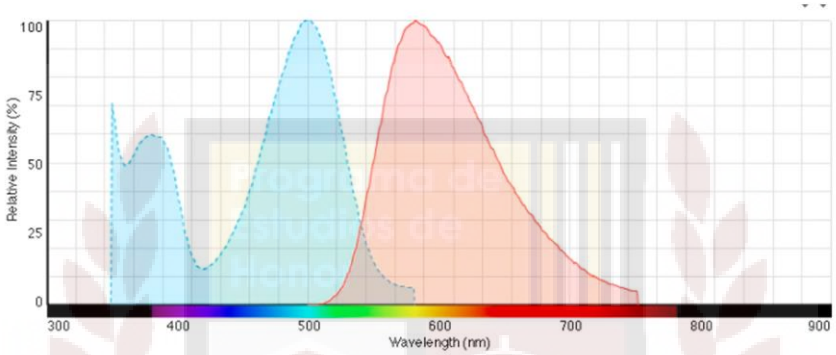


Figure 7. MitoSox molecule and spectra. Upper panel; structure of theMitoSox molecule. Lower panel; MitoSox specta; absorbtion is approximatelyat 510nm (blue graph) and emission at 580nm (red graph). (Figure obtained from ThermoFisher Scientific).

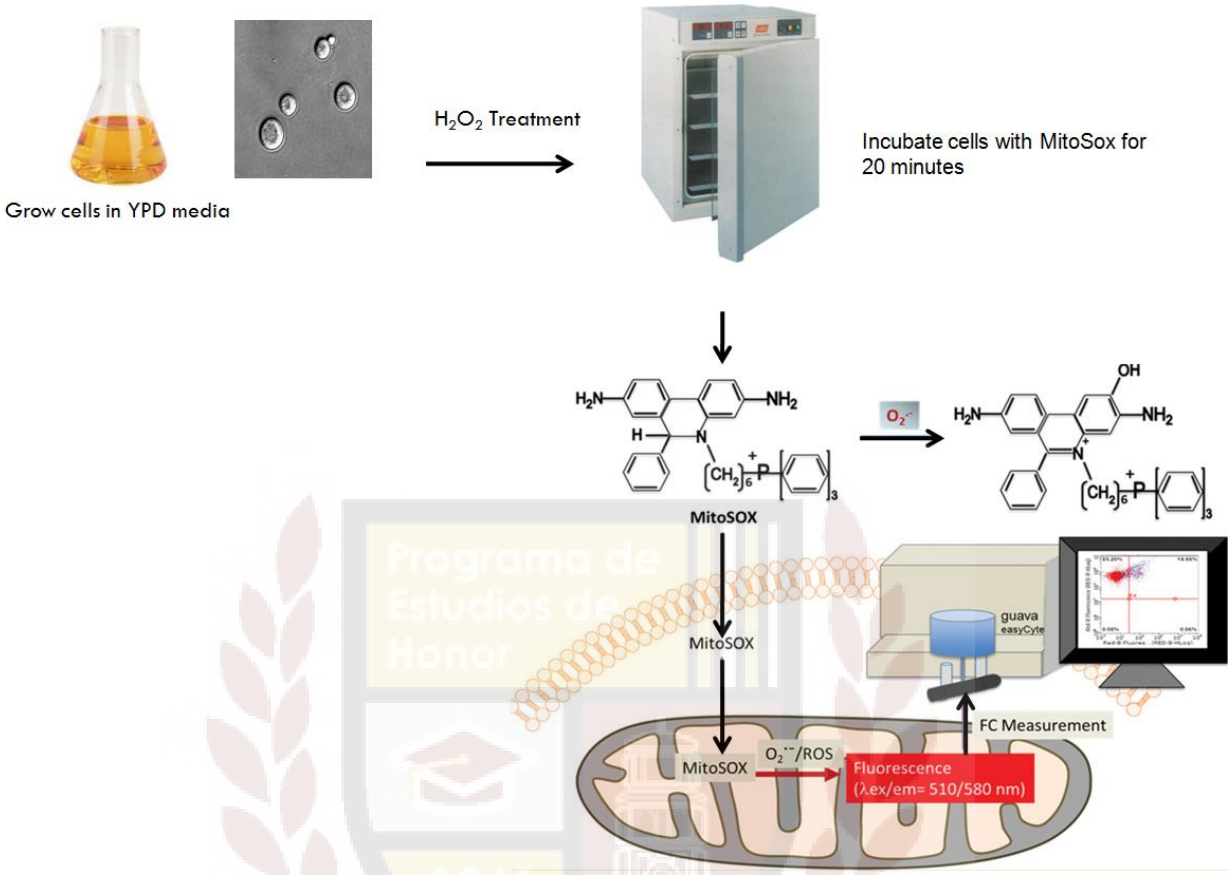


Figure 8. Schematic illustration of the protocol. Cells (1.0 x10^7 cells/mL) were treated with H_2O_2 treatment for 1h at 30ºC. After H_2O_2 treatment , MitoSox dye was added. Lastly, a FACS instrument was used to measure fluorescence intensity. The image also shows the MitoSox oxidation by superoxide. (Part of the image was obtained from Kauffman, M. 2016 and the other is original).

