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Mitochondrial and nuclear genomic integrity after oxidative damage in *Saccharomyces cerevisiae*

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1. ABSTRACT

All cells have the ability to adjust their metabolism to their changing environment to be able to survive. This adaptation is coordinated by various systems in the cell and mitochondria seem to play a unique and important role. Most endogenous oxidative damage to cells is actually generated as a byproduct of the mitochondrial function, which in turn damages mitochondrial structures more extensively due to their proximity to the source. Excessive damage to mitochondria leads to loss of parts or all of mtDNA, but unlike other organisms, *S. cerevisiae* cells are able to survive without mtDNA or respiration when grown on fermentative carbon sources. This allows studies of the role of mitochondria in the maintenance of cellular integrity, since lack of mitochondrial DNA frequently leads to genomic instability. Mitochondria are known for their role in respiration, ATP production and apoptosis, but it is now becoming clear that their function is intimately connected to diverse processes such as calcium and iron homeostasis and amino acid metabolism, and thus their dysfunction is not well tolerated. In this review, we discuss the mechanisms by which mitochondrial dysfunction can lead to genomic instability and the effect of the carbon source on this process.

2. INTRODUCTION

All cells require an intact genome to function properly; this includes the genomes of mitochondria and chloroplasts in eukaryotic cells. Mitochondria provide the cell with specialized metabolic activities, some of which generate toxic byproducts, making the mitochondria a challenging place to maintain a genome. There are multiple pathways to ensure that both nuclear and mitochondrial genomes are protected from internal or external damage. Similarly, there are multiple mechanisms that detect other cellular damage, activate repair pathways, and halt cell cycle progression. Several recent studies indicate that the mitochondrial genome is subject to higher levels of damage than the nuclear genome, and we discuss the mechanisms employed to maintain mitochondrial genome stability along with potentially deleterious metabolic activities. Mitochondrial dysfunction leads to a series of events that can lead to nuclear genomic instability. In addition, the state of the electron transport chain seems to have an influence on how much stress cells can tolerate. Here, we summarize the evidence that supports the role of mitochondria in damage sensing, and the communication between mitochondria and the nucleus in the unicellular fungi *Saccharomyces cerevisiae*. This yeast provides an

excellent system to study mitochondrial processes as it is viable on fermentable carbon sources even after losing its mitochondrial DNA, thus allowing the study of mitochondrial-specific processes that are involved in damage sensing and genome maintenance.

3. GENOMIC ARCHITECTURE OF *SACCHAROMYCES CEREVISIAE*

3.1. The nuclear genome

The nuclei of haploid *S. cerevisiae* cells contain approximately 12.1 million base pairs of DNA and about 6,000 genes within 16 chromosomes (1). *S. cerevisiae* chromosomes contain approximately 332 origins of replication that are uniformly distributed along all its chromosomes. Unlike in higher eukaryotes, the replication origins in yeast, referred as ARS (autonomously replicating sequences), contain specific sequence elements that are recognized by initiator proteins (2). The centromeres of budding yeast also differ from those of other eukaryotes in that they are of well-defined length (125 base pairs) and form kinetochores that each binds only one microtubule during division (2-4).

Similar to other eukaryotes, *S. cerevisiae* chromosomes contain transcriptionally active euchromatin, as well as silent telomeric and centromeric heterochromatin. However *S. cerevisiae* differs from other eukaryotes in that it does not establish and maintain heterochromatin using an RNAi-mediated pathway, nor does it use Heterochromatin Protein-1 (HP-1) or H3K9 methylation (5). The heterochromatin in *S. cerevisiae* is instead formed by a Sir (silent information regulator)-mediated transcriptional silencing mechanism (6-8) and is specifically referred to as either yeast heterochromatin or silent chromatin. Regardless of details of its mode of formation and composition, heterochromatin in yeast and other eukaryotes is involved in similar processes including gene silencing, protection of the chromosome ends and anchoring the chromosome segregation machinery during cell division (9).

The activity and compaction states of chromatin are determined by the physical association between histone proteins and the DNA. The basic unit of chromatin is the nucleosome, which is composed of eight highly conserved core histones, two each of H2A, H2B, H3 and H4. Besides providing a basic structural unit, posttranslational modifications on the tails and cores of histones influence many DNA transactions, including transcriptional activity, chromosome segregation, DNA damage recognition and repair (10-14). In addition, incorporation of several core histone variants, such as CenH3 (a.k.a. Cse4 in the budding yeast and CENP-A in human) at centromeric regions or H2AZ in the promoters of repressed genes, allows functional specialization (3, 4, 15, 16). Chromatin remodelers reposition nucleosomes or alter the extent of histone-DNA interactions so that the availability of a particular region of DNA can be altered upon demand (17, 18). Besides the core histones, chromatin includes linker histones, non-histone structural proteins such as the high mobility group proteins (HMGs), as well as the associated modifying and remodeling enzyme complexes and myriad RNAs, all of which determine the overall state of chromatin in a given time (19).

3.2. The mitochondrial genome

Mitochondria carry out many cellular functions including respiration, calcium and iron homeostasis, amino acid metabolism, and control of apoptosis (20). The mitochondrial genome in *S. cerevisiae* encodes several subunits of the respiratory chain and the ATPase complex, several mitochondria-specific tRNAs, one of the mitochondrial large ribosome subunit proteins and two mitochondrial rRNAs (21, 22). The mitochondrial genome is thus critical for many of the mitochondrial functions.

S. cerevisiae mitochondria maintain an 80-kilobase circular genome (about 5 times larger than animal mitochondrial genomes due to extended regions of non-coding DNA) that is 82% AT rich and has eight origins of replication (23). The AT-rich origins exhibit low melting temperatures and low sequence complexity, which likely accounts for the high levels of illegitimate recombination observed between origins. Inter- and/or intra-genome recombination events frequently lead to loss of segments of protein-coding mtDNA and result in formation of rho$^-$ strains that lack the respiratory function. Such strains are called "petite" due to the small size of the colonies they form (23). Formation of petite colonies and the inability of such cells to grow on non-fermentable carbon sources such as glycerol and ethanol are frequently used as a measure for partial or complete loss of mtDNA and mitochondrial respiratory function.

