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Dynamic Compartmentalization of Base Excision Repair Proteins in Response to Nuclear and Mitochondrial Oxidative Stress

By

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By

Lyra Maria Griffiths
B.A., Agnes Scott College, 2002

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Lyra Maria Griffiths

Both nuclear and mitochondrial DNA can be damaged by reactive oxygen species that are generated during cellular metabolic processes and exogenous insults. Base excision repair (BER) is the primary pathway for the repair of oxidative DNA damage and abasic sites, the most frequently occurring lesions in DNA. Certain eukaryotic BER proteins are capable of localizing to both nuclei and mitochondria and mediate DNA repair in both organelles. It was of interest to determine how BER proteins that occupy both nuclei and mitochondria are regulated. To address this issue, we chose to study Ntg1, one of two *Saccharomyces cerevisiae* homologs of bacterial endonuclease III and human hNTH1. The localization of Ntg1 and Ntg2 in response to nuclear or mitochondrial oxidative stress was analyzed using fluorescence microscopy. While Ntg2 remained statically localized to the nucleus, Ntg1 is capable of dynamic localization to the organelle sustaining greatest oxidative stress. Additionally, oxidative DNA damage likely produces the signal for Ntg1 localization to both nuclei and mitochondria. The critical amino acid residues necessary for localization of Ntg1 to nuclei and mitochondria were identified. A bipartite classical nuclear localization signal sequence, a mitochondrial matrix targeting sequence, and putative sumoylation sites were determined, and introduction of mutations in these sequences influenced the localization of Ntg1. Additional biochemical analysis revealed that Ntg1 associates with the classical nuclear transport proteins importin α/β in order to enter the nucleus. Hence, importin α/β have a novel role in the regulation of mitochondrial and nuclear oxidative DNA damage repair through Ntg1. Without dynamic localization of Ntg1 to nuclei or mitochondria, nuclear or mitochondrial mutation rates, respectively, were elevated, indicating that this novel mode of BER regulation is important for preserving the integrity of the nuclear and mitochondrial genomes and preventing mutagenesis. As accumulation of nuclear and mitochondrial DNA mutations is associated with numerous human diseases, including cancer, neurological disorders, and other degenerative disorders, this mechanism of BER regulation is likely to play a very important role in prevention of these diseases in higher eukaryotes.

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TABLE OF CONTENTS

Chapter 1	General Introduction	1
	References	27
Chapter 2	Dynamic Compartmentalization of Base Excision Repair Proteins in Response to Nuclear and Mitochondrial Oxidative Stress	71
	Abstract	72
	Introduction	73
	Materials and Methods	76
	Results	83
	Discussion	92
	References	98
Appendix 1	Cell Cycle Regulation of Localization of Ntg1	126
	Abstract	127
	Introduction	127
	Materials and Methods	130
	Results	131
	Discussion	133
	References	135

Chapter 3	Functional Significance of Ntg1 Nuclear and Mitochondrial Dynamic Localization	143
	Abstract	144
	Introduction	144
	Materials and Methods	148
	Results	152
	Discussion	160
	References	168
Chapter 4	Discussion and Future Directions	194
	References	209

FIGURES AND TABLES

Chapter 1	General Introduction	1
Table 1.	Mitochondrial DNA Repair Proteins in Eukaryotic Cells	58
Table 2.	Comparison of BER DNA-glycosylases in *E. coli*, Mammals, and *S. cerevisiae*	60
Figure 1.	Oxidative DNA Lesions	61
Figure 2.	Nuclear and Mitochondrial DNA Repair Pathways	62
Figure 3.	DNA Repair in *S. cerevisiae*	63
Figure 4.	Base Excision Repair	64
Figure 5.	Structures of AP Sites and Breaks Created by AP Endonucleases and AP Lyases	65
Figure 6.	The Nuclear Pore Complex	66
Figure 7.	Classical Nuclear Import	67
Figure 8.	Mitochondrial Protein Import	68
Figure 9.	Ubiquitin and Sumoylation	69
Figure 10.	Sumoylation of Thymine DNA Glycosylase	70
Chapter 2	Dynamic Compartmentalization of Base Excision Repair Proteins in Response to Nuclear and Mitochondrial Oxidative Stress	71
Table 1.	Strains and Plasmids Used in this Study	110
Figure 1.	Subcellular Localization of Ntg1 and Ntg2 Under Normal Growth Conditions	112
Figure 2.	Flow Cytometric Analysis of Cells to Determine Intracellular ROS Levels Following Nuclear or Mitochondrial Oxidative Stress	113

Figure 3.	Subcellular Localization of Ntg1 Following Exposure to Nuclear and Mitochondrial Oxidative Stress	114
Figure 4.	Mitochondrial Localization of Ntg1 is Influenced by Mitochondrial Oxidative DNA Damage	116
Figure 5.	Amino Acid Sequences of Ntg1 and Ntg2	118
Figure 6.	Post-translational Modification of Ntg1 and Ntg2 by SUMO	119
Figure 7.	Sumoylation of Nuclear Ntg1 Increases in Response to Oxidative Stress	120
Figure 8.	Subcellular Localization and Function of the Ntg1 K364R Mutant	122
Figure 9.	Proposed Model for Regulation of BER Proteins in Response to Oxidative Stress	124
Appendix 1	Cell Cycle Regulation of Localization of Ntg1	126
Table 1.	Quantification of the Localization of Ntg1-GFP in Alpha Factor Synchronized	140
Figure 1.	Flow Cytometry of Cells Released from Alpha Factor Arrest	141
Figure 2.	Localization of Ntg1 Throughout the Cell Cycle	142
Chapter 3	Functional Significance of Ntg1 Nuclear and Mitochondrial Dynamic Localization	143
Table 1.	Strains and Plasmids Used in this Study	179
Table 2.	Ntg1 Localization Motifs	181
Table 3.	Rates of Nuclear and Mitochondrial Mutations with Different DNA Excision Repair Capacities	182

Figure 1.	Definition of Functional Intracellular Targeting Signals within Ntg1	183
Figure 2.	The Bipartite cNLS of Ntg1 is Sufficient to Direct Nuclear Localization of Ntg1	186
Figure 3.	The Classical Nuclear Protein Import Pathway is Required for Nuclear Localization of Ntg1	188
Figure 4.	Functional Intracellular Targeting Signals are Required for Dynamic Localization of Ntg1 in Response to Oxidative DNA Damage	189
Figure 5.	Functional Analysis of the Dynamic Localization of Ntg1	190
Figure 6.	Amino Acid Substitutions within Intracellular Targeting Signals do not Affect the Catalytic Activity of Ntg1	191
Figure 7.	Model of Ntg1 Dynamic Localization in Response to Nuclear and Mitochondrial Oxidative DNA Damage	192
Chapter 4	Discussion and Future Directions	194
Figure 1.	The Amino Acid Sequence of Ung1 and hNTH1	214
Figure 2.	A Model for Dynamic Localization of Ntg1 as a Major Mode of BER Regulation	215

CHAPTER 1

GENERAL INTRODUCTION

1. Oxidative Stress and Oxidative DNA Damage

Reactive oxygen species (ROS) are a byproduct of normal cellular metabolic processes, such as mitochondrial electron transport. Normal levels of ROS can act as beneficial signaling molecules in cells; however, high levels of ROS can cause oxidative damage to DNA, lipids, and proteins (197). Oxidative DNA damage, which occurs frequently in all cells, is linked to aging and human disease, such as cancer and various degenerative disorders (11, 36, 105, 200, 204). Examples of commonly occurring oxidative DNA damage are thymine glycol and 8-oxoguanine (8OG) (Figure 1). Oxidative DNA damage is thought to be the most frequently occurring spontaneous DNA damage, and it is estimated that 10,000 oxidative hits occur per cell per day in the mammalian genome (11). Unrepaired oxidative DNA lesions can result in mutations and lead to arrest of both DNA replication and transcription (86), leading to the deleterious consequences of cell death and human disease.

2. Nuclear and Mitochondrial Oxidative DNA Damage

2.1 Occurrence of nuclear and mitochondrial oxidative DNA damage. The two organelles in non-plant eukaryotic cells that contain DNA are nuclei and mitochondria. DNA in both of these organelles is damaged by ROS and other deleterious agents on a regular basis. Several labs have attempted to quantify the deleterious impact of oxidative stress on each genome. It is thought that the proximity of mitochondrial DNA to electron transport chain-generated ROS results in an increased vulnerability of mitochondrial DNA to oxidative damage and a higher rate of mitochondrial DNA mutagenesis

compared to nuclear DNA (124). Although mitochondrial DNA is not associated with histones or packaged into nucleosomes, it is coated with other DNA-binding proteins which may shield it from endogenous and environmental DNA damaging agents (25). However, it has been suggested that the lack of histones and nucleosomal packaging may make mitochondrial DNA more susceptible to such oxidative insults (153). Efforts to quantify the number of lesions that occur spontaneously and in response to exogenous ROS demonstrate that DNA in both organelles is regularly oxidatively damaged. The results also suggest that mitochondrial DNA accumulates more oxidative damage than nuclear DNA. For example, in rat liver it is estimated that the number of spontaneous 8OG lesions in nuclear DNA (2.75 billion bp) is one in 130,000 bases; while lesions in mitochondrial DNA (16.3 thousand bp) are estimated to occur at a level of one in 8000 bases (153). Furthermore, in human and *Saccharomyces cerevisiae* cells, mitochondrial DNA is thought to contain two to three times more oxidative lesions than nuclear DNA following oxidative stress induced by various agents (163, 212).

2.2 Repair of nuclear and mitochondrial DNA. Because of the large number of oxidative lesions that can accumulate, all cells have evolved the ability to repair both nuclear and mitochondrial DNA damage (Figure 2). Repair of damaged nuclear DNA is carried out through six major DNA damage handling pathways: direct reversal, nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), recombination repair (RER), and translesion synthesis (TLS) (103) (Figure 3). Although direct reversal has been observed in *S. cerevisiae* and several other eukaryotic organisms, not all eukaryotic organisms are known to have direct reversal mechanisms (162). NER primarily removes DNA lesions that cause a structural deformation of the DNA helix,

while BER removes small lesions. MMR repairs mismatched bases following replication. RER and TLS are considered tolerance pathways, allowing replication fork progression in the presence of a lesion so that the cell can progress through the cell cycle and survive. Various repair proteins have been shown to localize to mitochondria; however, to what extent and how these proteins recognize and repair mitochondrial DNA damage is largely unknown. A list of DNA repair and damage-resistance proteins that localize to *S. cerevisiae* mitochondria are presented in Table 1. In 1974, it was shown that UV light-induced cyclobutane pyrimidine dimers (CPDs), substrates of NER, are not repaired in mammalian mitochondria (35). Since then, no evidence has emerged indicating that NER occurs in mitochondria. MMR and RER have been demonstrated in *S. cerevisiae* mitochondria (33, 61); however, the activity of MMR and RER in the repair of mammalian mitochondrial DNA remains a controversial area (120). Oxidative DNA damage is primarily repaired by BER, and it has been clearly demonstrated that this repair pathway exists in mitochondria from *S. cerevisiae* to humans (16, 46, 118, 140, 148, 172).

3. Base Excision Repair

3.1 Mechanism of base excision repair. BER is considered to be the predominant repair pathway by which small DNA lesions resulting from exposure to either environmental agents or cellular metabolic processes are recognized and repaired (Figure 4) (122, 139). Importantly, BER is the primary process by which oxidative DNA damage and alkylation is repaired (173, 221). BER is initiated by the recognition and

excision of a base lesion by a lesion-specific DNA glycosylase, resulting in an apurinic/apyrimidinic (AP) site (108, 109). These oxidative DNA glycosylases are bi-functional with an associated AP lyase function, and can function in short-patch BER (only one nucleotide is excised) or long-patch BER (a stretch of 2-13 nucleotides is removed). Following lesion removal, the resulting AP site is processed by an AP endonuclease or an AP lyase, which cleaves the sugar-phosphate DNA backbone on the 5´ side or 3´ side of the AP site, respectively (Figure 5) (9). DNA cleaved by bifunctional DNA glycosylase/AP lyase enzymes results in a 3´ terminal unsaturated aldehyde, which requires further processing prior to polymerization by DNA polymerase. The removal of the 3´ terminal unsaturated aldehyde is completed by a 3´ phosphodiesterase. AP endonuclease leaves a 5´ terminal deoxyribose-phosphate residue, which also cannot be processed by DNA polymerase. In this case, DNA-deoxyribophosphodiesterase (dRpase) removes the 5´ terminal deoxyribose-phosphate residue, creating a substrate for DNA polymerase (201). When the DNA is in the correct form, DNA polymerase hydrolyzes the 5´ deoxyribose-phosphate moiety and replaces the excised nucleotides (17). In cases of short-patch BER, DNA polymerase β (pol-β) completes this process, while during long-patch BER, DNA polymerase ε, δ, or β in conjunction with proliferating cell nuclear antigen (PCNA), replication factor C (hRCF), and Fen1 endonuclease complete the filling process (68, 150, 151). Finally, ligation of the DNA backbone occurs by either DNA ligase I or a complex of DNA ligase III and XRCC1 (24, 195).