Chapter 3: Results

One of the targets that is the subject of this research are the components of the BER machinery, which repairs the mtDNA lesions and thus preserve the integrity of the mitochondrial genome (Kaniak-Golik, A. 2015). Currently, the information available about mtDNA repair and mitochondrial dysfunction is scarce, thus studying the relationship between mtDNA repair and ROS, production is of great interest. Therefore, we proposed that if mtDNA repair is important to prevent mitochondrial dysfunction, then strains of yeast that have the BER system deficient will have higher production of ROS than WT strain. To answer this question, we studied the role of the genes *APN1* and *APN2* in the BER machine after a lesion in the mtDNA by an oxidizing agent, H_2O_2, measuring with the FACS instrument the fluorescence emitted by the molecule of MitoSox when oxidized by ROS.

The results of a typical experiment are shown in Figures 9-12. Cells that were treated with H_2O_2 followed by MitoSox incubation were analyzed in a FACS instrument and the percent of cells that exhibit fluorescence above background was determined. Figure 9 shows the FACS profile of the WT strain that was not treated with H_2O_2 nor exposed to MitoSox. The purpose of this experiment was to have a control sample to determine if there was autofluorescence. A total of 10,000 events (cells) were analyzed in this and all subsequent experiments. An area (P3) in the FACS scan was defined as the area above autofluorescence threshold. In this experiment, it can be seen that only 0.1% of the cells exhibited autofluorescence above the threshold.

Figure 10 shows the results of cells that were not treated with H_2O_2 but incubated with MitoSox. It can be observed that the levels of fluorescence in the P3 area increased to 3.3% of the cells. Figures 11 and 12 show the results of cells treated with two different H_2O_2, doses. The percentage of cells with fluorescence in the P3 area were 2.9 and 4.6 for 0.3 mM and 1.0 mM H_2O_2, respectively. We performed a minimum of 3 independent experiments per strain and the data obtained was average and analyzed by one-way ANOVA.

The results of the experiments performed in the WT strain show that in untreated cells, 2.94% ± 1.15 (AVG ± SD) of the cells exhibited a MitoSox signal above background (Figure 13). When cells were exposed to 0.3 or 1.0 mM H_2O_2, the values obtained were 2.88% ± 0.69 and 3.94% ± 1.00, respectively. ANOVA analysis shows that the differences were not statistically significant (p = 0.199).

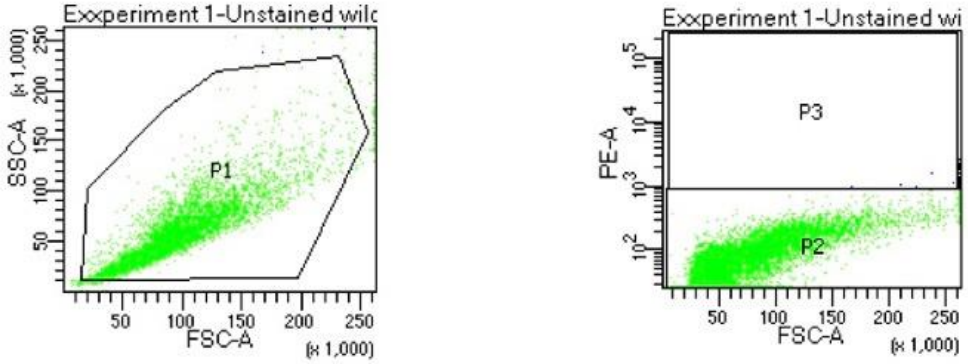
The results of the experiments performed in the *apn1Δ* strain show that in untreated cells, 6.07% ± 2.02 (AVG ± SD) of the cells had a MitoSox signal above background (Figure 14). When cells were exposed to 0.3 or 1.0 mM H_2O_2, the values obtained were 6.83% ± 2.10 and 8.07% ± 2.723, respectively. ANOVA analysis shows that the differences were not statistically significant (p = 0.59).