Mitochondria do not contain canonical histones. Instead mitochondrial DNA (mtDNA) is bound by Abf2, a histone-like HMG protein encoded in the nucleus that is capable of bending and wrapping the mtDNA (24). mtDNA is contained within nucleoids, and exists in chromatin-like rod-shaped complexes that also contain RNA and proteins with diverse functions, and are bound to the mitochondrial inner membrane (25, 26). Unlike nuclear DNA, the total amount of mtDNA in an organelle as well as the amount within individual nucleoids varies greatly depending on the growth conditions. It was estimated that aerobically grown stationary phase cells contain nucleoids with about 1.5 genome equivalents, while anaerobically grown cells can contain giant nucleoids with up to 20 genome equivalents. Not only the amount, but also the form of the mtDNA changes depending on the cell cycle stage and cell age; while monomeric mtDNA is predominant in the newly forming daughter cell, concatemers that are formed either through rolling-circle replication or recombination events are the major species in mother cells (27).

Interestingly, the nucleoid structure appears to undergo remodeling under different growth conditions. Nucleoids have a more open structure in cells grown on glycerol (where mitochondria are highly active) and more closed structure in glucose-grown cells where the mitochondria exhibit reduced activity (24). The amount of mtDNA contributes to this remodeling, as cells grown in

glycerol have about two-fold more mtDNA compared to those grown in glucose while Abf2 levels stay the same, resulting in a decrease in the Abf2 to mtDNA ratio. The presence of the mitochondrial chaperone Hsp60 increases several fold in nucleoids from glucose-grown cells, and a similar increase of Ilv5 (an amino acid biosynthetic enzyme) in nucleoids occurs in cells starved for amino acids (24). This suggests that the mitochondrial nucleoids recruit specific proteins in response to metabolic cues.

3.3. Nuclear and mitochondrial communication

Cells maintain close communication between nuclei and mitochondria in order to preserve various cellular functions. As mentioned previously, mitochondria are integral in many processes, including respiration, calcium homeostasis, amino acid metabolism and apoptosis (20). However, only a small fraction of the 851 proteins recently identified in highly purified mitochondria are encoded by the mtDNA. These nuclear-encoded proteins include subunits of complexes required for oxidative phosphorylation and respiration (28). Therefore, it is not surprising that the mitochondrial function is tightly linked to the regulation of a number of nuclear genes (29). In fact, a screen for mutants that show altered mitochondrial morphology and inheritance in *S. cerevisiae* identified 119 nuclear genes (30). These genes include those involved in ergosterol biosynthesis, mitochondrial protein import, actin-dependent transport, vesicular trafficking and ubiquitin/26S proteasome dependent protein degradation (30). While the roles that nuclear genes play in mitochondrial maintenance are complicated and as yet poorly understood, these data indicate that mitochondrial structure and inheritance are under complex control via several cellular activities influenced by nuclear gene expression.

There are three known pathways of communication between the mitochondria and the nucleus. One pathway of communication, referred to as "retrograde regulation", is activated when respiration is compromised. Retrograde regulation controls the activity of nuclear genes that encode proteins involved in maintaining glutamate levels, as well as nitrogen and carbohydrate metabolism. Retrograde signaling thus allows cells to readjust their metabolism to the altered mitochondrial state. "Hypoxic signaling" occurs in cells that have reduced oxygen levels. Under these conditions, mitochondria produce reactive oxygen species (ROS), nitric oxide (NO) and reactive nitrogen species (RNS). This triggers the induction of genes that allow survival under hypoxic conditions (31). The absence of mtDNA (as in the case of petites), but not simply the lack of respiration or oxidative phosphorylation, triggers a third pathway referred to as "intergenomic signaling". Intergenomic signaling leads to down regulation of several nuclear genes, including the components of cytochrome c oxidase, succinate dehydrogenase, cytochrome c reductase, ATP synthase and genes involved in amino acid and nucleotide metabolism (31, 32). These signaling pathways coordinate activity between the nucleus and the mitochondria and allow cells to adapt their metabolism to wide changes in their environments.

4. CELLULAR OXIDATIVE DAMAGE

Cells generate ROS as a result of aerobic metabolism and must actively detoxify these compounds to avoid cellular damage (33). The major source of ROS generation is the leakage of electrons from the mitochondrial electron transport chain (ETC). These high-energy electrons react with molecular oxygen forming superoxide ($O_2^{\cdot -}$) (34-36). The superoxide is quickly converted to hydrogen peroxide (H_2O_2) by the main mitochondrial superoxide dismutase (Sod2), and then to water by the action of catalases or peroxidases. DNA and proteins are not very reactive to H_2O_2. However, in the presence of iron or copper, H_2O_2 is converted to hydroxyl free radical ($\cdot OH$), which causes numerous forms of DNA damage, including DNA single- and double-stand breaks, oxidatively modified bases, depurination, depyrimidation, chemical modification of the sugar moiety, and DNA-protein cross-links (37, 38). Damage inflicted on proteins in the presence of metals includes oxidative scission, loss of histidine residues, bityrosine cross links, introduction of carbonyl groups, and the formation of protein-centered alkyl, alkxyl and alkylperoxly radicals. Proline, histidine, arginine, lysine and cysteine residues are predominantly affected by ROS, as they are highly sensitive to oxidation (38). Reactive nitrogen (RNS) species are another group of potent modifiers of proteins. Reaction of superoxide with nitric oxide yields peroxynitrite, which leads to nitration of tyrosine residues (39). Another extremely sensitive target of oxygen radicals are the polyunsaturated fatty acid moieties of phospholipids, especially those in the mitochondria due to their proximity to the sites of generation of these radicals (39). As with DNA, proteins are not very reactive to the simple presence of H_2O_2 unless transition metals are present.

In *S. cerevisiae* the type of carbon source utilized for growth influences the amount of endogenous ROS generated. *S. cerevisiae* is a facultative anaerobe; it derives its energy from fermentation regardless of the presence of oxygen, but depends on respiration and oxygen availability when grown on non-fermentable carbon sources like glycerol. Glucose is a preferred fermentable carbon source and glucose availability leads to downregulation of respiratory activity. When the electron flow is disrupted and oxygen is present, higher amounts of ROS are generated from mitochondria (39). In the presence of high glucose levels (2%), *S. cerevisiae* cells in stationary phase generate significant amounts of ROS and undergo high levels of cysteine oxidation of proteins. This effect is not seen when the cells are under conditions that require increased respiration and high ETC activity, such as on low glucose (0.5% glucose supplemented with glycerol), or on glycerol as the sole carbon source (40).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphopyruvate hydratases are two glycolytic enzymes that are inhibited by oxidation. Treatment of cells with the alkylating agent methyl methanesulfonate (MMS), which generates ROS in both the nucleus and the mitochondria, results in a rapid inhibition of two major glycolytic enzymes GAPDH and pyruvate