3.2 Significance of BER to cellular function. The importance of BER has been illustrated through various studies. Haploid *S. cerevisiae* cells possessing functional BER

alone (a NER/TLS/RER-deficient mutant) are viable, but any other combination (BER/NER/TLS, BER/TLS/RER, or BER/NER/RER) of repair deficiencies in *S. cerevisiae* are lethal (128). These results suggest that BER is essential for the DNA damage resistance pathways in *S. cerevisiae* and may be responsible for the removal of either the majority of spontaneously occurring DNA damage or specifically those damages that are potentially lethal. Furthermore, animal model systems have shown that mice carrying null mutations for DNA glycosylases are normal, but mice with null mutations in BER proteins acting subsequently to the glycosylase (Polβ and mouse AP endonuclease, for example) are embryonic or perinatal lethal (63), suggesting that the BER-related activity of these proteins may be important for normal development. Additionally, defects in numerous BER proteins are associated with various tumor types. Mutations in many of the BER glycosylases, including 8OG DNA glycosylase (hOGG1), thymine DNA glycosylase (hTDG), uracil DNA glycosylase 2 (hUNG2), and hMYH are associated with specific types of cancer (2, 171, 185). Overexpression of the major human AP endonuclease, hAPE1, is associated with ulcerative colitis, colon cancer, and ovarian carcinomas and are associated with microsatellite instability in these tumors (29, 30, 73, 81, 188). An XRCC1 variant is associated with tobacco-related tumorigenesis (85). These numerous examples of diseases linked to BER aberrations exemplifies the importance of BER to the health of eukaryotic cells and illustrates the importance to understand how BER is regulated and orchestrated in order to maintain the health of the cell.

4. *Saccharomyces cerevisiae* as a Model System for DNA Repair Studies

The budding yeast, *Saccharomyces cerevisiae,* has been utilized extensively to investigate the mechanisms that underlie DNA repair as the DNA damage management pathways are conserved between *S. cerevisiae* and humans (49, 122). This eukaryotic model system is a reliable and informative system for the evaluation of biochemical activities, localization, mutagenesis, and overall function of BER proteins as yeast grow rapidly and are inexpensive and relatively easy to manipulate (169). In addition, *S. cerevisiae* can live with high levels of mitochondrial genomic instability or no mitochondrial DNA at all (rho^0 cells), conferring the ability to evaluate how mitochondrial DNA is maintained (54, 57, 169).

5. Role of Ntg1 and Ntg2 in the Process of Base Excision Repair

5.1 Ntg1 and Ntg2: DNA damage recognition. DNA glycosylases execute BER in species from *Escherichia coli* to *S. cerevisiae* to humans (Table 2). DNA glycosylases can be subdivided into five groups according to their main substrate: uracil (Ung1/hUNG, hSMUG1), uracil-containing mismatches (hTDG, hMBD4), alkylated bases (hANPG, also known as hAAG), methylated bases (Mag1) and lesions resulting from an oxidation event (Ogg1/hOGG1, hMYH, Ntg1/hNTH1, Ntg2, hNEIL1/2/3) (60). Ntg1 and Ntg2 are the *S. cerevisiae* homologs of *Escherichia coli* endonuclease III (Nth), and these two proteins are bi-functional oxidative DNA glycosylase/AP lyase enzymes (Figure 5), capable of recognizing a number of base damage products including thymine glycol, dihydrouracil, 5-hydroxy-6-hydrothymine (5-OH-6-HThy), 5-hydroxy-6-hydrouracil (5-

OH-6-HUra), 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxyuracil (5-OH-Ura), 5-hydroxycytosine (5-OH-Cyt), 2,6-diamino-4-hydroxy-5-(N-methylformamido) pyrimidine (Fapy-7MeGua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 4,6-diamino-5-formamidopyrimidine (FapyAde) (165, 220). In addition, Ntg1 and Ntg2 can repair alkylation damage caused by MMS (74) and the AP site repair intermediate (121). While Ntg1 and Ntg2 have overlapping substrate specificities, recognition of certain substrates is restricted to Ntg1 or Ntg2. For example, 8OG, the most frequently occurring DNA base oxidation product, opposite a guanine (8OG:G) can be recognized by Ntg1 *in vitro*, while Ntg2 cannot recognize this lesion (22, 165). In contrast, Ntg2 can recognize the oxidation products resulting from the incorporation of 8-hydroxyguanine, while Ntg1 cannot (96). Ntg1 and Ntg2 also excise common substrates at different rates (165).

5.2 Ntg1 and Ntg2: general features. Ntg1 and Ntg2 are encoded by genes present on *S. cerevisiae* chromosomes I and XV, respectively (10). The amino acid sequences of Ntg1 and Ntg2 are closely related to each other (41% identity, 63% similarity) and to *E. coli* Nth (Ntg1: 24% identity, 46% similarity; Ntg2: 25% identity, 51% similarity) (7). The presence of two Nth homologs in *S. cerevisiae* suggests that the two genes are the result of a duplication event (166), as few other prokaryotic or eukaryotic species contain more than one Nth homolog (41). Consistent with the nuclear and mitochondrial localization of Ntg1 and nuclear localization of Ntg2 (4, 221), the amino acid sequence of Ntg1 contains a putative nuclear localization signal (NLS) and a putative mitochondrial transit sequence (MTS), whereas Ntg2 only contains a putative NLS (7, 220). Another characteristic that distinguishes Ntg1 from Ntg2 is that Ntg2

contains an iron-sulfur center, like most Nth homologs, while Ntg1 does not (7, 220). It is thought that the lack of an iron-sulfur center evolved in Ntg1 to avoid the negative consequences of high levels of ROS produced by the reaction of iron-sulfur centers in the Fenton pathway. Because Ntg1 localizes to mitochondria, it is thought that the lack of an iron-sulfur center is necessary in order to prevent the exposure of mitochondrial DNA to additional ROS (221).

5.3 Ntg1 and Ntg2: significance in* S. cerevisiae *cellular function. Ntg1 and Ntg2 are important proteins for BER function and nuclear and mitochondrial DNA maintenance following oxidative stress. Studies have suggested that synthesis of Ntg1 may be regulated by levels of ROS (H_2O_2 specifically) (48, 220), while expression of Ntg2 does not appear to be ROS inducible (220). While single mutations in Ntg1 and Ntg2 or triple mutations in Ntg1, Ntg2, and Apn1 (BER-defective cells) do not affect cell survival of *S. cerevisiae* exposed to oxidative stress, quadruple mutations in Ntg1, Ntg2, Apn1, and Rad1 (BER-/NER- defective cells) decreases the viability of *S. cerevisiae* cells exposed to H_2O_2 (184), emphasizing the redundancy of repair protein function within BER and between BER and NER. In the absence of Ntg1 and Ntg2, Apn1 is capable of completing the BER process, and in the absence of Ntg1, Ntg2, and Apn1 (BER-defective cells), the NER pathway (and possibly TLS and RER) repairs the oxidative lesions. Although BER-defective cells do not display increased sensitivity to oxidative stress, these cells do exhibit increased nuclear mutation rates and nuclear chromosomal rearrangements, indicating that BER is important to the elimination of nuclear oxidative DNA damage and prevention of nuclear genomic instability (39, 184). As Ntg1 localizes to nuclei and mitochondria, the role of Ntg1 in maintaining mitochondrial DNA has also

been evaluated. Cells lacking Ntg1 contain twice as much mitochondrial oxidative DNA damage as wild type cells (46, 140). Additionally, cells with mutant Ntg1 and either mutant Pif1 (DNA helicase) or mutant Abf2 (mitochondrial DNA binding protein) display increased mitochondrial DNA damage levels and produce respiration incompetent cells (46, 140). These results indicate an important interplay between Ntg1, Pif1, and Abf2 in the maintenance of mitochondrial DNA, specifically organized to eliminate oxidative mitochondrial DNA damage caused by ROS in mitochondria. As nuclear and mitochondrial genome instability is associated with cancer and numerous degenerative disorders, nuclear and mitochondrial genomic maintenance facilitated by Ntg1 and other BER proteins is important for the prevention of these diseases.

6. Mechanisms of Regulation of Repair Proteins

It is a goal of this dissertation to determine the mechanisms of regulation of nuclear and mitochondrial BER. In an effort to consider the possible modes of regulation, the regulatory mechanisms of various DNA repair proteins are reviewed.

6.1 Regulation at the level of transcription. Regulation of repair proteins has been observed at the transcriptional level in response to induced oxidative stress and other types of DNA damage-inducing agents (64, 66). The expression of Ntg1 is DNA damage inducible following exposure to H_2O_2, methyl methane sulfonate (MMS), 4-nitroquinoline-1-oxide (4-NQO), and menadione (4, 48), while Ntg2 is not induced by any of these (4). Other *S. cerevisiae* proteins induced by genotoxic agents are the BER 3-methyladenine DNA glycosylase (Mag1), which is induced by MMS, ultraviolet

irradiation (UV), 4-NQO, and hydroxyurea (27, 28, 209). Additionally, the *S. cerevisiae* direct reversal photolyase, Phr1, is induced by MMS, *N*-Methyl-*N*'-Nitro-*N*-Nitrosoguanidine (MNNG), UV irradiation, 4-NQO and γ-ray (154, 164). Transcription of *S. cerevisiae* ribonucleotide reductase is regulated by the cell cycle and can be induced with UV, MMS or 4-NQO (51, 52). In addition to the induction of individual proteins, DNA damage is capable of inducing entire stress response pathways. One way that this mass transcriptional response can be accomplished is through the interaction of *S. cerevisiae* Dun1 and Crt1. Following DNA damage, Dun1 phosphorylates Crt1, preventing its ability to repress transcription of numerous DNA damage response proteins (83). Similarly, interaction of the *S. cerevisiae* Yap1 transcription factor with the promoters of a number of DNA damage response proteins is thought to induce transcription of these genes following DNA damage. In fact, it is predicted that Yap1 is capable of promoting the transcription of Ntg1 as evidenced by two studies that screened for genes containing potential Yap1 transcription factor binding sites (127, 161, 190, 194).

6.2 Regulation by post-translational modification. Post-translational modifications, especially ubiquitination and sumoylation, have been implicated in the regulation of numerous DNA repair proteins (71, 77). For example, ubiquitination of *S. cerevisiae* PCNA has been implicated in polymerase switching during replication to facilitate TLS (104). Also, the *S. cerevisiae* TLS polymerase, polymerase η, is regulated by a mechanism of ubiquitination that is cell-cycle dependent (143). Sumoylation of the human BER protein, thymine DNA glycosylase (TDG), regulates the ability of TDG to detach from damaged DNA (75, 180). Post-translational modification may play a role

during RER in the regulation of *S. cerevisiae* Rad52 protein stability (6). Activation of mammalian p53 in response to DNA damage is associated with a rapid increase in its levels, post-translational modification, and with an increased ability of p53 to bind DNA and mediate transcriptional activation (100). Mammalian BRCA1, which plays a role in several types of DNA repair, including NER, RER, and nonhomologous endjoining, is an E3 ubiquitin ligase (178), suggesting that many DNA repair proteins may be regulated by BRCA1 via ubiquitination. See "Post-translational modification by sumoylation" below for more information on post-translational modification.