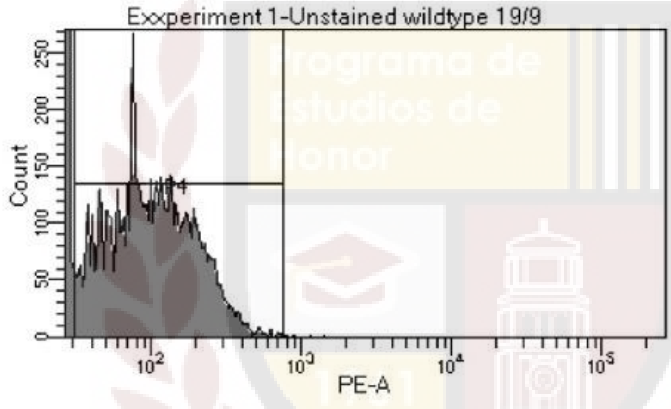
The results of the experiments performed in the *apn2Δ* strain show that in untreated cells, 3.03% ± 0.97 (AVG ± SD) of the cells had a MitoSox signal above background (Figure 15). When cells were exposed to 0.3 or 1.0 mM H_2O_2, the values obtained were 3.18% ± 0.29 and 3.6% ± 0.62, respectively. ANOVA analysis shows that the differences were not statistically significant (p = 0.59).

The results of the experiments performed in the *apn1Δapn2Δ* strain show that in untreated cells, 5.54% ± 1.28 (AVG ± SD) of the cells had a MitoSox signal above background (Figure 16). When cells were exposed to 0.3 or 1.0 mM H_2O_2, the values obtained were 5.76% ± 1.76 and 5.88% ± 1.75, respectively. ANOVA analysis shows that the differences were not statistically significant (p = 0.94).

While analyzing the results of the individual strains, we noticed that the ROS levels of the untreated strains were different among each other. Thus, we performed an ANOVA analysis of the untreated strains. The analysis indicates that there is a significant difference within the strains (p=0.0074). We then performed a multiple comparisons Tukey test to determine which strains exhibit this difference. The WT strain when compared to the *apn1Δ* strain shows a statistically significant P value (p=0.0292). However, when compared to the apn2Δ strain, the WT strain shows no significant difference (p=0.9997). When comparing the WT strain to the double *apn1Δ apn2Δ* double mutant strain there was a statistically significant difference (p=0.0368). A comparison of the individually mutated strains shows a statistically significant difference between the *apn1Δ* and *apn2Δ* strains (p=0.0438). No statistically significant differences were found between the single mutant apn1Δstrain and the double mutant *apn1Δ apn2Δ* strain (p=0.9463). Similarly, there was no statistically significant difference between the single mutant apn2Δ and the double mutant apn1Δ apn2Δ strain (p=0.06). The results of these experiments are summarized in Figure 17.

The results shown in this section indicate that although H_2O_2 treatment did not cause significant increases in ROS, there were significant differences in the basal levels of mitochondrial ROS within the strains.

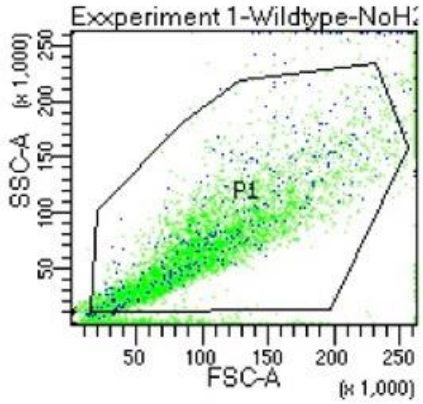


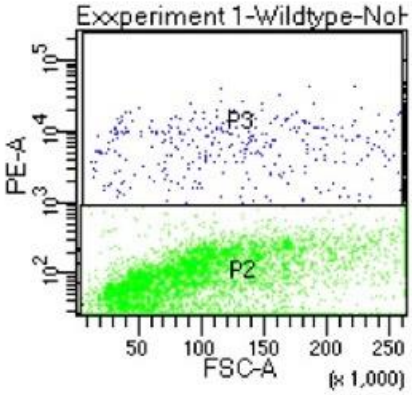


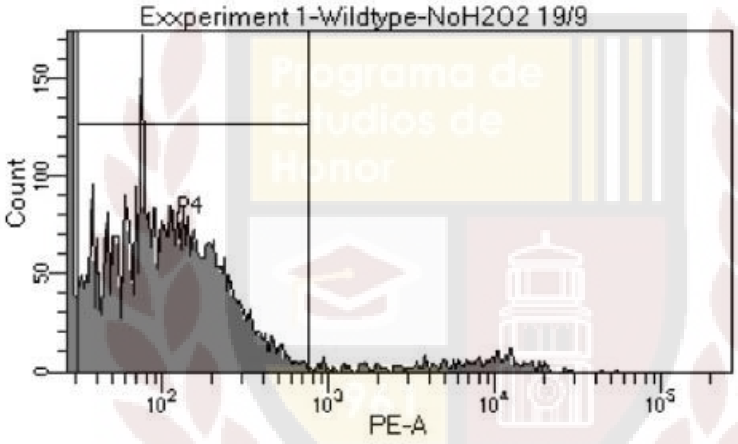
Experiment Name:	MitoSox - Sacharomyces- Dr Ca...
Specimen Name:	Exxperiment 1
Tube Name:	Unstained wildtype 19/9
Record Date:	Sep 19, 2019 3:42:59 PM

Population	#Events	%Parent	PE-A Median
All Events	10,000	####	62
P1	9,200	92.0	68
P2	9,977	99.8	62
P3	5	0.1	1,040
P4	6,714	67.1	96

Figure 9. Detection of fluorescence in WT cells that were not treated with H_2O_2 nor incubated with MitoSox.