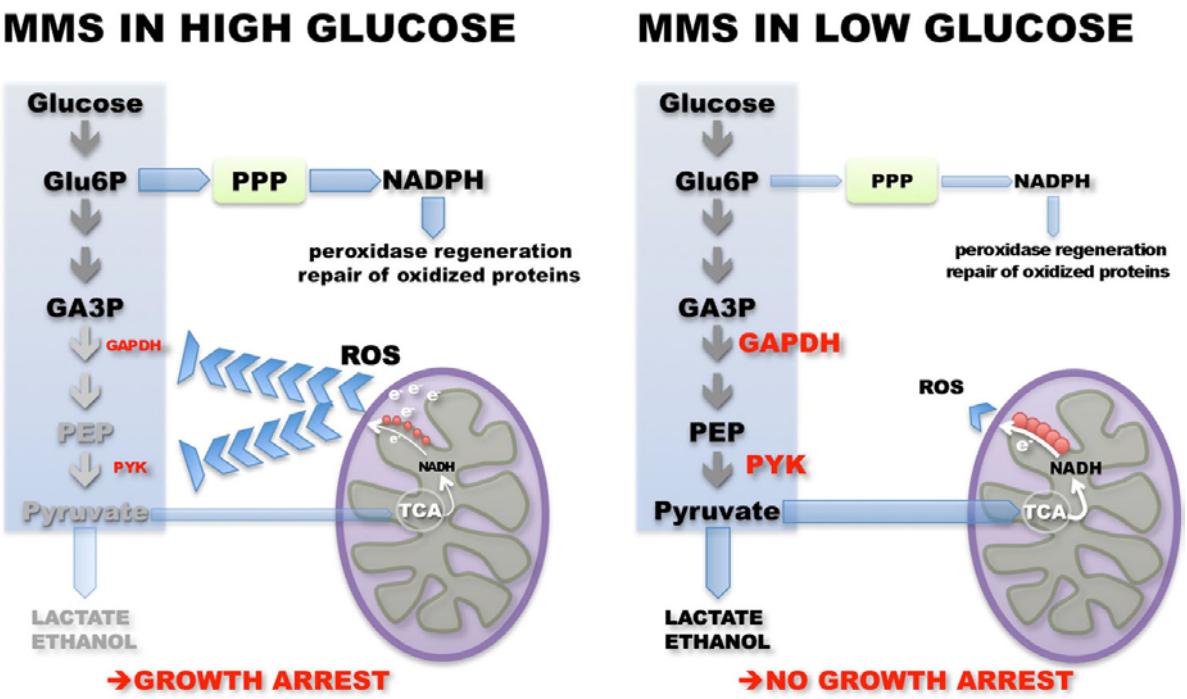

Figure 1. The effects of MMS treatment are amplified in the presence of high levels of glucose. Left panel: Mitochondria generate additional reactive oxygen species (ROS) upon treatment with MMS in the presence of high levels of glucose (2%). Cellular damage triggered by MMS treatment leads to leakage of electrons from the repressed electron transport chain (small red circles), generation of additional ROS, and inhibition of the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PYK) by oxidation. Diminished glycolysis leads to growth arrest and metabolic shift to the pentose phosphate pathway (PPP), which generates NADPH to replenish peroxidases and allow repair of oxidized proteins. Right panel: in the absence of the respiratory repression by glucose, a highly active electron transport chain (larger red circles) is less prone to electron leakage and allows continued growth in the presence of MMS.

kinase (PYK). Damage to electron transport chain components causes a rapid decline in respiratory activity and a drastic increase in ROS production (Figure 1, left panel). Pre-culturing cells under low glucose conditions preserves respiratory activity, reduces electron leakage from the ETC, and therefore reduces ROS production upon MMS treatment (Figure 1, right panel) (41). These results suggest that preservation of the mitochondrial respiratory activity is important for protection of cells from ROS generation and damage.

The above examples indicate a role for oxidant-sensitive glycolytic enzymes in the cell's control of ROS production (42, 43). This suggests that ROS directly feedback-inhibit metabolic pathways that support ROS production. GAPDH may be a key player in this defense as a sensor of the oxidative state. Oxidative modification of GAPDH represses glycolysis, and metabolic flux shifts from glycolysis to the pentose phosphate pathway, which generates the reduced electron carrier nicotinamide adenine dinucleotide phosphate (NADPH) (44). NADPH is used to reduce two classes of peroxidases, glutathione peroxidases and the thioredoxin peroxidases, which in turn repair oxidized cysteines and methionines, and regulate proteins containing sulphydryl groups (39). We speculate that NADPH may reactivate glycolysis by counteracting oxidative inactivation of GAPDH.

Proteins with complex structural organization, especially those containing cofactors or metal-containing prosthetic groups such as those involved in glycolysis and respiration, are especially prone to oxidative damage. When damaged beyond repair, these proteins are subjected to proteolytic degradation by the ATP-dependent protease Pim1 (45). In addition to removing oxidatively damaged macromolecules, the cell also responds to oxidative stress by transcribing genes involved in damage removal. The transcription factors Yap1, Skn7, Hsf1 and Msn2/4 are involved in upregulation of genes in damage removal pathways. Similar to the regulation of GAPDH by oxidation, Yap1 is activated by oxidation of its cysteines, leading to nuclear accumulation and activation of Yap1-dependent genes (39). However, we will not discuss the transcriptional aspects of the response to oxidative damage in this review.

5. CONSEQUENCES OF OXIDATIVE DAMAGE TO THE GENOME

Not only does respiration produce ROS, but mitochondria also contain stores of iron and other metals. As mentioned earlier, the presence of iron promotes the conversion of peroxide to hydroxyl free radicals that damage not only mitochondrial proteins and lipids, but also

the mitochondrial DNA. Ironically, peroxide is formed by the detoxification of superoxide by superoxide dismutases. This occurs in mitochondria primarily by the activity of the mitochondria-specific Mn-dependent superoxide dismutase Sod2. A small fraction of the mainly cytosolic Cu,Zn-dependent Sod1 also localizes at the mitochondrial intermembrane space and aids in removal of superoxide (39). Deletion of *SOD2* (*sod2delta*) results in an approximately three-fold increase in fluorescence of the oxidant-sensitive fluorescent probe DCFHDA (46). This is similar to the increase seen in cells treated with antimycin A, a specific inhibitor of the ETC, which causes electron leakage and a significant increase in ROS. Using a gene-specific DNA damage detection assay, Doudican *et al.* measured a three-fold increase in oxidized pyrimidines in the mitochondrial gene *COB1* in cells of a *sod2delta* strain compared to the wild type control (46). These results demonstrate that Sod2 suppresses ROS production and prevents mtDNA damage. In spite of these powerful ROS scavenging enzymes, the mitochondrial genome experiences higher levels of oxidative damage compared to the nuclear genome (47). It is estimated that mtDNA undergoes 3-10 fold more oxidative damage compared to nuclear DNA, and this imbalance is observed in many cell types and in organisms from yeast to humans (47). The proximity of mtDNA to the main source of ROS could explain this difference; however compartmentalization of ROS inactivation and damage repair mechanisms favoring the mitochondria could negate this effect. We will discuss the mechanisms that have evolved to minimize and repair oxidative damage to nuclear and mtDNA.