6.3 Regulation via localization. While regulation of protein activity can be accomplished through various means, sequestration of proteins to certain sub-compartments of the cell is a common mode of protein regulation. A pool of protein that can be relocated to the appropriate subcompartment allows the cell to rapidly respond to a stimulus as the generation of new mRNA and protein is not required. To accomplish this, certain post-translational modifications are associated with changes in localization of certain proteins. Several enzymes involved in various DNA repair pathways are regulated by such dynamic localization. Some, such as human APE1, hRP2, and damage-specific DNA binding protein (hDDB), are maintained in the cytoplasm and only localize to the nucleus or mitochondria following cellular exposure to a DNA damaging agent. Human APE1 (also known as Ref-1) relocalizes to the nucleus and mitochondria following exposure to oxidative stress (H_2O_2) in lymphocyte cells (65, 191). hRP2 is a DNA-binding protein that exhibits exonuclease activity with a preference for single-stranded or nicked DNA substrates that occur as intermediates of BER. hRP2 undergoes re-localization into the nucleus upon treatment of HeLa cells with DNA damaging agents

that induce oxidative stress, most notably solar simulated light and UVA irradiation (218). Nuclear translocation of the hDDB was seen after human fibroblast cells were treated with UV irradiation (112). Some proteins, such as mammalian c-Abl and *S. cerevisiae* ribonucleotide reductase localize in response to DNA damage. c-Abl is translocated into the nucleus in response to DNA damage, where it causes apoptosis (219). Two subunits make up the *S. cerevisiae* ribonucleotide reductase holoenzyme; the R2 subunit is nuclear, while the R1 subunit is maintained in the cytoplasm. By changing the localization of the nuclear R2 subunit following DNA damage, the holoenzyme can form, allowing ribonucleotide reductase to reduce cytoplasmic ribonucleotides to generate the deoxyribonucleotides needed for repair (225). The localization of other enzymes is regulated in a cell cycle-dependent manner. Mammalian flap endonuclease-1 (hFEN-1) and hMYH localize to the nucleus in a cell cycle-dependent manner during S phase to facilitate replication (18, 152).

6.4 ROS mediated BER regulation. Recent studies have shown that DNA damage, including oxidative DNA damage, alkylation damage, and UV-induced DNA damage, induces the production of certain subspecies of ROS in *S. cerevisiae* (157). These ROS are then thought to be capable of activating certain DNA damage response pathways, such as those regulated by Yap1 (38, 70, 91, 142). It is thought that moderate levels of these types of ROS are capable of inducing a DNA repair response; however, at high levels, these ROS are thought to be deleterious and lethal to the cell. Signals produced by ROS could control DNA repair by modifying proteins in the pathway, modulating protein levels, promoting or preventing interactions between specific proteins, and changing localization of proteins.

6.5 A final note on the regulation of DNA repair. Repair proteins might also be regulated by mechanisms of post-transcriptional regulation in which mRNA stability is changed. Lastly, stability of the DNA repair protein could be regulated in such a way that the protein is degraded when it is not needed.

It is evident from this review that little is known regarding the mechanisms by which BER proteins are regulated. This is an important area of study given the deleterious consequences of defects in the BER pathway. Localization and post-translational modification of Ntg1 are possible targets for regulation of Ntg1, as Ntg1 localizes to both nuclei and mitochondria and Ntg1 contains seven potential sumoylation sites. Therefore, it is a goal of this dissertation to determine if these modes of regulation could regulate a BER protein such as Ntg1. Regulation of DNA repair functions by regulation of subcellular localization requires specialized transport systems described in sections 7 and 8. Sumoylation as a mode of regulation is described in section 9.

7. Nuclear Protein Transport and Classical Nuclear Import in *S. cerevisiae*

7.1 Nuclear protein localization. It is estimated that 27% of *S. cerevisiae* proteins are nuclear (98), and up to 45% of *S. cerevisiae* proteins could use the classical nuclear protein import pathway to enter the nucleus (102). Additionally, of the *S. cerevisiae* proteins that localize to the nucleus, about 57% of steady-state nuclear proteins are predicted to use classical nuclear import, whereas about 43% may use other mechanisms to enter the nucleus (102). The alternative pathways have been implicated in

the transport of proteins involved in mRNA export, tRNA processing, and ribosomal assembly (147).

7.2 Classical nuclear protein import. Proteins enter the nucleus from the cytoplasm via the nuclear pore complex (NPC). NPCs allow passive diffusion of ions and small proteins (<40 kDa) but restrict passage of larger molecules to those containing an appropriate targeting signal (19, 144). The NPC is made up of three substructures: the cytoplasmic filaments, a central core, and the nuclear basket (Figure 6) (183). The pores are constructed from a class of proteins called nucleoporins, a subset of which contains a tandem series of phenylalanine-glycine (FG) repeats that line the central transport channel of the pore (3, 181, 183, 196). This central core contains eight spokes sandwiched between the cytoplasmic and nuclear rings. The spoke structures collectively encircle the central region through which all active transport occurs (56). Karyopherins are a family of proteins that transport cargo from the cytoplasm through the NPC (Figure 7). Karyopherin-β proteins interact with the NLS of cargo directly or indirectly through an adapter protein. Approximately 14 karyopherin-β proteins exist in *S. cerevisiae* cells, including Kap95 (importin-β1) (175). The most studied adaptor protein is karyopherin-α (importin α). Importin α recognizes the classical nuclear localization sequence (cNLS) of its cargo. After importin α binds to the cNLS cargo, it heterodimerizes with the karyopherin-β.

Nuclear import takes place when the α/β complex interacts with the NPC. An interaction occurs between the karyopherin-β portion of the heterodimer and the nucleoporin protein, RanBP2 (Nup358), which is a component of the cytoplasmic

filaments (208, 217). Following docking, the cytoplasmic filaments bend, bringing the docked heterodimer-cargo complex further into the NPC, and rapid hydrolysis of GTP occurs, stabilizing the interaction between the α-β dimer, but releasing the heterodimer-cargo complex into the central channel (40, 59, 146). It is then thought that the complex binds to the nucleoporin, Nup62, and nuclear transport factor, NTF2, which takes the complex across the central gated channel (175). Finally, the heterodimer-cargo complex docks onto the nuclear basket via interactions between Nup62 and Nup153, and due to the presence of high levels of RanGTP, the interaction between karyopherin-β and importin α/cargo is compromised (59, 167). Following this dissociation, the cargo is released from importin α into the nucleus.

8. Mitochondrial Protein Transport in *S. cerevisiae*

8.1 General features. It is estimated that 10-15% of eukaryotic nuclear genes encode proteins that localize to mitochondria (138). Proteins destined for mitochondria are generally translated in the cytoplasm and are quickly bound by cytoplasmic chaperone proteins, such as Hsp70 and Hsp90, which keep the proteins in an unfolded conformation so that interaction with the mitochondrial translocation machinery is possible (123, 222). It is possible for nuclear encoded proteins to be imported cotranslationally (137, 198). Mitochondrial translocation involves protein recognition by receptors, threading of the protein through pores in the outer and inner membranes of the mitochondrion, proteolytic processing of the protein by MIP (mitochondrial intermediate

peptidase) and/or MPP (mitochondrial processing peptidase) proteins, protein folding, and then possible insertion into mitochondrial membranes.

8.2 Mitochondrial matrix targeting sequences. Most proteins whose intended destination is the mitochondrion contain an N-terminal targeting sequence, which in many cases contains a cleavage sequence, known as a presequence. These N-terminal mitochondrial targeting sequences (MTSs), are made up of 10-80 amino acid residues and are characterized by a series of basic amino acids that have the potential to form amphipathic helices with one hydrophobic and one positively charged face (21, 138, 203). These faces are important for mitochondrial protein interaction with the import machinery. Some proteins do not contain N-terminal mitochondrial targeting sequences, possessing internal ones instead (138). These internal sequences are associated with protein insertion into mitochondrial membranes. Various computer-based algorithms have been generated to predict whether or not a particular protein has a putative MTS. These programs include MitoProt (34), PSORT (134), and TargetP (53), and the accuracy of predicting the sequences responsible for protein localization by these programs is 90%, 57%, and 40%, respectively .

8.3 Mitochondrial transport. In order for a protein to pass from the cytoplasm into the mitochondrial matrix, the protein must pass through both an outer mitochondrial membrane and an inner membrane. Protein transport machinery resides in both membranes of mitochondria: the TOM complex in the outer membrane and the TIM complex in the inner membrane (Figure 8). The TOM complex recognizes the amphipathic helixes of the target protein and contains binding pockets for the hydrophobic face of the helix (1). The TIM complex resides in the inner membrane of

the mitochondrion. There are two types of TIM complexes, the TIM23 complex is the inner membrane complex that is responsible for interacting with proteins destined for the matrix and some mitochondrial membrane proteins, and the TIM22 complex is responsible for interacting with the majority of proteins that become incorporated into mitochondrial membranes. For proteins containing an MTS, the MTS is recognized by subunits of TIM23. The protein is then directed to the protein-conducting channel of the TIM23 translocase, and the presequence passes through the channel in an electrical membrane potential ($\Delta\psi$)-dependent manner. Next, the protein encounters a chaperone, mtHsp70, that uses ATP hydrolysis to help direct the matrix-bound protein through the channel in a ratchet-like manner (129, 138). TIM22 also transports proteins in a $\Delta\psi$-dependent manner; however, from the TIM22 complex, the protein is directly inserted into the inner membrane (138). Proteins which have entered the mitochondrial matrix are proteolytically cleaved by MPPs (67), which remove the presequence from the protein. Additionally, some proteins are cleaved a second time subsequent to MPP cleavage by MIP.

9. Post-translational Modification by Sumoylation

9.1 Ubiquitination and sumoylation are post-translational modifications. Post-translational modification is a mode of regulation that allows the diversification of protein function. These covalent modifications include ubiquitination and ubiquitin-like post-translational modifications (Ubls). One Ubl is small ubiquitin-like modifier (SUMO), which, like ubiquitin, covalently and reversibly binds its substrate.

SUMOylation is involved in many biological pathways, including maintenance of genome integrity, chromosome packaging, various aspects of signal transduction, meiosis, and mitosis (31, 76, 88). As sumoylation is implicated in numerous cellular processes, its dysregulation has also been linked to various diseases, including cancer, diabetes, neurodegenerative disorders, viral infections, and developmental defects (226).

9.2 The SUMO moiety. SUMO proteins are ubiquitously expressed throughout eukaryotic species and are essential in most organisms (69). While some eukaryotes have only one SUMO protein (*S. cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*), others express more than one SUMO protein (human has four) (69). All SUMO proteins are expressed in an immature pro-form, in which they carry a C-terminal stretch of variable length (2–11 amino acids) following a Gly-Gly motif that marks the C-terminus of the mature protein. In order to be capable of sumoylation, the C-terminal extension must be removed by SUMO-specific proteases (69).

9.3 Conjugation. The process of sumoylation is analogous to the process of ubiquitination; however, the SUMO and ubiquitin conjugation pathways are distinct from one another (Figure 9) (47, 95). In the initial step, SUMO forms a thioester bond with an E1 activating enzyme (heterodimer AOS1–UBA2) in an ATP-dependent manner. The activated SUMO is then passed to the E2 conjugating enzyme, Ubc9 (ubiquitin-conjugating enzyme 9), which will either act on its own or together with an E3 ligase to covalently attach the C-terminal Gly residue of SUMO to the ε-amino group of a Lys residue in the substrate through an isopeptide bond. The target Lys residue(s) for sumoylation are often found within a consensus tetrapeptide, ψKXE/D (ψ denoting a hydrophobic residue), which positions the catalytic site of Ubc9 on the target lysine

residue (12, 155). Whereas specificity for substrate is dictated by the E2 and E3 during ubiquitination, the SUMO system is thought to utilize only a single E2 enzyme, and probably many fewer E3 ligases, making Ubc9 important for specificity of SUMO-substrate interactions. In fact, for some SUMO substrates, the binding to Ubc9 is enhanced by additional interactions that occur outside the consensus sequence (5, 12). In most cases, one sumoylation event occurs per individual substrate Lys residue. However, the formation of polySUMO chains (chains formed by conjugation of one SUMO to another) has been observed both *in vivo* and *in vitro* in both *S. cerevisiae* and mammalian cells (23, 31, 130, 189). Desumoylating isopeptidases can cleave the SUMO from the substrate, allowing the SUMO to be recycled. The desumoylating isopeptidases in *S. cerevisiae* are Ulp1 and Ulp2 (43, 106), and the six Ulp homologues in humans are called sentrin-specific proteases (hSENP1-3 and hSENP5-7).