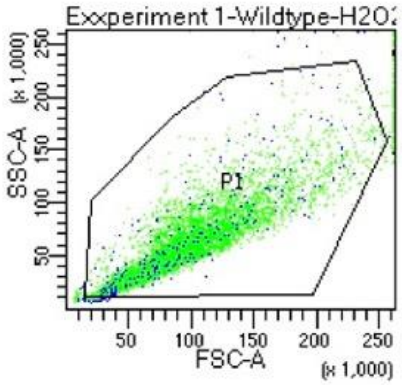


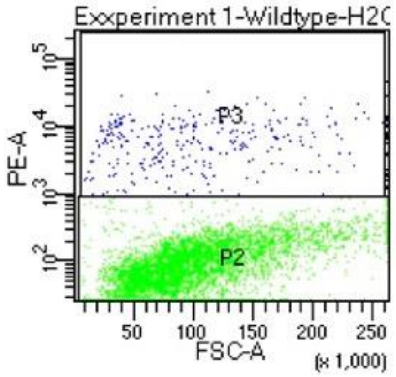


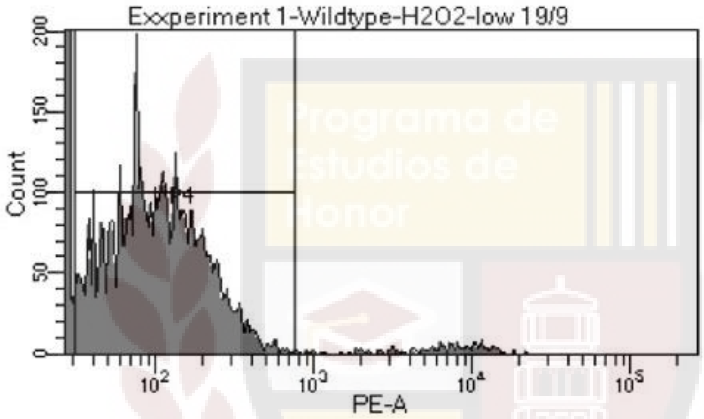
Experiment Name:	MitoSox - Sacharomyces- Dr Ca...
Specimen Name:	Exxperiment 1
Tube Name:	Wildtype-NoH2O2 19/9
Record Date:	Sep 19, 2019 3:43:52 PM

Population	#Events	%Parent	PE-A Median
All Events	10,000	####	29
P1	7,932	79.3	35
P2	9,519	95.2	23
P3	327	3.3	6,954
P4	4,443	44.4	100

Figure 10. Detection of fluorescence in WT cells that were not treated with H_2O_2 but incubated with MitoSox.