5.1. Some of the mitochondrial and nuclear DNA repair components overlap

In addition to direct detoxification, which prevents damage to DNA, the cell also detects and repairs oxidatively-modified nucleotide bases. One might expect the more damage-susceptible mitochondria to carry sophisticated DNA repair mechanisms. However, compared to nuclear DNA repair pathways, mitochondrial DNA repair appears to be quite simple. For example, mitochondria do not perform nucleotide excision repair (NER), a nuclear DNA repair pathway that requires a large number of specialized proteins to function (48). This raises the possibility that some kinds of mtDNA lesions might even escape repair (48). The relative simplicity of mtDNA repair may be of some advantage though. For instance, mtDNA repair does not require the import and assembly of large protein complexes.

The majority of oxidative damage to DNA is repaired by the short- or long-patch base excision repair (BER) systems, which are present in the nucleus and mitochondria (49). In BER, most damaged DNA bases are recognized and removed by DNA *N*-glycosylases, resulting in apurinic/apyrimidinic (AP) sites that are acted on either by AP endonucleases or by DNA *N*-glycosylases/lyases. The resulting single strand breaks are then repaired by DNA polymerase and DNA ligase (50). The *N*-glycosylases of the BER system in *S. cerevisiae*, Ntg1, Ntg2 and Ogg1, have AP lyase activity, which yield 3'-blocked single stranded breaks that are more toxic than AP sites themselves. These blocked ends are processed by the Apn1, Apn2 or 3'-flap endonucleases, but when these enzymes are absent or overwhelmed, toxic 3'-blocked single strand breaks accumulate and lead to the formation of double strand breaks (DSB) and replication fork collapse (51). If mitochondria, lacking NER, rely more heavily on BER, then they may require more active DSB repair and mechanisms to deal with replication fork collapse. Since DSBs and replication fork collapse are dealt with via homologous recombination repair, the presence of several mitochondrial genomes per organelle may counteract the lack of NER (52).

Considering that the activities of mitochondria that influence the production of ROS are under metabolic regulation, the relative amount of damage accrued in nuclear or mitochondrial DNA probably changes with metabolic state. There is also evidence that *S. cerevisiae* cells control the relative activity of DNA repair in the nucleus and mitochondria. Several DNA repair proteins are shared between the nucleus and the mitochondria, and their distribution changes depending on the severity of the damage to these organelles. Apn1 is the major AP endonuclease in the cell; it has both a mitochondrial and a nuclear localization signal and localizes to both organelles. However, the mitochondrial signal sequence of Apn1 is not strong and mitochondrial translocation of Apn1 takes place only when its stronger nuclear localization signal is blocked by association with Pir1 (Figure 2A). In the absence of Pir1, Apn1 accumulates in the nucleus, is depleted in the mitochondria and the mitochondria more readily accrue MMS-induced mutations (51). The *N*-glycosylase/lyase Ogg1 is specific for oxidized purines (53). Ogg1 has mitochondrial and nuclear targeting sequences that, when deleted, prevent translocation of the protein into the respective organelle (Figure 2B). The mitochondrial targeting sequence of the yeast Ogg1 seems to be weak, as a GFP fusion of the complete protein localizes to the cytoplasm (54). This might be a similar situation to Apn1, where the weak N-terminal mitochondrial targeting sequence is effective only when the C-terminal nuclear targeting sequence is blocked by binding of another protein in a regulatory manner. Ntg1 is an *N*-glycosylase/lyase specific for oxidized pyrimidines. Similar to Apn1, Ntg1 has nuclear and mitochondrial targeting sequences. Both of these sequences are required for the dynamic localization of Ntg1 to either the nucleus or the mitochondria (Figure 2C). And, as predicted, the absence of either sequence increases the mutation rates in the corresponding organelle (55).

There is evidence that the localization of Ntg1 is responsive to damage. Treatment of cells with H_2O_2 in combination with the ETC inhibitor antimycin leads to increased mitochondrial ROS, and an increase in the mitochondrial localization of GFP-tagged Ntg1 (Figure 2C) (55). Interestingly, relocalization of GFP-Ntg1 to mitochondria appears to be in response to DNA damage, rather than ROS levels. Indeed, GFP-Ntg1 relocalization in response to ROS is weaker in rho^0 cells that lack mtDNA but still show elevated ROS levels after treatment (56). Additionally, when ROS are generated in the nucleus by treatment with H_2O_2 or MMS treatment, GFP-Ntg1