9.4 The functions of sumoylation. Sumoylation has been implicated in a variety of cellular processes, outlined below:

- **Cell cycle regulation**. Both members of the E1 activating enzyme heterodimer, AOS1 and UBA2, are essential for transition from G2 to M in *S. cerevisiae* (45, 89). Additionally, in *S. cerevisiae*, sumoylation is implicated in sister chromatid separation and mitotic exit (44).
- **Genomic stability**. Several proteins involved in DNA repair and maintenance are sumoylated (133). Sumoylation is important for telomere maintenance in *Schizosaccharomyces pombe* (187). In *S. cerevisiae*, sumoylation has been shown to play a role in damage-tolerance and damage-induced interchromosomal homologous recombination (117) and non-homologous end-joining through the

protein Yku70 (145). Additionally, mitochondrial fusion and stability are influenced by sumoylation in mammalian cells (229). A number of mammalian proteins involved in DNA repair and genomic stability are sumoylated, including thymine DNA glycosylase (hTDG), bloom syndrome protein (hBLM), topoisomerase 1/2 (hTOP1/2), Werner syndrome protein (hWRN), hXRCC4, and the DNA damage response histone acetyltransferase, hTIP60, (8, 32, 50, 75, 87, 93, 119, 223). In all of these cases, the enzymes are made more efficient by sumoylation. Also, see paragraph below regarding the role of sumoylation in handling cellular stress.

Thymine DNA glycosylase. The human BER glycosylase hTDG is sumoylated, and sumoylation plays a role in the catalytic turn-over rate of this enzyme in BER (Figure 10). hTDG primarily recognizes guanine mismatched with thymine or uracil. The rate-limiting step for BER is release of the glycosylase after hTDG removes a damaged base. Sumoylation of hTDG causes a conformational change in hTDG that allows it to be released from its DNA product (75, 180). Thus, sumoylation causes a faster rate of BER. Additionally, sumoylation of hTDG is thought to help it to localize to progressive multifocal leukoencephalopathy (PML) subnuclear regions (186).

- **Competition with other post-translational modifications**. Sumoylation has been shown in some instances to modify on the same locus as ubiquitination, acetylation, and phosphorylation. It is thought that the two post-translational modifications compete in order to change the function of the protein. For example, sumoylation of *S. cerevisiae* and human PCNA occurs at the same

residue where ubiquitination by Rad18 and Rad5 occurs, and the different modifications allow for a differential response to DNA damage following replication (80). Mammalian IκBα, Rad52, superoxide dismutase (hSOD1), and Huntingtin proteins are also modified by both SUMO and ubiquitin at the same residues, and it has been suggested that SUMO prevents degradation of these proteins by preventing ubiquitination (42, 55, 159, 179). In contrast, sumoylation of certain proteins is thought to contribute to ubiquitin ligase recruitment to the substrate, which could result in enhanced protein degradation (210). For many of the proteins which contain sites where SUMO and ubiquitin compete, phosphorylation at a nearby site serves as a mechanism to prevent sumoylation. For example, if S32 and S36 are phosphorylated in mammalian IκBα, then the protein cannot be sumoylated and is consequently ubiquitinated and degraded (42). On the other hand, phosphorylation of a protein can facilitate its sumoylation and is required in some cases. This is the case with hPPARγ2 and hHSF1 (78, 213). A specific SUMO motif has been described for this phosphorylation-dependent sumoylation event: PDSM (phosphorylation dependent sumoylation motif, yKxExxS/T) (79, 215). Sumoylation and acetylation compete for the same residues in mammalian nucleosomal core histones, hMEF2, and hELK-1 (135, 214, 227). In each of these cases sumoylation results in transcriptional repression of the genes controlled by these proteins (226).

- **Transcriptional regulation**. Sumoylation has been implicated in both increased and decreased protein expression. Numerous transcription factors are

sumoylated. Those sumoylated transcription factors with increased activity include human heat shock factors, hHSF1 and hHSF2, Nuclear factor of activated T-cells (hNFAT), and p53 (72, 82, 156, 193). Those transcription factors with decreased activity following sumoylation include mammalian Smads, c-Jun, c-Myb, Lef1, and androgen receptor, (14, 113, 131, 149, 158). Sumoylation of these transcription factors enhance activity by either changing protein localization or altering the folding of the protein.

- **Localization**. The majority of sumoylated proteins are found in nuclei; however, sumoylated proteins can be found throughout the cell, such as in the cytoplasm, the plasma membrane, mitochondria, and the endoplasmic reticulum (ER) (69). A few examples of proteins which enter the nucleus upon sumoylation are eukaryotic RanGAP1 and human NF-κB essential modulator (hNEMO) (84, 90). As RanGAP1 is important for the nuclear localization of many proteins, its own sumoylation allows RanGAP1 to interact with the nuclear pore complex. It has been hypothesized that proteins in *S. cerevisiae* which enter the nucleus via classical nuclear transport require sumoylation to interact with importin α for nuclear import (177). Additionally, sumoylation has been implicated in localization of proteins to mammalian subnuclear compartments, such as PML bodies, Cajal bodies, DNA damage foci, centrosomes, and centromeres (15, 226). It is thought that these compartments serve as reservoirs for proteins that are not being used or that they are centers for high protein activity. Not only is sumoylation important for nuclear import, but it is also involved in the nuclear export of certain human proteins, including hMEK1 and hTEL (174, 206). While

numerous nuclear proteins are sumoylated, SUMO is not necessary for nuclear localization of every nuclear sumoylated protein.

9.5 Cellular stress and sumoylation. Cellular stress has been implicated in global sumoylation of proteins in both humans and *S. cerevisiae* (160, 228). These stresses include osmotic stress, oxidative stress, and heat shock. Numerous examples of sumoylation events associated with oxidative stress have been documented. In particular, oxidative stress is associated with decreased sumoylation of human proteins at low doses (below 1 mM H_2O_2), but increased sumoylation at high doses (100 mM) (20). Interestingly, high doses of oxidative stress cause inactivation of desumoylating enzymes by creation of an intra- or inter-molecular disulfide bridge, and this causes the accumulation of sumoylated proteins (211). hNEMO is an example of a protein whose sumoylation is elevated following oxidative stress (115). hHIF1 is sumoylated following hypoxia (168), which is interesting considering the high levels of oxidative stress associated with the hypoxic state. Additionally, sumoylation has been associated with survival in the presence of other genotoxic agents. For example, *S. cerevisiae* Rad52, hTIP60, hXPC, and hNEMO demonstrate increased sumoylation under genotoxic stress conditions (32, 84, 159, 202). In *S. cerevisiae*, inhibition of the SUMO-conjugating pathway or SUMO-specific proteases leads to increased sensitivity to a wide range of genotoxic agents (87, 107). In human cells, inhibition of the SUMO pathways, either by a dominant-negative Ubc9 (125) or by the viral protein Gam-1, which targets E1 for degradation (207), sensitizes cells to genotoxic stress-induced apoptosis. Genotoxic stress induced by chemotherapeutic agents (etoposide, doxorubicin, cisplatin, camptothecin etc.), ionizing radiation, and UV exposure has been associated with

increased sumoylation of certain proteins (192). Additionally, heat shock induces the sumoylation of some proteins. Examples of these sumoylated proteins include mammalian hHSF1, hTOP1, c-Myb, and hPML (promyelocytic leukemia) (82, 126, 136, 176).

How is nuclear and mitochondrial base excision repair regulated in *S. cerevisiae*?

Maintenance of nuclear and mitochondrial genomic integrity is important for cellular survival. Oxidative stress increases the occurrence of oxidative DNA damage in both nuclei and mitochondria, and unrepaired nuclear and mitochondrial oxidative DNA damage is deleterious to the cell, as evidenced by increased mutation rates and arrest of DNA replication and transcription (86). Ultimately, in humans, the accumulation of oxidative DNA damage can lead to multiple mutations leading to activation of oncogenes, inactivation of tumor suppressors, and gross chromosomal rearrangements. The consequences of these genomic disturbances includes tumorigenesis, other degenerative disorders, and aging (11, 36, 105, 200, 204). Thus, it is important for cells to have functional DNA repair systems, such as BER, to remove oxidative lesions from nuclear and mitochondrial DNA in a timely manner. This dissertation explores how BER is regulated so that both nuclear and mitochondrial genomes are maintained during oxidative stress. The work presented in this dissertation addresses the following questions. (1) Are the *S. cerevisiae* BER proteins, Ntg1 and Ntg2, regulated by a mechanism of differential subcellular localization? (2) Is the localization of Ntg1 cell cycle-dependent? (3) To what extent are Ntg1 and Ntg2 post-translationally modified by

SUMO, and does sumoylation play a role in the regulation of Ntg1 and Ntg2? (4) How does Ntg1 enter nuclei or mitochondria in order to repair nuclear or mitochondrial oxidative DNA damage? (5) What is the impact of eliminating nuclear or mitochondrial DNA repair by Ntg1 on nuclear and mitochondrial genomic integrity? By elucidating the answers to some of these questions, we will provide insight into how DNA repair proteins are capable of balancing signals from two organelles and maintaining a level of repair in both nuclei and mitochondria. The regulation of *S. cerevisiae* BER may suggest modes of regulation that are utilized in BER of other eukaryotic organisms. Considering the association between genomic instability and human disease, understanding the ways in which DNA repair pathways are regulated will provide information about how many human diseases are naturally prevented. This information could also be utilized in order to establish new treatments that are capable of preventing disease.

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Table 1: Mitochondrial DNA Repair Proteins in Eukaryotic Cells. Known repair proteins that localize to mitochondria and the known or proposed *in vivo* and/or *in vitro* function(s) for each. BER: base excision repair; MMR: mismatch repair; RER: recombination repair; TLS: translesion synthesis.

***S. cerevisiae* Protein Name**	**Human Homolog**	**Repair Pathway**	**Function**	**Reference**
Polγ	hPolγ		DNA polymerase	(37)
	hLIG3		Ligase	(101)
Phr1	-	Direct reversal	Photolyase	(216)
Apn1	hHAP1; hREF1	BER	AP endonuclease, 3´diesterase	(199)
Ogg1	hOGG1	BER	DNA glycosylase, AP lyase	(172)
Ntg1	hNTH1	BER	DNA glycosylase, AP lyase	(46, 221)
Ung1	hUNG1	BER	Uracil DNA glycosylase	(26)
-	hMYH	BER	MutY homolog; DNA glycosylase	(37)
-	MtTGendo	BER	DNA glycosylase	(182)
Pif1	hPIF	RER	5´-to-3´ DNA helicase; works with Ntg1p to reduce oxidative damage in mtDNA	(13, 140)
Rrm3	hPIF	RER	DNA helicase	(13, 141)

Rad23	-	NER	DNA damage binding protein	(170)
Mlh1	-	MMR	Molecular matchmaker	(170)
Msh1	-	MMR	*E. coli* MutS homologue	(33)
Mre11	-	RER	Nuclease	(170)
Cce1	-	RER	Cruciform cutting endonuclease	(97)
Nuc1	EndoG	RER	Mitochondrial nuclease, DNase, RNase	(224)
Mhr1	-	RER	Maintains rho status of mtDNA	(110)
Abf2	h-mtTFA	RER	High mobility group (HMG) protein; maintains rho status of mtDNA	(116)
Rad50	-	RER	dsDNA binding protein	(170)
Rad18	-	TLS	Ubiquitin-conjugating enzyme	(170)
Din7	-		5´-to-3´ Exonuclease	(58)
-	hMTH1		Triphosphatase	(92)
Rim1	hRIM1		ssDNA binding protein	(37, 170)
Top2	-		Topoisomerase	(170)
-	DUT1		dUTPase	(99)
Hmi1	-		DNA helicase	(170)
Mgm101	-		Mitochondrial genome maintenance	(170)

Table 2: Comparison of BER DNA Glycosylases in *E. coli*, Mammals, and *S. cerevisiae* (modified from (94)).