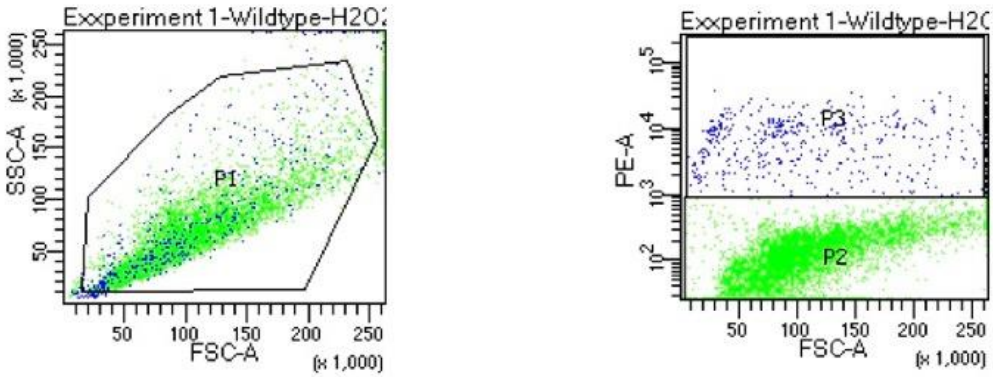


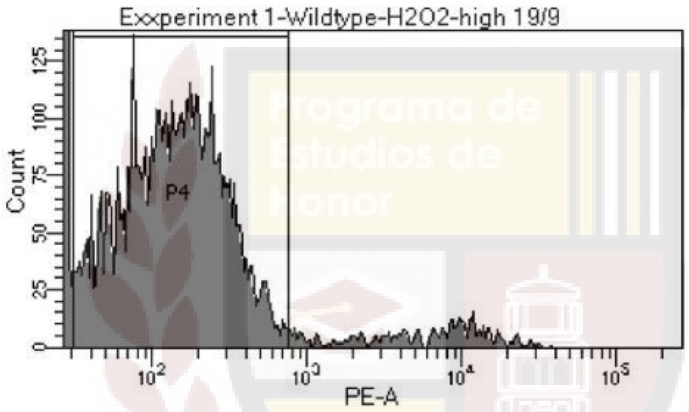


Experiment Name:	MitoSox - Sacharomyces- Dr Ca...
Specimen Name:	Exxperiment 1
Tube Name:	Wildtype-H2O2-low 19/9
Record Date:	Sep 19, 2019 3:44:23 PM

Population	#Events	%Parent	PE-A Median
All Events	10,000	####	41
P1	9,349	93.5	43
P2	9,685	96.9	38
P3	286	2.9	6,319
P4	5,131	51.3	97

Figure 11. Detection of fluorescence in WT cells that were treated with 0.3 mM H_2O_2 and incubated with MitoSox.





Experiment Name:	MitoSox - Sacharomyces- Dr Ca...
Specimen Name:	Exxperiment 1
Tube Name:	Wildtype-H2O2-high 19/9
Record Date:	Sep 19, 2019 3:44:42 PM

Population	#Events	%Parent	PE-A Median
All Events	10,000	####	72
P1	9,500	95.0	68
P2	9,450	94.5	64
P3	462	4.6	7,422
P4	5,687	56.9	135

Figure 12. Detection of fluorescence in WT cells that were treated with 1.0mM H_2O_2 and incubated with MitoSox.

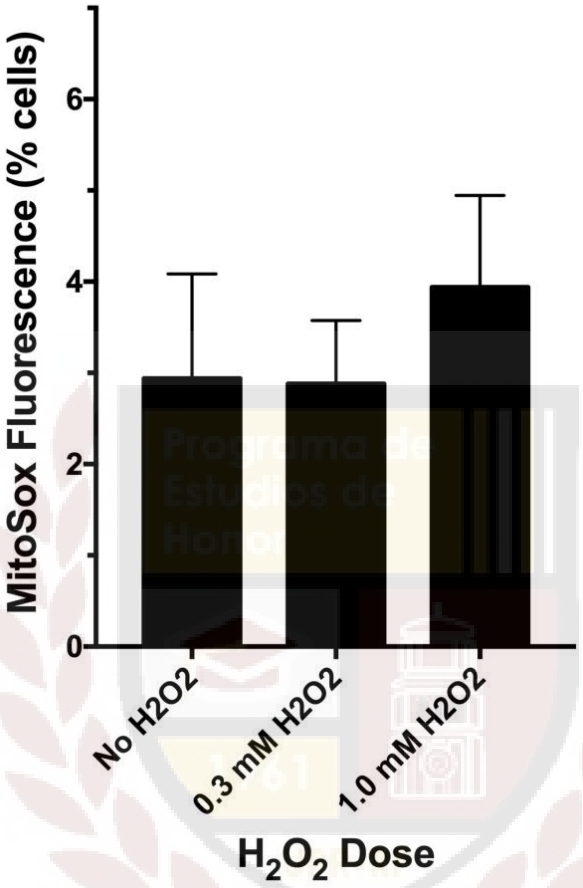


Figure 13. Effects of H_2O_2 in ROS production in Wild Type strain. The strain was exposed to the indicated H_2O_2 doses for 1 hr and ROS were detected immediately after using MitoSox and FACS. Results are expressed in terms of % positive cells. N= 5 experiments.