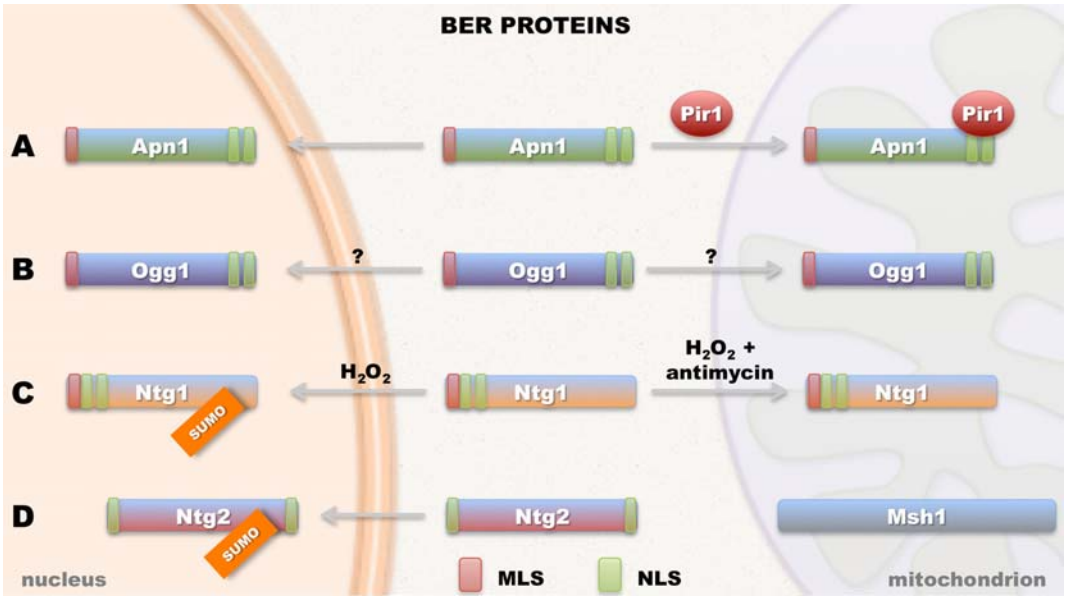

Figure 2. Dynamic localization of base excision repair (BER) proteins in *S. cerevisiae*. The base excision repair proteins Apn1, Ntg1 and Ogg1 have both mitochondrial (MLS) and nuclear localization signals (NLS) and their localization to either organelle depends on conditions. A) Apn1 localization to mitochondria requires the presence of Pir1, which bind and block the NLS, upon which the weaker MLS can direct the protein into the mitochondria. In the absence of Pir1, Apn1 localizes to the nucleus and is depleted in mitochondria. B) Ogg1 also contains both MLS and NLS, and deletion of either signal depletes the protein in the respective organelle. GFP-tagged protein containing both signals localizes to the cytoplasm, suggesting neither signal dominates. The conditions for localization of Ogg1 to either organelle have not been determined. C) Ntg1 localization to nuclei or mitochondria depends on the amount of oxidative damage to the DNA. In the presence of H_2O_2, which generates DNA damage mainly in the nucleus, Ntg1 localizes to the nucleus and this localization depends on its sumoylation. H_2O_2 plus the ETC inhibitor antimycin treatment generates mostly mitochondrial DNA damage, and the Ntg1 localization becomes mainly mitochondrial, suggesting Ntg1 localization depends on the level of DNA damage in the respective organelle. D) Ntg2 is an exclusively nuclear protein, and similar to Ntg1, is sumoylated. Msh1 is a mismatch repair protein but has a role in BER, and is mitochondria-specific.

accumulates in the nucleus and decreases in mitochondria. Ntg1 localized to the nucleus is sumoylated on lysine 364, and when this residue is mutated, Ntg1 localization to nucleus decreases after treatment with either H_2O_2 or MMS. This work therefore identifies a posttranslational modification that controls dynamic localization of this enzyme. Taken together, these data strongly suggests that *S. cerevisiae* controls DNA repair activity in the mitochondria and nucleus by differentially localizing DNA repair proteins.

Even though some of the components of the BER pathway are shared between the mitochondria and the nucleus, the relative effect of their absence differs in the organelles. Deletion of either *NTG1* or *APN1* leads to an increased mutation rate in the nucleus, but a decrease in point mutation rate in mtDNA as measured by the erythromycin resistance assay (57-59). On the other hand, deletion of *OGG1* causes almost 5-fold increase in mitochondrial point mutations, and a significant increase in nuclear DNA damage (54). Deleting *APN1* or *NTG1* in cells lacking *OGG1* significantly increases mitochondrial mutational rates, whereas the *apn1 ntg1* double mutant is not significantly different than either single mutant (57). This data suggests that Ogg1 functions in a BER pathway distinct from *APN1* and *NTG1* (58). In addition to genetic interactions between enzymes of similar DNA repair pathways, components of different repair pathways also interact with each other. For example, Msh1, a *S. cerevisiae* homolog of the bacterial mismatch repair protein MutS, functions in mitochondrial BER (Figure 2D). Msh1 binds to DNA substrates containing nucleotide mismatches and unpaired nucleotides (58, 60). One model is that Msh1 may aid Ogg1, but not Ntg1 or Apn1, in lesion recognition through its DNA-binding activity (58). On the other hand, Ntg1 and Apn1 likely work in a separate complex and require Msh1 to complete the repair in the long patch pathway. Involvement of a mitochondria-specific BER component in more than one step in the pathway suggests that repair pathways in mitochondria function differently than their nuclear counterparts. Indeed, this would help explain the different mutation rates observed between the mitochondrial and nuclear DNA upon deletion of a shared BER component.

5.2. Increased ROS cause mitochondrial genomic instability

Despite the fact that mitochondria have potent defenses for inactivating ROS and eliminating damage caused to their macromolecules, damage still accumulates when these systems become saturated, especially when there is also a need for nuclear DNA repair. It is believed

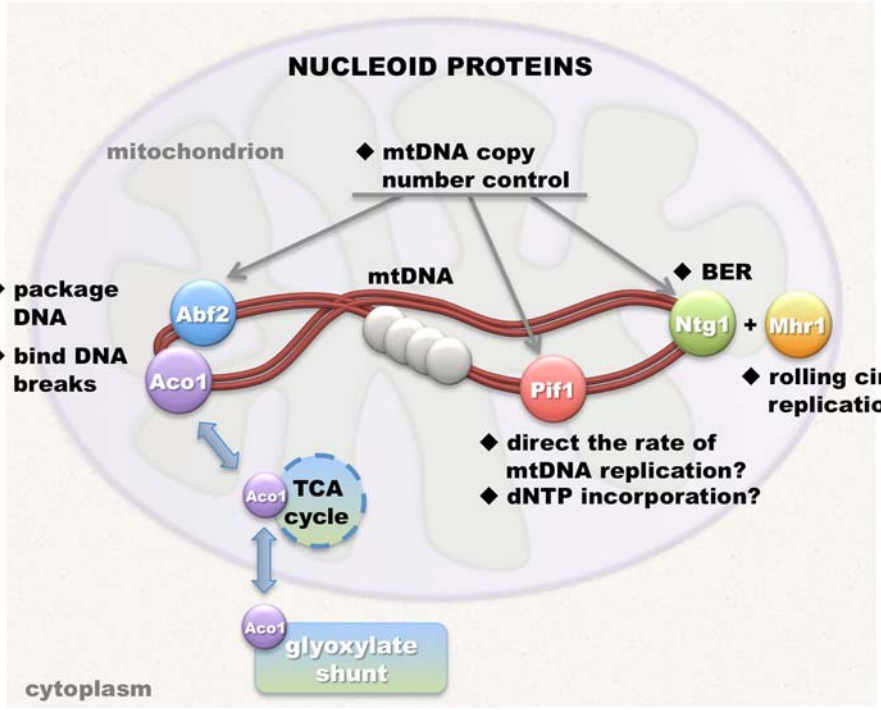

Figure 3. Mitochondrial DNA (mtDNA) is bound by several multifunctional proteins that link DNA damage repair and mtDNA replication. Ntg1 (green circle) is a base excision repair (BER) protein that is also involved in initiation of the Mhr1-dependent rolling circle mtDNA replication by introducing double stranded breaks at replication origins. Pif1 (red circle) is a DNA helicase that is implicated in mtDNA maintenance, repair, replication and recombination, but it's mode of action remains unclear; it is proposed to direct the rate of replication and dNTP incorporation into DNA. The mtDNA is packaged by a histone-like protein Abf2 (blue circle), which also functions in binding double strand breaks in DNA and in stabilization of recombination junction intermediates. In low levels of glucose, Aco1 (purple circle) also packages mtDNA, binds single and double stranded DNA breaks and protects mtDNA. Aco1 also functions in the TCA/Krebs cycle (in the mitochondrial matrix) and in the glyoxlate shunt (in the cytoplasm) that generates four carbon compounds from two-carbon substrates. Gray circles represent other nucleoid proteins, see (68) for an extensive list.