E. coli	Mammals	*S. cerevisiae*	Substrates
NTH1	NTH1	NTG1 and NTG2	Thymine glycol, 5-hydroxy- and 6-hydroxy-dihydrothymine (DHT), uracil glycol, 5-hydroxycytosine, 5-hydroxyuracil, ß-ureidoisobutyric acid, urea
NEI	NEIL1	Absent	Same as NTH1. In addition, endo VIII also recognize 8-oxoG, in particular when 8-oxoG is paired with A or G.
UNG	UNG	UNG	Uracil in both single and double stranded DNA
MUG	DUG	Absent	Uracil and thymine (in T/G mismatch) in double stranded DNA. Ethenocytosine
FPG	OGG1	OGG1	8-oxoG:C, 8-oxoG:G, 8-oxoG:T, FapyG, FapyA
Absent	Absent	MAG1	3-methyl adenine
Mut Y	MYH	Absent	8-oxoG:A
Mut T	MTH	Absent	8-oxo-dGTPase
Absent	AAG	Absent	Alklated bases
Absent	MBD4	Absent	G:FU, G:T, G:U
Absent	SMUG1	Absent	Uracil from single- and double-stranded DNA in nuclear chromatin
Absent	TDG	Absent	Thymine from G/T mismatches

Figure 1

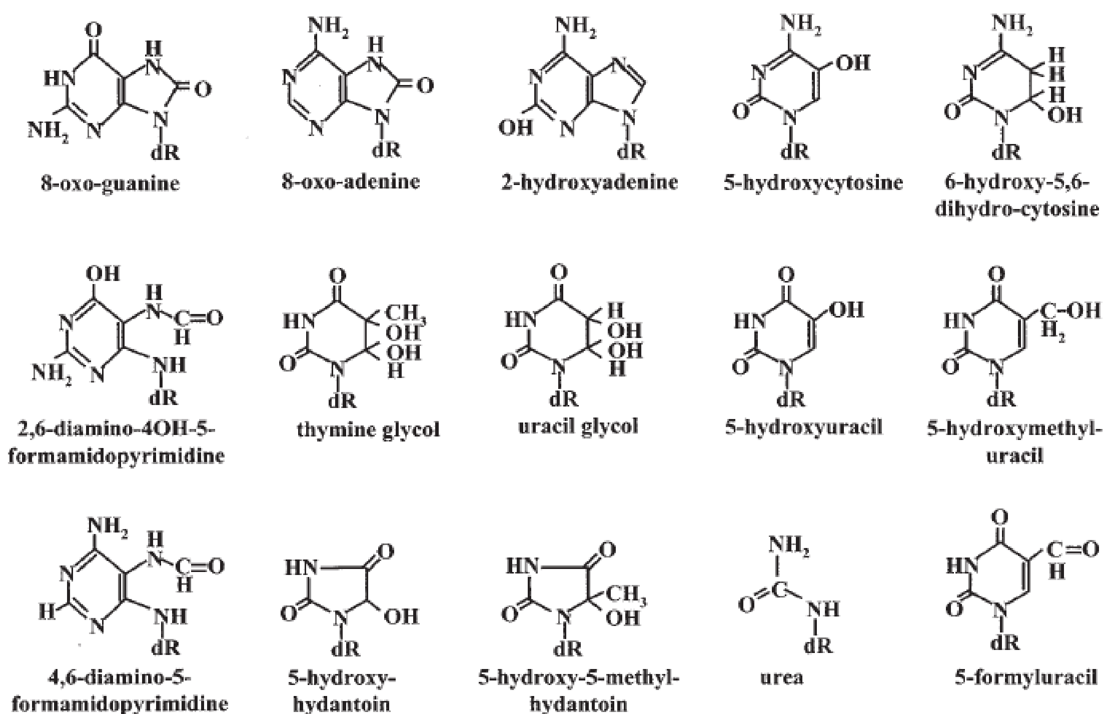

Figure 1. Examples of Oxidative DNA Lesions. ROS caused by oxidative stress that is produced during cellular metabolism and in the environment of the cell are capable of damaging DNA, lipids, and proteins (197). A number of oxidized DNA bases can result from deleterious interactions with ROS. The lesions listed include fragmented or ring-opened forms and oxidized aromatic derivatives and represent only a subset of the 40 to 60 known oxidative lesions. This figure was adapted from Lu, et al, 2001 (114).

Figure 2

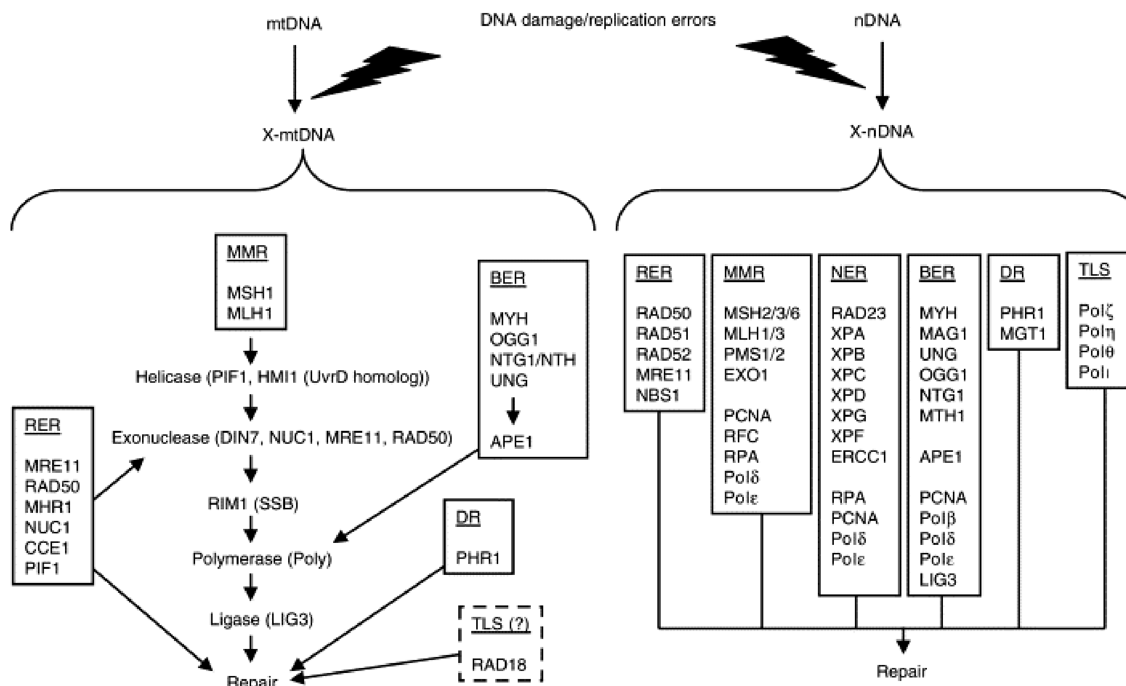

Figure 2. Nuclear and Mitochondrial DNA Repair Pathways. Both nuclear and mitochondrial DNA are subject to damage. Nuclear DNA repair and tolerance pathways include recombination (RER), mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), direct reversal (DR), and translesion synthesis (TLS). Less is known about the repair processes that occur in mitochondria, but BER, MMR, RER, DR, and TLS are likely to function to maintain mitochondrial genomic stability. Proteins from both *S. cerevisiae* and mammalian cells that are known to play a role in each pathway and organelle are listed. Importantly, some proteins appear to function in repair of both nuclear and mitochondrial DNA. This figure was adapted from Larsen, et al, 2005 (103).

Figure 3

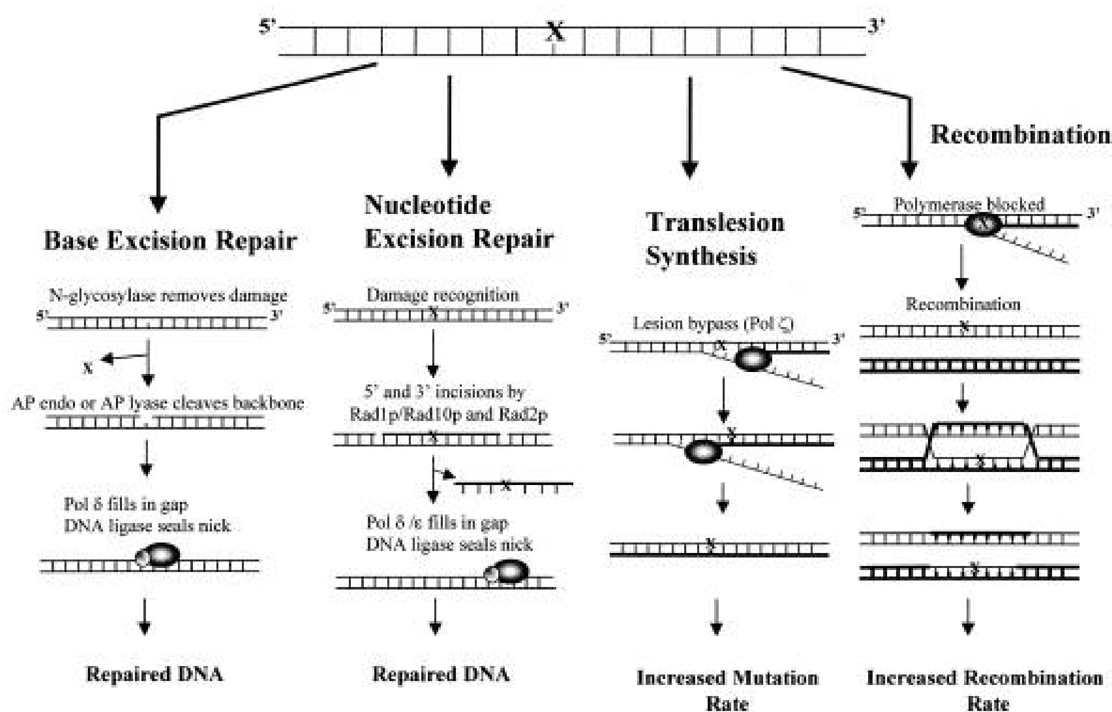

Figure 3. DNA Repair in *S. cerevisiae*. Four of the DNA repair pathways utilized by *S. cerevisiae* are outlined. In BER, one or a short stretch of nucleotides is removed and replaced with the correct sequence. During NER, a large stretch (25-30 nucleotides) of damage is excised. Both BER and NER repair with fidelity. TLS utilizes a polymerase switch to bypass a lesion during DNA replication. RER utilizes homologous sequence in a sister chromosome to allow bypass of a lesion during replication. Both TLS and RER can result in the incorporation of mutations. The DNA lesion to be repaired is indicated by an "X". This figure was adapted from Swanson, et al, 1999 (184).