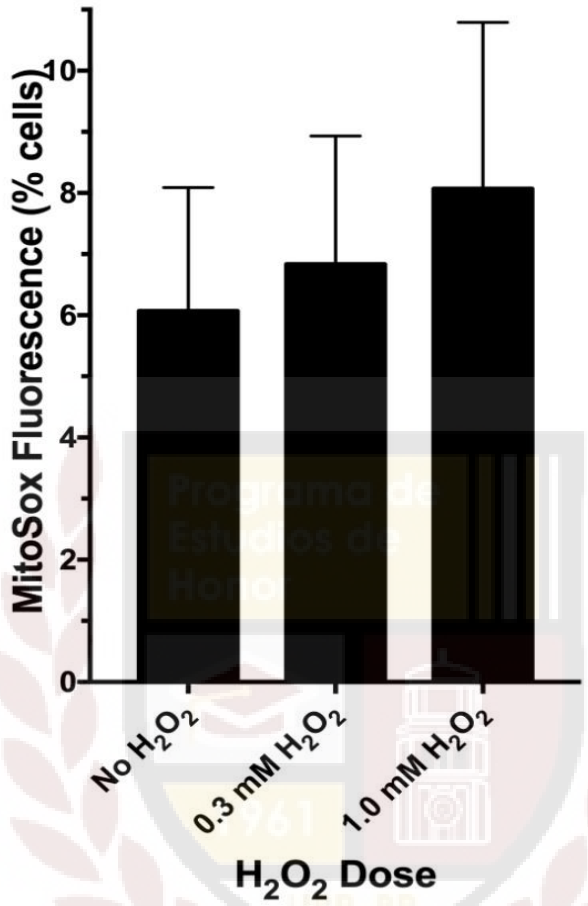


Figure 14. Effects of H_2O_2 in ROS production in apn1Δ strain. The strain was exposed to the indicated H_2O_2 doses for 1 hr and ROS were detected immediately after using MitoSox and FACS. Results are expressed in terms of % positive cells. For each strain, N= 4 experiments.

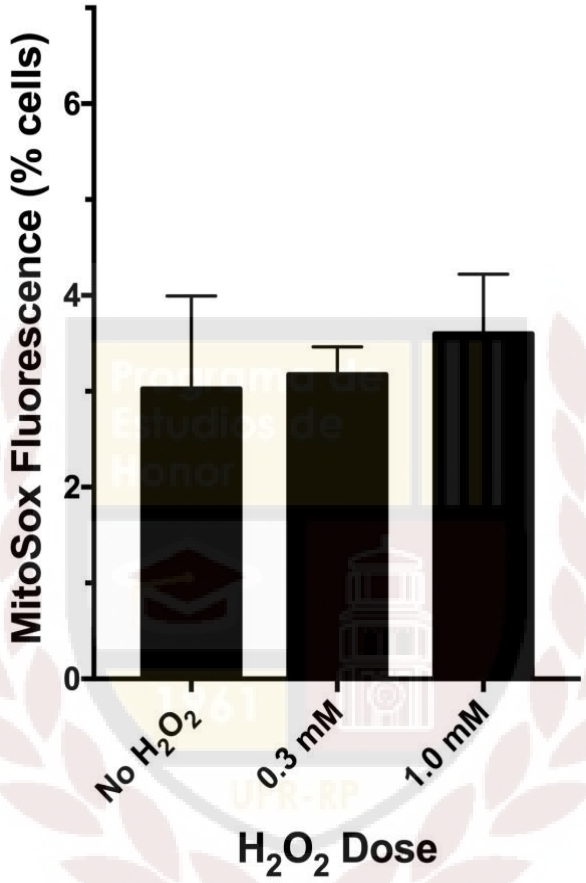


Figure 15. Effects of H_2O_2 in ROS production in apn2Δ strain. The strain was exposed to the indicated H_2O_2 doses for 1 hr and ROS were detected immediately after using MitoSox and FACS. Results are expressed in terms of % positive cells. For each strain, N= 3 experiments.

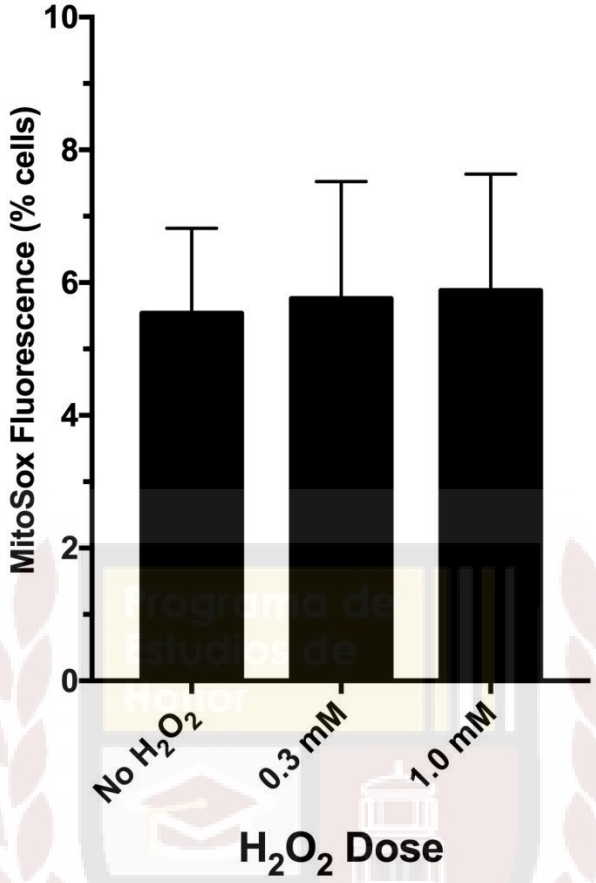


Figure 16. Effects of H_2O_2 in ROS production in apn1Δ apn2Δ strain. The strain was exposed to the indicated H_2O_2 doses for 1 hr and ROS were detected immediately after using MitoSox and FACS. Results are expressed in terms of % positive cells. For each strain, N= 5 experiments.