that damage to mtDNA is well tolerated because there are about 20-50 copies of mitochondrial genome in each yeast cell, so healthy mitochondria can replicate and compensate for the loss of the damaged ones. Many of the proteins of the BER system are also involved in the replication of mtDNA, separate from their repair activity, supporting the idea that damage repair and increasing the number of undamaged mitochondria by replication are tightly interconnected. Ntg1 and Pif1 seem to be involved in both of these processes (61). Besides its role in BER as a lyase, Ntg1 also initiates the Mhr1-dependent rolling circle mtDNA replication by introducing a double stranded break at the mtDNA replication origins (Figure 3, green circle) (27, 62). Pif1 is a 5'-to-3' DNA helicase that is localized to both mitochondria and nuclei. Pif1 function is less clear, but it has been implicated in telomere length regulation and prevention of gross chromosomal rearrangements in the nucleus, and in mtDNA maintenance, repair, replication and recombination, and is a part of the nucleoid structure in mitochondria (Figure 3, red circle) (63). The *pif1delta* strain shows an increase in Ntg1-recognizable mtDNA lesions. These cells gradually lose segments of their mtDNA, leading to mixed rho^+ and rho^- cell populations (46). Similarly, in strains that lack the Ntg1 *N*-glycosylase/lyase, there is a slight increase in Ntg1-recognizable lesions, but they maintain complete mitochondrial genomes (46, 64). When the *ntg1delta* mutation is combined with *pif1delta* mutation, the amount of lesions increases synergistically and the mtDNA becomes unstable. In a triple mutant of *pif1delta ntg1delta sod2delta*, genomic instability occurs very rapidly and cells lose all mtDNA and become rho^0. These results suggest that these three proteins are components of an interactive network of mtDNA damage resistance pathways (46).

The non-histone mtDNA binding protein Abf2 is also implicated in this network (Figure 3, blue circle). Deletion of Abf2 leads to about two-fold increase in petite formation, but the *ntg1delta abf2delta* double mutant generates almost all petites within a 24-hour period. Furthermore, 2 to 3-fold overexpression of Abf2 results in about a two-fold decrease in petite formation, confirming the role of Abf2 in protecting the cells from spontaneous mtDNA damage (64). It is not clear what role Pif1 or Abf2 plays in mtDNA damage protection. Both proteins are implicated in mtDNA copy number control, which may be a part of the protection they provide. However, it is speculated that Pif1, based on its role in inhibition of DNA

replication in the nucleus, might direct the rate of mtDNA replication, allowing time for repair to take place, similar to the role for a checkpoint protein (64). It is also suggested that Pif1 plays a role in incorporation of dNTPs into mtDNA during replication since some of damage to mtDNA in a *pif1delta* strain can be reduced if the levels of mitochondrial dNTPs are increased (61). Abf2, on the other hand, might shield mtDNA (similar to the way nucleosomes protect nuclear DNA) and stabilize recombination junction intermediates (65). However, a similar damage protection effect is also seen in *abf2delta* cells when the *de novo* production of dNTPs is increased by overexpression of the ribonucleotide reductase (RNR), which results in an increase in mtDNA copy number to near wild-type levels and to reduction of the petite formation rates, albeit not to the wild type levels (66). RNR is generally activated after DNA damage or entry into S-phase by the Mec1/Rad53 kinase pathway, when the demand for dNTPs is high, making it an appropriate regulatory system for mtDNA copy number control (67). Reducing the dNTP pools by overexpression of the RNR inhibitor Sml1 leads to three-fold increase in the rate of petite formation (66). The fact that increased dNTP levels can reduce some of the defects seen in either the *abf2delta* or the *pif1delta* strains brings out the question whether Abf2 or Pif1 are truly involved in mtDNA copy number control, or whether loss of their function indirectly results in changes in the mtDNA copy number.

Even though overexpression or loss of Abf2 leads to an approximately two-fold change in mtDNA copy number, the mtDNA nucleoids still form in the *abf2delta* strain, which can be propagated in non-fermentative media (68). Under these conditions, the aconitase, Aco1, that catalyzes the conversion of citrate to isocitrate in the TCA cycle, replaces Abf2 in nucleoids such that the mtDNA can be maintained (Figure 3, purple circle) (68). However, if the cells are grown in glucose, the Aco1-dependent metabolism is repressed and mtDNA is lost in as little as six generations (68). Aco1 is a multifunctional enzyme; the majority of Aco1 is located in mitochondria and is a component of the Krebs cycle but is also found in the nucleoids. A smaller fraction of Aco1 is present in the cytoplasm and is involved in the glyoxylate pathway, which allows cells to make four-carbon compounds from two-carbon substrates such as ethanol, acetate or oleate, and thus allows cells to grow on these simple carbon sources (69). Disruption of Aco1 activity leads to the loss of protection of mtDNA due to accumulation of single stranded breaks and illegitimate recombination events in both rho^+ and rho^- cells and leads to a respiratory-deficient phenotype. Interestingly, *ACO1* overexpression suppresses the mtDNA instability phenotypes in cells lacking the mtDNA packaging factors Pif1 or Abf2, and this function requires its DNA binding activity (63). Aco1 is present in nucleoids even in the presence of Abf2 and is believed to protect mtDNA from both double and single stranded breaks, whereas Abf2 has the ability to bind to double stranded breaks only. This suggests that Aco1 has a broader role in protecting the stability of the mitochondrial genome.

The mtDNA protection activity of Aco1 (as well as its enzymatic activity in the Krebs cycle) seems to depend on the presence of its iron-sulfur cluster (68). Enzymes containing oxidation-sensitive prosthetic groups such as iron sulfur clusters represent the majority of ROS-modified protein targets degraded by the ATP-dependent Pim1 protease in the mitochondrial matrix (45). Thus, upon increased oxidative stress, the combined effects of an increased number of mtDNA lesions that overwhelm the repair enzymes (63, 68), plus the loss of critical protective proteins such as Aco1 to selective degradation (45), may lead to increased mutagenesis, single or double stranded breaks, stalled replication forks and loss of parts or all of the mtDNA.