Figure 4

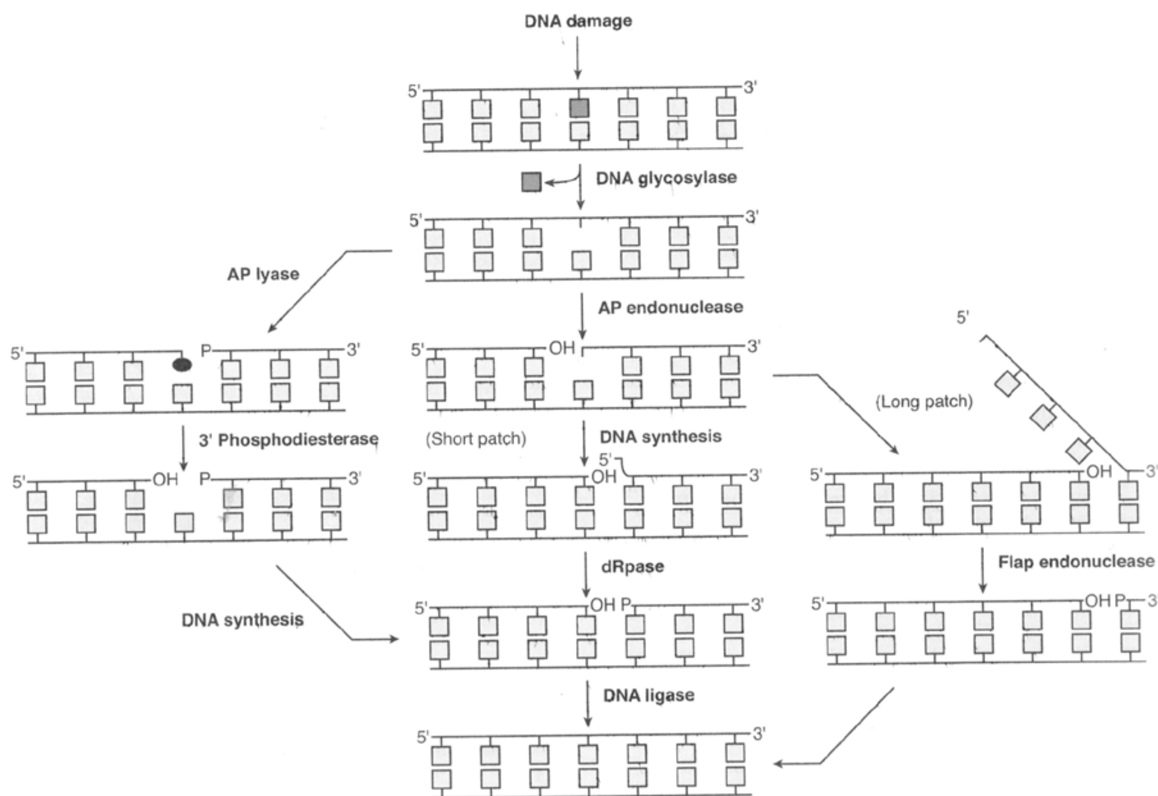

Figure 4. Base Excision Repair. The process of BER begins with recognition and removal of a damaged base by a DNA glycosylase, creating an AP site and leaving the sugar phosphate backbone intact. Next, either an AP lyase or an AP endonuclease cleaves the sugar-phosphate DNA backbone on the 3´ side or 5´ side of the AP site, respectively. Subsequent trimming, polymerization, and ligation processes occur to complete BER and restore the sequence of the DNA. Short patch BER results in the removal of one nucleotide, while long patch BER results in the removal of 2 to 13 nucleotides. This figure was adapted from Friedberg, 2006 (62).

Figure 5

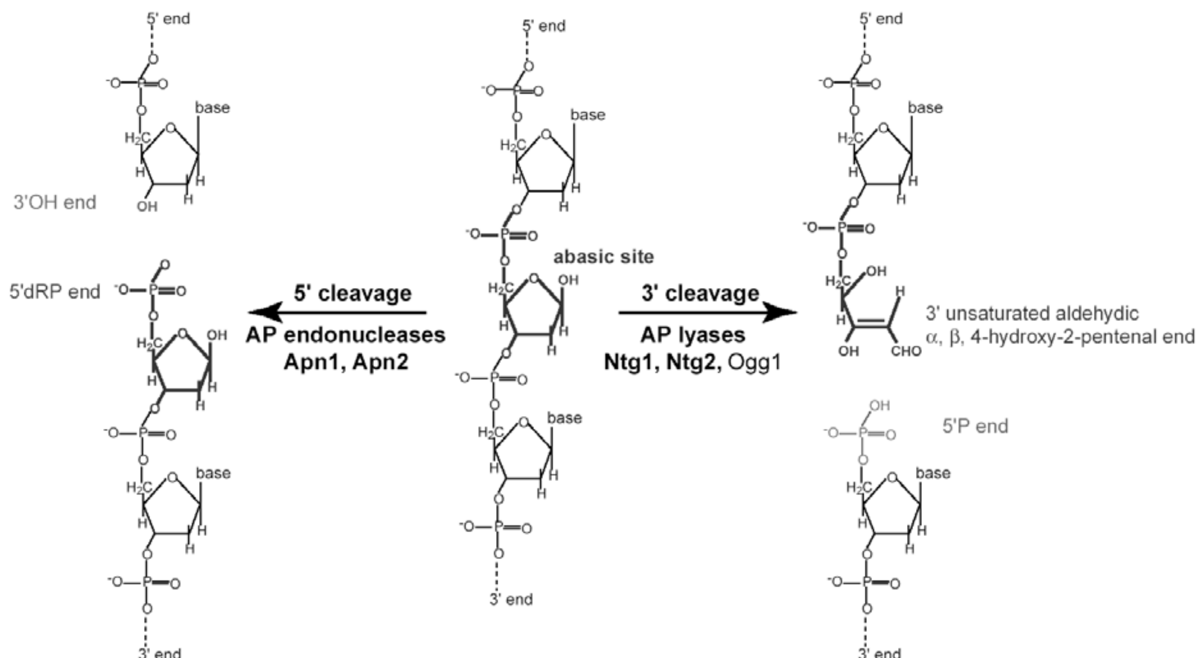

Figure 5. Structures of AP Sites and Breaks Created by AP Endonucleases and AP Lyases. AP endonucleases recognize AP sites and cleave to the 5´ side of the site, leaving a 5′-blocked single stranded break with a 5′-deoxyribose-phosphate end (5′-dRP). AP lyases cleave 3´ to the AP site, leaving a 3′-blocked single stranded break with a 3′-unsaturated aldehydic (α,β-4-hydroxy-2-pentenal) end (3′-dRP). As this substrate is not recognized by DNA polymerase, the activity of a 3´ phosphodiesterase is necessary to cleave the 5´ phosphate terminus in order for BER to progress. Ntg1 and Ntg2 have AP lyase function. This figure was adapted from Boiteux and Guillet, 2004 (17).

Figure 6

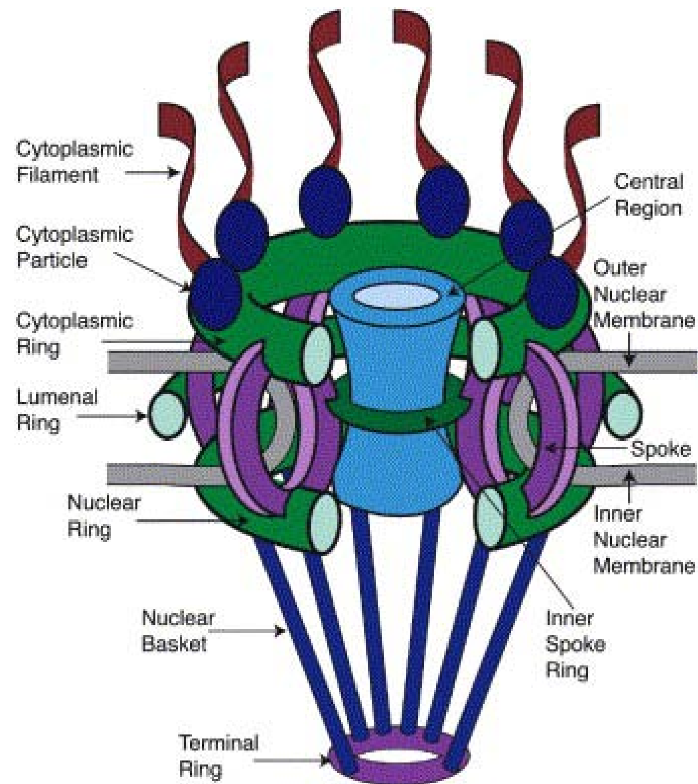

Figure 6. The Nuclear Pore Complex. Nuclear proteins enter the nucleus from the cytoplasm via transport through the NPC. The NPC is made up of three substructures: the cytoplasmic filaments, a central core, and the nuclear basket. This figure was adapted from Suntharalingam and Wente, 2003 (183).

Figure 7

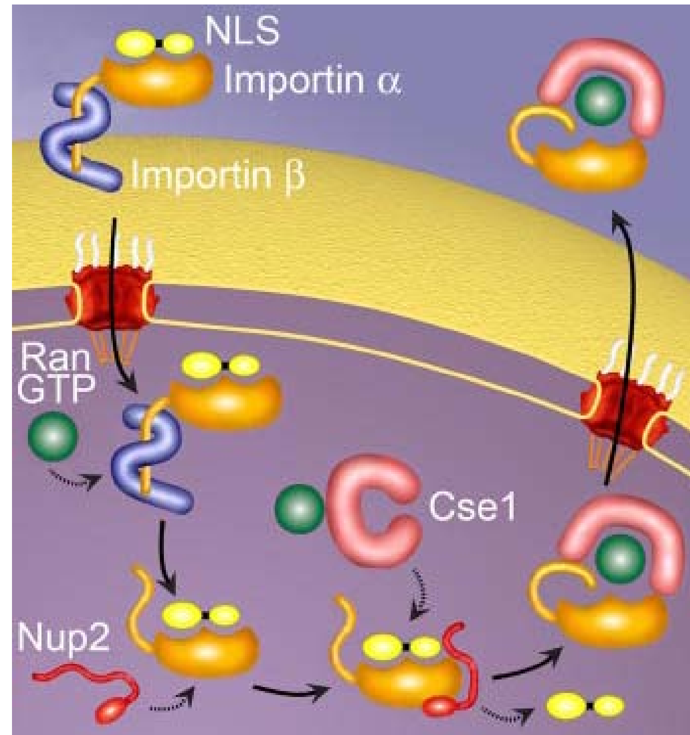

Figure 7. Classical Nuclear Import. In the cytoplasm, proteins destined for the nucleus and containing a classical NLS are bound by importin α, which recognizes the classical NLS of the cargo. Importin β then heterodimerizes with importin α and, via interactions with the nuclear pore, facilitates entry of the cargo into the nucleus. Once inside the nucleus, RanGTP binds the complex, causing dissociation of the cargo. The importins are then recycled back into the cytoplasm in order to facilitate transport of more proteins. This figure was adapted from Lange, et al, 2006 (102).

Figure 8

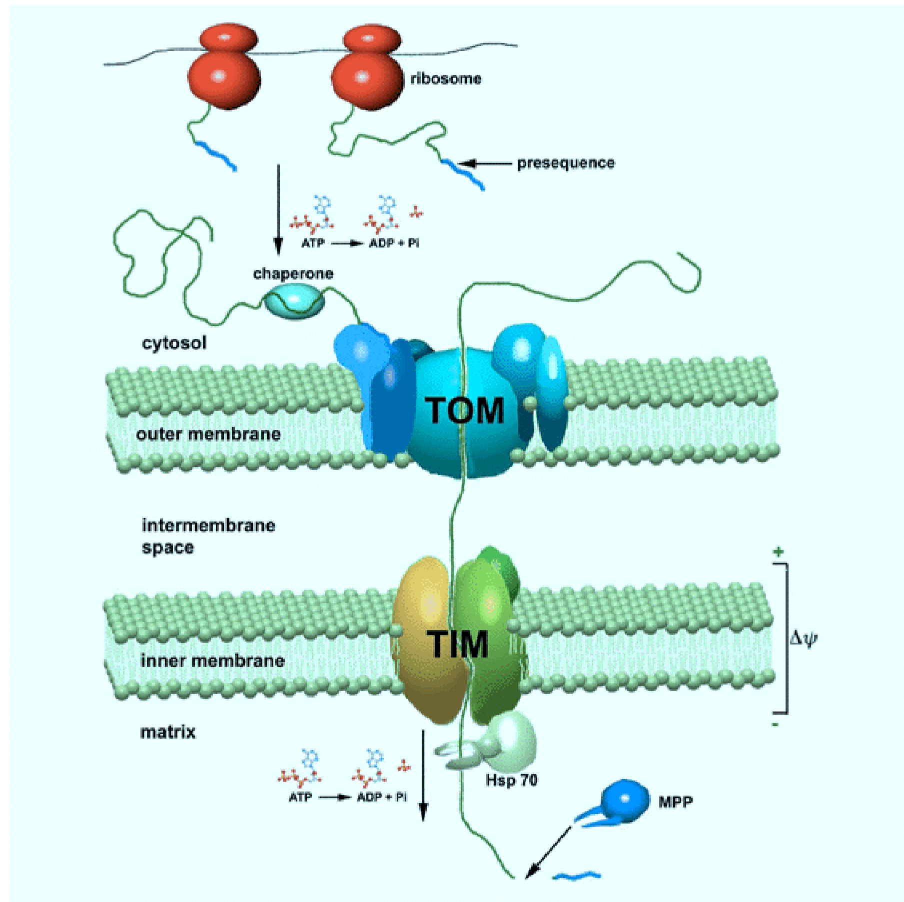

Figure 8. Mitochondrial Protein Import. Mitochondrial matrix proteins are imported into mitochondria through the outer and inner mitochondrial matrix membranes (TOM and TIM) via associations between the amphipathic helix of the target protein and components of the TOM and TIM complex. The chaperone protein, mtHsp70, directs the protein into the matrix using ATP hydrolysis. Finally the target protein is cleaved by mitochondrial processing peptidase (MPP). This figure was adapted from the following website: www.biochem.biomedchem.uwa.edu.au (111, 205).