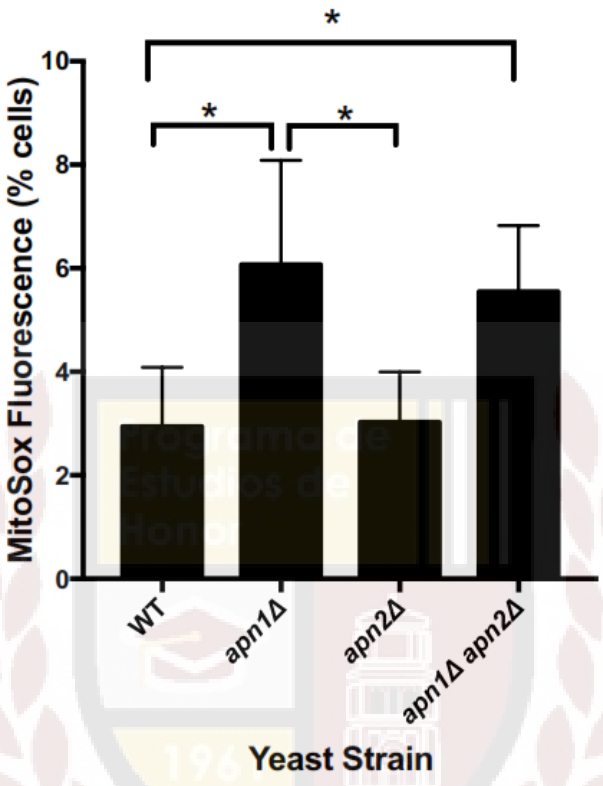


Figure 17. ROS detection in untreated yeast strains. The asterisks (*) denotes the strain that had statistical significance (WT vs. *apn1*Δ; WT vs. a*pn1*Δ *apn2*Δ*; apn1*Δ vs. *apn2*Δ).

Chapter 4: Discussion and Conclusion

To understand the role of BER DNA repair genes, *APN1* and *APN2*, in preventing mitochondrial dysfunction, we employed a method to assess the generation of mitochondrial ROS after oxidative stress in the yeast *Saccharomyces cerevisiae.* Oxidative DNA damage was induced by H_2O_2 treatment. Our hypothesis predicted that cells deficient in mtDNA repair would exhibit increased ROS production than a WT strain.

We focused our attention on the APN genes since they have a prominent role during BER, the main mechanism for the repair of oxidative DNA lesions in mitochondria. AP sites (the substrate for AP endonucleases) are not only an intermediate during BER but also one of the most common spontaneous DNA lesions. Because they are noncoding, these sites would present a block to the DNA replicational machinery inducing mutations via DNA translesion synthesis (Johnson, R.E. 1998). The *APN1* and *APN2* genes encode AP endonucleases that cleaves the 5' end of an AP site created when the damaged base is removed by monofunctional DNA glycosylase. They also have a 3' phosphodiesterase and exonuclease activities and can remove and digest the 3' blocking end created by bifunctional glycosylases (Abedin, Z. 2013). In our study we exposed yeast strains carrying single and double null mutations in the APN genes (WT, *apn1Δ*, *apn2Δ, and apn1Δ apn2Δ*) to an oxidizing agent and measured the production of mitochondrial ROS using MitoSox, a molecule that upon oxidation by the superoxide anion emits fluorescence. If there is an increase in mitochondrial ROS it should be reflected in the amount of fluorescence emitted by MitoSox. The results obtained in our study show than in none of the strains analyzed there was an increased in ROS production after H_2O_2 treatment (Figures 13 to 16). Thus, the results obtained do not

support our original hypothesis. However, when analyzing the data, we noticed the levels of untreated cells vary in each of the strain. We found that the single *apn1Δ* and the double *apn1Δ apn2Δ* mutant strains have elevated basal levels of mitochondrial ROS. These results indicate that the *APN1* gene is required for preventing mitochondrial dysfunction under basal levels.

We used to different H_2O_2 doses; 0.3 mM and 1.0 mM. It is well established that cell viability at 0.3 mM H_2O_2 is hardly affected (>80% viability) (Guzner et al 1998). However, the 1.0 mM H_2O_2 induces more that 50% lethality in all the strains, particularly in the *apn1Δ apn2Δ* double mutant. In addition, unpublished studies in our laboratory show that 0.3 mM H_2O_2 induces mtDNA damage (Torres-Arroyo et al, manuscript in preparation). Furthermore, we have previously determined that *APN1* is required for repair of mitochondrial alkylation damage (Acevedo-Torres, et al 2009). Thus, we were expecting to detect increased ROS production in the BER mutant strains.