5.3. Mitochondrial DNA loss leads to nuclear genomic instability

Yeast cells that lose most or all of their mtDNA, i.e., rho^- and rho^0 respectively, are still viable on a fermentable carbon source. However, these cells display a variety of changes other than simply lack of respiration. For example, respiratory-deficient petite cells fail to upregulate nuclear-encoded diauxic shift-associated genes, which normally allows cells to adapt to growth on respiratory carbon sources after depleting glucose in the media by increasing mitochondrial biogenesis (70). Along the same lines, petite strains cannot upregulate expression of Tar1, a nuclear-encoded mitochondrial gene that is required for the biosynthesis of Coenzyme Q, required for proper functioning of the electron transport chain upon diauxic shift (71). These examples illustrate the fact that signals from a defective mitochondrial genome prevent upregulation of nuclear genes that function in respiration when other components of the respiratory machinery are missing.

As early as 1976 it was recognized that petite strains are prone to enhanced spontaneous mutations of nuclear genes. For example, there is a significant increase in the mutation rate for the nuclear nonsense allele *lys1-1* and the missense allele *his1-7* in petite strains (72). Subsequent work showed that this mitochondria-mediated nuclear mutator phenotype can be suppressed by inactivating the error-prone translesion synthesis (TLS) pathway, and that even though petite strains generate lower amounts of endogenous ROS, they accumulate more mutations and have reduced survival rates when treated with oxidizing agents (48, 73, 74). A more recent study implicated defects in iron-sulfur cluster metabolism resulted in genomic instability (75). These researchers use controlled expression of a dominant-negative mutant of the mitochondrial DNA polymerase gene *MIP1* to force loss of mtDNA, and then trace loss of heterozygosity (LOH) at two chromosomal loci. Cells that lose their mtDNA initially have comparable growth rates to their rho^+ counterparts (about a 75 minute doubling time), but then progressively slow down to about a 400 minute doubling time, and at about 22 hours, 60% of the cells arrest growth in G_1. Some of the cells go through the arrest and reenter the cell cycle, in a process defined as crisis, but exhibit increased genomic instability. Interestingly, by 30 hours following the loss of mtDNA, cells start to form colonies faster and display fewer LOH events, suggesting the cells compensate for the loss of mtDNA by selection for nuclear

mutations that improve growth in the absence of mtDNA. The clones that go through the crisis and reenter the cell cycle show defects in iron-sulfur cluster biogenesis. In fact, inhibition of cytoplasmic iron-sulfur protein assembly alone can lead to a progressive decline in growth rate and increased incidence of LOH in the nuclear genome (75).

Why do cells that lose their mtDNA have defects in iron-sulfur metabolism, and how does this lead to genomic instability? Part of the answer is linked to the mitochondrial membrane potential. As stated earlier, mtDNA encodes some of the subunits of the electron transport chain complexes III and IV, the F_0 segment of the F_0F_1 ATPase, and complement the other subunits encoded by the nuclear DNA. When some or all of the mitochondrial subunits are missing, the electron transport chain is not functional and only the F_1 segment of the ATPase is assembled. The F_1 ATPase utilizes ATP to generate a membrane potential, albeit at a much lower level than that achieved by fully functional oxidative phosphorylation. This lowered membrane potential is believed to be responsible for the defects leading to genomic instability. Indeed, increasing the membrane potential in rho^0 cells by use of a hyperactive F_1 ATPase that generates a larger membrane potential prevents the crisis and alleviates the loss of heterozygosity (75).

One way that the lowered mitochondrial membrane potential affects genomic instability is through its effects on the assembly of iron-sulfur clusters in the cell. Assembly of all iron-sulfur clusters is initiated in mitochondria, utilizing more than 20 protein components. Proper levels of mitochondrial membrane potential and matrix ATP are required for the import of iron, as well as the assembly and export of the iron-sulfur clusters into the cytoplasm (76-78). Reduction of exported iron-sulfur clusters into the cytoplasm due to insufficient membrane potential in turn leads to accumulation of iron in the mitochondria. Reduced mitochondrial iron-sulfur cluster synthesis results in activation of the iron regulon, increasing iron uptake, and changes in iron metabolism (79, 80). Iron is crucial for the activity of numerous enzymes and misregulation of its levels has multiple downstream effects on the physiology of the cell. One consequence of the increased mitochondrial iron concentration is the incorporation of iron, instead of manganese, into Sod2, resulting in the inactivation of Sod2, the major mitochondrial defense against ROS; Sod2 inactivation can be detrimental to mitochondrial proteins and lipids (81). Outside the mitochondria, increased iron concentration could affect the activity of critical enzymes as well. It is known that the activity of the RNR complex in the cytoplasm increases after DNA damage due to the activation by the checkpoint proteins Mec1/Rad53. Increased RNR activity leads to increased dNTP levels, which are essential for DNA damage repair. The presence of a diiron cluster is essential for the activity of the RNR complex (82, 83). It is not known whether the intracellular iron concentration has a regulatory effect on formation of the active RNR complex, but if so, it could lead to overactivation of the complex when iron levels rise. Chabes *et al.* showed that an RNR complex with defective feedback inhibition activity generates excessive amounts of dNTPs and results in increased survival, but in increased mutation rates as well, likely because of the reduced fidelity of replicative polymerases and/or activation of the error-prone translesion repair pathway (84). These are examples of two proteins whose activities are tightly linked to changes in the iron availability. It is easy to understand how altered activity of Sod2 would lead to increased ROS levels and resulting increase in damage. Similarly, the RNR activity is central for the control of the fidelity of the DNA repair process and changes in its activity can have widespread effects.