Figure 9

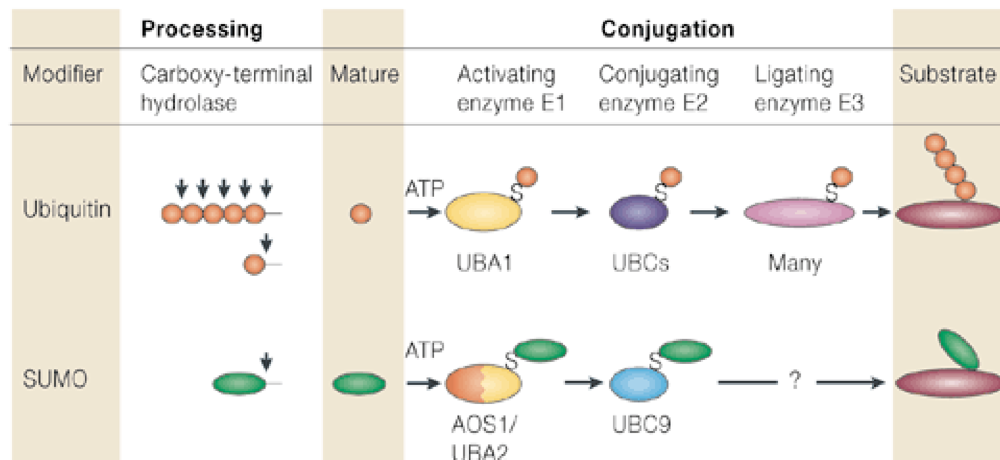

Figure 9. Ubiquitin and Sumoylation. Post-translational modification by ubiquitin and small ubiquitin-like modification (SUMO) is a covalent linkage at a lysine on the substrate protein. The processes of modification by ubiquitin or SUMO are very similar in that there are activating (E1), conjugating (E2), and sometimes ligating (E3) enzymes that facilitate the process. This figure has been adapted from Müller, et al, 2001 (132).

Figure 10

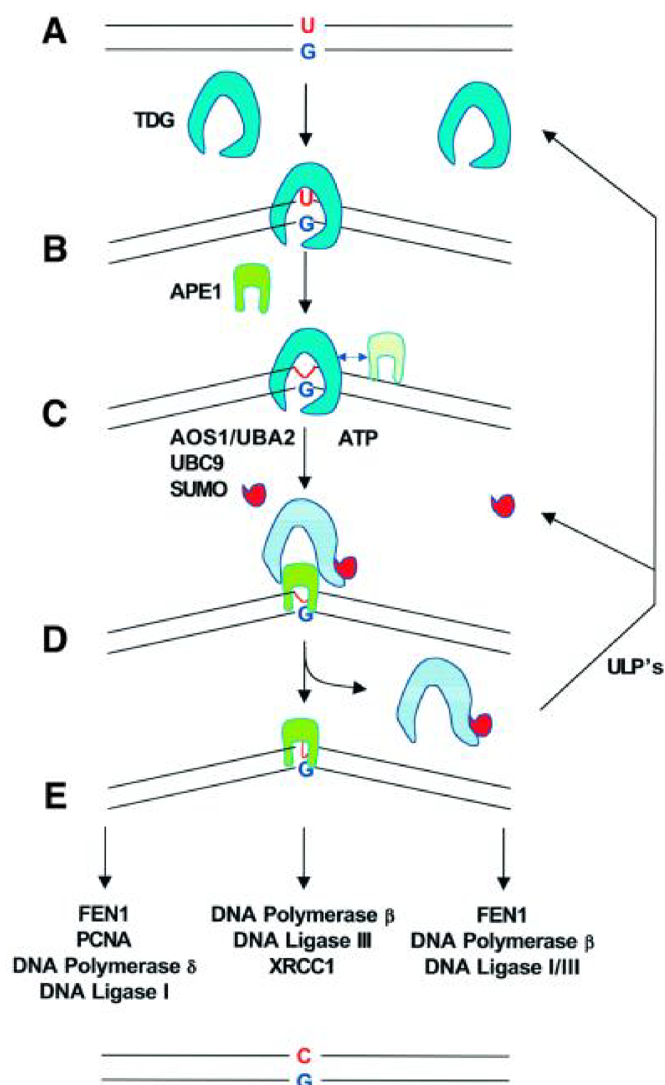

Figure 10. Sumoylation of Thymine DNA Glycosylase. During BER, TDG recognizes and removes uracil or thymine across from guanine; however, following its glycosylase activity, TDG remains associated with the DNA. In order to facilitate the removal of TDG from its DNA product, TDG is sumoylated and AP endonuclease arrives at the AP site. The accomplishment of these two steps allows the removal of TDG from of the DNA so that BER can be completed. This figure was adapted from Hardeland, et al, 2002 (75).

CHAPTER 2

DYNAMIC COMPARTMENTALIZATION OF BASE EXCISION REPAIR PROTEINS IN RESPONSE TO NUCLEAR AND MITOCHONDRIAL OXIDATIVE STRESS

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1. Abstract

DNA harbored in both nuclei and mitochondria of eukaryotic cells is subject to continuous oxidative damage resulting from normal metabolic activities or environmental insults. Oxidative DNA damage is primarily reversed by the base excision repair (BER) pathway initiated by N-glycosylase apurinic/apyrimidinic (AP) lyase proteins. To execute an appropriate repair response, BER components must be distributed to accommodate levels of genotoxic stress that may vary considerably between nuclei and mitochondria, depending on the growth state and stress environment of the cell. Numerous examples exist where cells respond to signals resulting in relocalization of proteins involved in key biological transactions. To address whether such dynamic localization contributes to efficient organelle-specific DNA repair, we determined the intracellular localization of the *Saccharomyces cerevisiae* N-glycosylase/AP lyases, Ntg1 and Ntg2, in response to nuclear and mitochondrial oxidative stress. Fluorescence microscopy revealed that Ntg1 is differentially localized to nuclei and mitochondria likely in response to the oxidative DNA damage status of the organelle. Sumoylation is associated with targeting of Ntg1 to nuclei containing oxidative DNA damage. These studies demonstrate that trafficking of DNA repair proteins to organelles containing high levels of oxidative DNA damage may be a central point for regulating BER in response to oxidative stress.

2. Introduction

Oxidative DNA damage, which occurs frequently in all cells, is linked to aging and human disease, such as cancer and various degenerative disorders (6, 13, 45, 81, 83). Reactive oxygen species (ROS) are a byproduct of normal cellular metabolic processes that can cause oxidative damage to DNA, lipids, and proteins (79). Unrepaired oxidative DNA lesions can result in mutations and lead to arrest of both DNA replication and transcription (34). In order to combat such continuous insults to the genome, cells have evolved DNA repair and DNA damage tolerance pathways (2).

Base excision repair (BER) is the primary process by which oxidative DNA damage is repaired (74, 90). BER is initiated by the recognition and excision of a base lesion by an *N*-glycosylase resulting in an apurinic/apyrimidinic (AP) site (47, 48). The resulting AP site is processed by an AP endonuclease or an AP lyase, which cleaves the sugar-phosphate DNA backbone on the 5´ side or 3´ side of the AP site, respectively (5). Subsequent processing involving DNA repair polymerases replaces the excised nucleotides, and DNA ligase completes the repair process (8).

Very little is known about how eukaryotic cells regulate events that initiate BER in response to oxidative stress. Deleterious oxidative DNA damage can occur in both nuclear and mitochondrial genomes, adding a level of complexity to this cellular response. In this case, the intracellular localization of BER proteins would be dynamically regulated in response to the introduction of either nuclear or mitochondrial DNA damage. Controlled protein localization has been implicated in regulation of a number of critical cellular processes (24, 29, 37, 58). For example, under normal growth

conditions, the human c-Abl protein tyrosine kinase is cytoplasmic, but in response to cellular stress that results in DNA damage, c-Abl translocates into the nucleus, where it induces apoptosis (88). Yap1 is a critical transcription factor in the oxidative stress response in budding yeast that is imported from the cytoplasm into the nucleus, where it regulates many stress-response genes in response to oxidative stress (43). Human DJ-1 protein, mutations in which are implicated in Parkinson's disease (9), translocates to mitochondria following oxidative stress in order to protect against cytotoxicity (11, 46). As subcellular localization is a regulatory component of numerous non-DNA repair pathways, it is possible that DNA repair is regulated in a similar manner.

If subcellular localization of BER proteins is regulated, then such events might be modulated through post-translational modification. Phosphorylation, myristoylation, and numerous other modifications affect nuclear localization of certain proteins, such as c-Abl, FoxO proteins, and p53 proteins (10, 71, 88). Another post-translational modification that has been implicated in modulation of intracellular localization, especially of nuclear proteins, is modification by the ubiquitin-like protein, SUMO (22, 30). Several proteins involved in DNA repair and maintenance are sumoylated, conferring a range of functions (55). For example, sumoylation of human thymine DNA glycosylase affects its glycosylase activity and localization to sub-nuclear regions (28, 54). SUMO modification also affects the nuclear localization of proteins such as mammalian heat shock transcription factor (HSF1) and the repressor of transcription, TEL, in response to environmental stress (27, 33, 42, 82). Sumoylated HSF1 colocalizes with nuclear stress granules, facilitating transcription of specific heat-shock genes (33). SUMO modification of TEL is required for TEL export from the nucleus in response to

cellular stresses, such as heat shock and exposure to UV radiation (27). Thus, SUMO modification is a major mechanism for regulation of subcellular protein localization.

The budding yeast, *Saccharomyces cerevisiae,* has been utilized extensively to investigate the mechanisms that underlie DNA repair as the DNA damage management pathways are conserved between yeast and humans (18, 53). To determine whether targeting of BER proteins to the appropriate organelle harboring oxidative DNA damage is likely to represent a general regulatory component of DNA repair, we evaluated the localization of BER proteins in response to oxidative stress. This study focused on the *S. cerevisiae* BER proteins, Ntg1 and Ntg2, which are both homologs of *Escherichia coli* endonuclease III, possessing N-glycosylase/AP lyase activity that allows recognition and repair of oxidative base damage (primarily pyrimidines) as well as abasic sites (3, 25, 73, 89). Because Ntg1 and Ntg2 play an important role in the repair of oxidative DNA damage in *S. cerevisiae,* the aim of these studies was to determine how oxidative stress and sumoylation influence subcellular localization of these proteins. Consistent with the presence of predicted nuclear localization signals (NLS) and a mitochondrial targeting sequence (MTS) (4, 89), Ntg1 is found in both the nucleus and mitochondria (1, 90). In contrast, Ntg2, which contains only a putative NLS but no MTS, is localized exclusively to the nucleus (1, 90).

In this study, we evaluated the localization of Ntg1 and Ntg2 following exposure to nuclear and/or mitochondrial oxidative stress. Results show that the localization of Ntg1 is dynamically regulated in response to nuclear and mitochondrial oxidative stress. However, Ntg2 remains nuclear regardless of the oxidative stress state of the cell. Importantly, we provide evidence that dynamic localization of Ntg1 is a response to DNA

damage rather than a general response to ROS. Additionally, sumoylation is associated with nuclear localization of Ntg1 that occurs in response to oxidative stress. These results indicate that the localization of BER proteins can likely be regulated by the introduction of nuclear and mitochondrial oxidative DNA damage and suggest that sumoylation plays a role in modulating the localization of BER proteins.