The major pathway for removing AP sites in yeast is carried out by the AP endonucleases, Apn1 and Apn2, who are major components of the BER machinery (Ma, W. 2008). Apn1 accounts for >90% of the AP endonuclease activity in wild type yeast cells, suggesting the activity encoded by the *APN2* gene is relatively minor (Jonson, R.E. 1998). This is reflected in the results obtained in the *apn1Δ* strain and *apn2Δ* strain. Specifically, in the *apn1Δ* strain, we observed that the MitoSox fluorescence is higher than WT strain, indicative of increased ROS production at the basal state. However, in the apn2*Δ,* MitoSox fluorescence was similar to the WT strain. Similarly, the double

apn1Δ apn2Δ mutant strain has similar levels of MitoSox fluorescence than the *apn1Δ* strain. This confirms the importance of Apn1 in the BER machine.

On the other hand, *apn2Δ* strain results show that MitoSox fluorescence is less than the *apn1Δ* strain. As above said, that Apn1 endonuclease accounts for the main role in the BER machine. This means that when Apn1 is present (as it is in the apn2Δ strain) it is able to correct more efficiently the lesions induced by the endogenous oxidative damage. A similar explanation can be used to explain the results obtained in the double mutant *apn1Δ apn2Δ* mutant strain. Since Apn1 is absent in this strain, basal endogenous levels would accumulate and indices mitochondrial dysfunction, similarly to what we observed in the single apn1Δ strain.

Apn1 is required for preventing ROS formation because it is not only important for the repair of the DNA when there has been an insult to the DNA, but also its functionality reassures the integrity of the transcribed DNA. Thirteen polypeptides of the ETC are encoded in the mtDNA, hence any damage in the mtDNA will lead to decreased ETC proteins, affecting the functionality of the ETC. Therefore, an inefficient ETC could increase the escape of electrons increasing the formation of ROS. Hence, Apn1 is required for the prevention of ROS formation (Figure 18).

Future directions involve experiments using fluorogenic dyes with broader specificity for ROS, such as Rhodamine 123 or 2',7'-dichlorodihydrofluorescein diacetate to determine whether *APN1* or *APN2* can prevent ROS formation in the basal state or after H_2O_2 treatment. In addition, we will study bioenergetics parameters by performing

extracellular flux analyses (Seahorse analyses) to determine if *APN* mutants exhibit any defects.

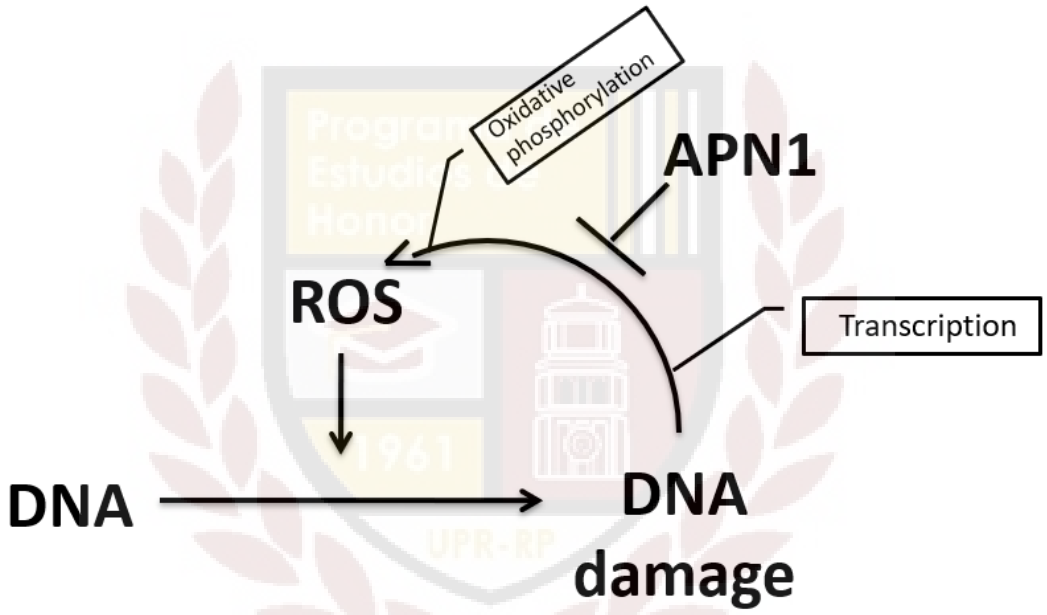


Figure 18. Proposed role of *APN1* in preventing mitochondrial dysfunction in the form of increased ROS formation. Our studies indicate that the *APN1* gene is required for preventing mitochondrial dysfunction induced in the basal state.

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