In addition to its effects on the iron levels, reduction in the assembly and export of iron-sulfur clusters from mitochondria can directly influence activities of those proteins that depend on the assembled iron-sulfur clusters. Many of these proteins are involved in a variety of cellular functions and are essential in several critical processes, including DNA synthesis and the damage response (85). Having a transition metal as a prosthetic group makes iron-sulfur cluster proteins highly sensitive to the redox state of the cell. To date, there are only a few iron-sulfur cluster proteins identified in yeast, and three of which seem to be involved in the DNA repair pathways. One of the repair enzymes in this category is Ntg2, the third *N*-glycosylase/lyase of the BER system identified in *S. cerevisiae* so far. Unlike Ntg1, which is both nuclear and mitochondrial, Ntg2 is exclusively nuclear and requires an iron-sulfur cluster for its activity. Genetic analyses of these two family members indicate that they act through different pathways and have slightly different substrate affinities and specificities (50, 56, 59, 86, 87). The second protein is Rad3, a helicase of the nucleotide excision repair pathway that is related to the human XPD protein. Rad3 contains an iron-sulfur cluster that is essential for its helicase activity and mutations abrogating its iron-sulfur cluster cause UV sensitivity in *S. cerevisiae* (88). The third and the final iron-sulfur cluster-containing repair protein is the large subunit of the DNA primase in *S. cerevisiae* (89). DNA primases are required for DNA synthesis and double strand break repair. Even though these enzymes were identified long ago, their dependence on iron-sulfur clusters is just recently discovered; it is therefore likely that there are more of these critical enzymes that have not yet been classified as iron-sulfur proteins.

Multiple repair pathways, such as base excision, nucleotide excision, recombination and translesion synthesis pathways form interconnected networks and work synergistically in removal of spontaneous or exogenous oxidative damage to DNA (90-92). It is not clear how removal of activities of some of the enzymes in the repair network would affect the overall efficiency and fidelity of the repair process. Furthermore, the effect of the iron-sulfur proteins on repair processes could be indirect. Regardless of the mechanism, it is clear that defects in the iron-sulfur metabolism initiated by mitochondria play a role in the nuclear genomic instability and will attract more attention for further research.

5.4. Effects of oxidative damage to the nuclear genome

Even though most of the endogenous oxidative damage is initiated at the mitochondria and affects

mitochondrial DNA more extensively, the nuclear DNA does not escape damage. This is especially true when cells are treated with reagents that generate oxidative stress, most commonly by menadione or H_2O_2, as well by indirect effects of alkylating agents such as MMS. Repair pathways in the nucleus are more diverse and complex than those found in the mitochondria (52). Access to DNA is a major challenge because it is highly compacted in nucleosome structures by its association with histone proteins. *In vitro*, the catalytic activities of the mammalian BER enzymes uracil DNA glycosylases (UDG) and AP endonucleases (APE) on nucleosome templates decrease to 10% of that on naked DNA (93, 94). Posttranslational modifications on histone tails provide a means for altering the level of compaction that hinders the repair process; for example the mammalian histone acetyl transferase CBP/p300 associates with thymine DNA glycosylase (TDG), and the resulting complex has activity for both the excision step of the repair and for histone acetylation (95). Other modifications on histones, such as phosphorylation, are known to mark the sites of damage. For example, phosphorylation of histone H2A at serine 129 at and around double stranded break sites is involved in the recruitment of repair and chromatin remodeling enzymes (96-99). Similarly, phosphorylation of histone H2A at serine 122 is observed upon treatment of cells with menadione, though the contribution of this modification to repair is not yet understood (100, 101). Unfortunately, most of the work on the effect of histone modifications and chromatin remodeling on the repair process has been studied for double stranded break repair and very limited information is available for their effect on the base excision repair of oxidatively damaged DNA in *S. cerevisiae*.

Beyond their involvement in repair functions, chromatin-associated proteins are being implicated in damage sorting. It turns out, even as a single celled organism, *S. cerevisiae* maintains a soma-like and germ cell-like division between the mother and the daughter cells in a process that depends on the NAD-dependent histone deacetylase Sir2 (102-105). This allows the newly emerged bud to generate a lineage of cells that is viable indefinitely, while the mother cells cease to replicate after about 20 generations. This has been attributed to the ability of the cells to selectively sort out damaged organelles, and even damaged proteins like Aco1, and retain them in the mother cell, while the newly generated organelles and active proteins are placed into the bud (104, 106). This raises the question, if damaged organelles and even proteins can be sorted out according to their damage levels, could chromosomes be sorted in a similar manner? It turns out that selective segregation of particular chromosomes indeed takes place in mouse and has been suggested as a developmental process (107). Perhaps, we will find out that chromosomes can also be sorted according to the level of damage they accrue, aided by the damaged DNA lesions, chromatin components or even by their associations with repair enzymes.

6. CONCLUDING REMARKS

It is evident that part of the response to oxidative damage is initiated by mitochondria, possibly because mitochondria receive more damage compared to nuclear DNA under oxidative stress conditions. This effect seems to be more pronounced when cells are grown aerobically in high glucose media, which leads to repression of electron transport chain activity and to formation of higher levels of endogenous ROS. The mitochondria have powerful defense mechanisms, mainly the superoxide dismutases that scavenge the generated ROS, the protein quality control mechanisms that quickly turn over damaged proteins, and the DNA repair enzymes that remove oxidative lesions in mtDNA. However, when these mechanisms get overwhelmed, a chain of events leads to the loss of parts or all of mtDNA, the reduction of the mitochondrial inner membrane potential, the misregulation of iron homeostasis, and collectively result in nuclear genomic instability. It seems as though once the cells begin to lose their mtDNA, the effects are devastating for the survival of the cell. This may not be true for cells that have the ability to mate and restore healthy mitochondria and perhaps successfully repair the damage in their nuclear genome, as would be the case in the wild, but it seems impossible for an unmated haploid laboratory culture. Petite strains are usually overtaken by their counterparts with fully functional mitochondria in the laboratory cultures, except in cases where they compensate for the loss of mitochondria function by gaining suppressive mutations and survive; generating stable rho^- or rho^0 respiratory-deficient cultures that can be maintained on fermentable carbon sources. Veatch *et al.* analyzed four of the stable clones that lost mtDNA and found that the suppressor mutations segregated as either one or two unlinked nuclear loci (75). It would be very interesting to identify what type of mutations can suppress the growth phenotypes of cells with no mtDNA, as these can have significant, unaccounted for effects on the phenotype of cells that are being studied.

Loss of mtDNA is a result of an extreme case of damage and is not tolerated by organisms other than yeast. Being able to remove the mtDNA allows us to discover the many levels of processes the mitochondria are involved in, in both adaptation to environmental changes such as carbon source or temperature, as well as how cells deal with treatments with agents that cause DNA damage. Most interestingly, the studies mentioned here suggest that the functional state of the mitochondria influences the extent of damage cells can tolerate. We might need to pay more attention to how we interpret our DNA damage assays; some of the phenotypes we observe might be exaggerated by defects in processes unrelated to the actual DNA repair process.

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Abbreviations: mtDNA: mitochondrial DNA, ROS: reactive oxygen species, BER: base excision repair, NER: nucleotide excision repair, TLS: translesion synthesis, dNTP: deoxyribonucleotide triphosphate

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