3. Materials and Methods

3.1 Strains, Media, and Growth Conditions. Haploid *S. cerevisiae* strains and all plasmids used in this study are listed in Table 1. Yeast cells were cultured at 30° C in rich YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 0.005% adenine sulfate, and 2% agar for plates) or YPGal medium (1% yeast extract, 2% peptone, 2% galactose, 0.005% adenine sulfate, and 2% agar for plates). In order to introduce plasmids or integrated chromosomal gene modifications, yeast cells were transformed by a modified lithium acetate method (35).

The pPS904 green fluorescent protein (GFP) expression vector (2 micron, *URA3*) was employed for generation of C-terminally tagged Ntg1-GFP and Ntg2-GFP fusion proteins (41). The *S. cerevisiae* haploid strain FY86 was utilized for all localization studies (84). Δ*NTG1* and Δ*NTG2* strains (DSC0282 and DSC0283) were generated by precisely replacing the *NTG1* or *NTG2* open reading frames in FY86 with the kanamycin antibiotic resistance gene (pFA-KMX4 (80), selected with 150 mg/L G418 [US Biological]) or blasticidin antibiotic resistance gene (Invitrogen, BsdCassette™ vector pTEF1/Bsd 3.6 kb, selected with 100 mg/L blasticidin S HCl [Invitrogen]), respectively.

Plasmids encoding Ntg1-GFP or Ntg2-GFP were introduced into Δ*NTG1* or Δ*NTG2* cells. Plasmid mutagenesis of Ntg1-GFP to create Ntg1 K364R-GFP was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene), and these plasmids were introduced into Δ*NTG1* cells.

For studies of cells lacking mitochondrial DNA, a rho^0 yeast strain (DSC0291) was generated by incubating 4 x 10^6 Δ*NTG1* cells in ethidium bromide as previously described (17). Following this incubation, cells were stained with 4' 6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) and MitoTracker Red CMXRos stain (Invitrogen) and evaluated via fluorescence microscopy in order to verify that no mitochondrial DNA was present.

Haploid yeast strains expressing integrated genomic copies of C-terminally tandem affinity purification (TAP)- tagged Ntg1 and Ntg2 were obtained from Open Biosystems [Ntg1-TAP (DSC0297); Ntg2-TAP (DSC0298)]. A tetracycline repressible promoter (tet off) from the plasmid, pCM225, was integrated at the N-terminus of the *NTG1* and *NTG2* genes using the kanamycin resistance gene to generate tetracycline-repressible Ntg1-TAP and tetracycline-repressible Ntg2-TAP strains (DSC0295 and DSC0296) as previously described (7). Cells expressing galactose inducible Smt3-HA and Ntg1-GST (DSC0221) or Smt3-HA and Ntg2-GST (DSC0222) were generated by integrating the HA tag from the vector, p1375, and the *GAL* promoter and the GST tag from the vector, p2245 (51), at the C-terminus of *SMT3* and *NTG1* or *NTG2* in the haploid strain ACY737 (70). ACY737 contains mutations in the sumoylation deconjugating enzymes, Ulp1 and Ulp2, which can aid in the isolation of sumoylated proteins (70).

3.2 Exposure to DNA Damaging Agents. Cells were grown in 5 mL YPD to a density of 5 x 10^7 cells/mL, centrifuged, and washed with water. Cells were then resuspended in 5 mL water containing the appropriate DNA damaging agent: 2-20 mM H_2O_2 (Sigma), 25-55 mM methyl methanesulfonate (Sigma), or 10 μg/mL antimycin A (Sigma). Cells were exposed to agent(s) for one hour at 30° C. Cytotoxicities of agents were evaluated by growing cells in agent, plating cells, and counting colonies.

3.3 Fluorescence Microscopy. For all experiments, cells (grown and treated as described above) were treated as follows: no treatment, 5 mM H_2O_2, 10 mM H_2O_2, 20 mM H_2O_2, 25 mM MMS, 55 mM MMS, 10 μg/mL antimycin, 5 mM H_2O_2 plus 10 μg/mL antimycin, 10 mM H_2O_2 plus 10 μg/mL antimycin, or 20 mM H_2O_2 plus 10 μg/mL antimycin. During exposure to DNA damaging agents, cultures were also incubated with 25 nM MitoTracker in order to visualize mitochondria. Following washes, cells were placed in 1 mL of water containing 1 μg DAPI to visualize DNA and incubated for 5 minutes at room temperature. Cells were washed and analyzed by direct fluorescence confocal microscopy, employing a Zeiss LSM510 META microscope. Images were analyzed using the Carl Zeiss LSM Image Browser software, and cells were evaluated for nuclear only or nuclear plus mitochondrial Ntg1-GFP or Ntg2-GFP localization. Mitochondrial only localization was negligible. At least 200 cells were counted for each strain and treatment condition, and each microscopic evaluation was repeated at least twice. Standard deviations were calculated for each strain and treatment condition. The image analysis software program, Metamorph 6.2, was utilized in order to quantify the intensities of GFP in nuclei and mitochondria of individual cells. Mitochondrial GFP intensities were determined by subtracting nuclear GFP intensity

from the total cellular GFP intensity. The fraction of cells with a mitochondrial GFP intensity score higher than 500 was determined for cells exposed to H_2O_2 and H_2O_2 plus antimycin, and the t-test was employed to determine p values.

3.4 Measurement of ROS Levels by Flow Cytometry. For all experiments, cells were grown and treated as described above. Following exposure to DNA damaging agents, cells were washed with water and resuspended in YPD at a density of 2 x 10^7 cells/mL. Dihydroethidium (DHEt) was added to the YPD to a concentration of 160 μM to detect cellular superoxide (67); MitoSox (Molecular Probes) was added to the YPD to a concentration of 5 μM as per manufacturer's instructions to detect mitochondrial superoxide; or cells were left untreated. Cells were incubated for 45 minutes in the fluorescent dye, washed, and resuspended in 2 mL phosphate buffered saline (PBS). Fluorescence intensity of 10,000 cells for each strain and condition was assessed by employing a BD™ LSR II flow cytometer (BD Biosciences). Excitation and emission wavelengths employed to evaluate cells were 488 nm and 595 nm, respectively, for DHEt and 488 nm and 575 nm, respectively, for MitoSox.

3.5 Ntg1 and Ntg2 Analysis. Purification of TAP-tagged Ntg1 and Ntg2 was achieved as follows. Four liters of tetracycline-repressible Ntg1-TAP (DSC0295) and Ntg2-TAP (DSC0296) were grown in YPD to a density of 5 x 10^7 cells/mL without tetracycline in order to overproduce Ntg1-TAP and Ntg2-TAP. Cells were then pelleted and washed with water. Cell pellets were frozen at -80° C. A version of the previously published tandem affinity purification was utilized (63) with the following modifications. Cell pellets were crushed with a mortar and pestle, and powdered yeast lysate was suspended in 10 mL buffer A (10 mM K-HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$,

0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM benzamindine, 1 μM leupeptin, 2.6 μM aprotinin) with 5 mM N-ethylmaleimide (NEM) (Sigma), 10 mM iodoacetamide (Sigma). Lysate was centrifuged at 3000 x g for 10 minutes, and supernatant was re-centrifuged at 88,000 x g for 1 and a half hour. Dialysis, incubation with IgG beads, and incubation with AcTEV protease was performed as instructed. Western analysis was performed on 50 μL of eluate. Anti-TAP antibody (Open Biosystems, 1:3333 dilution) was employed for Western analysis. Ntg2-TAP migrates as a slightly smaller species in Western analysis than the predicted TAP-tagged Ntg2 (~53 kDa), while Ntg1-TAP migrates at its expected size of 55 kDa.

In order to purify GST-tagged Ntg1 and Ntg2, one liter of cells expressing galactose-inducible Smt3-HA and Ntg1-GST (DSC0221) or galactose-inducible Smt3-HA and Ntg2-GST (DSC0222) was grown for each strain in YPGal to a density of 5 x 10^7 cells/mL. Cells were centrifuged, and pellets were washed and frozen at -80° C. Cell pellets were crushed with a mortar, and powdered lysate was suspended in 500 μL PBS with 0.5 mM PMSF and 3 μg/mL each leupeptin and aprotinin. Lysate was centrifuged at 3000 x g for 10 minutes, and supernatant was applied to 150 μL washed Glutathione Sepharose™ 4 Fast Flow beads (Amersham Biosciences). Beads and lysate were incubated at 4° C overnight. Beads were washed 3 times with 1 mL PBS, and then 50 μL of 1 x SDS-PAGE loading buffer (50% w/v glycerol, 10% w/v SDS, 1% v/v 2-mercaptoethanol, 0.1 M Tris-HCl, pH 6.8, 0.1% bromophenol blue) was applied to beads to elute the bound fraction. Western analysis was performed on 20 μL of eluate. Anti-HA (1:1000 dilution, Covance) and anti-GST antibodies (1:1000 dilution, Oncogene) were employed for immunoblotting.

3.6 Sucrose Gradient Subcellular Fractionation. In order to fractionate yeast cells into nuclear and mitochondrial preparations, one liter of cells expressing tetracycline- repressible Ntg1-TAP (DSC0295) or tetracycline-repressible Ntg2-TAP (DSC0296) was grown in YPD to a density of 5 x 10^7 cells/mL without tetracycline in order to overproduce Ntg1-TAP and Ntg2-TAP. Crude mitochondrial and nuclear protein lysate fractions were generated using a differential centrifugation protocol as described previously (14). Following this procedure, mitochondrial fractions were further purified using sucrose gradient centrifugation (61). Solutions of 20%, 40%, and 60% (w/v) sucrose in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1 mM PMSF were prepared. The crude mitochondrial pellet was resuspended in the 20% sucrose solution. The 60% sucrose solution was placed in the bottom of a Beckman Ultraclear centrifuge tube, followed by the 40% sucrose solution and the 20% sucrose solution containing mitochondria. The tubes were centrifuged at 100,000 x g for 1 hour at 4° C. The mitochondria were removed from the 40%/60% interface, concentrated, resuspended in storage buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4, 20% glycerol, 5 mM NEM, 10 mM iodacetamide, 5 mg/ml aprotinin, 5 mg/ml leupeptin, and 0.1 M PMSF), and stored at -80°C.

The crude nuclear pellets were further purified using sucrose step gradient purification as previously described (66). The step gradient contained solutions of 58.2%, 68.8%, 71.9%, and 78.7% sucrose in sucrose buffer (8% PVP-40, 11.5 mM KH_2PO_4, 8.4 mM K_2HPO_4, 0.75 mM $MgCl_2$, pH 6.53). The nuclei were removed from the 71.9%/78.7% sucrose interface, concentrated and resuspended in storage buffer. Western analysis was performed using 10 μg of nuclear or mitochondrial protein lysate.

Anti-TAP, anti-Por1 (1:25,000 dilution, MitoSciences), and anti-Nop1 (1:25,000 dilution, EnCor) antibodies were employed for Western analysis. Anti-Nop1 and anti-Por1 antibodies were used to ensure enrichment of nuclear (Nop1) or mitochondrial (Por1) fractions (62). To optimize visualization, Western blot exposures were variable for each protein analyzed. Analysis of Western blots by chemiluminescence was employed in order to determine the fold-change of sumoylated Ntg1 in the nuclear fraction. The ratio of modified to unmodified Ntg1 in the nuclear fraction was determined, and values for each condition were normalized to the no treatment condition. Standard error of the mean was calculated for each strain and treatment condition, and the t-test was employed to determine p values.

***3.7 Functional Analysis of Ntg1* in vivo.** To assess the function of Ntg1 *in vivo*, we utilized BER-/NER- (*ntg1 ntg2 apn1 rad1)* cells (SJR1101/DSC0051) that are highly sensitive to oxidative stress (75). BER-/NER- cells containing Ntg1-GFP or Ntg1 K364R-GFP plasmids were assessed for the ability of the episomal Ntg1 to function *in vivo* and decrease the sensitivity of these cells to treatment with H_2O_2. Cytotoxicity assays were carried out as described above for exposure to DNA damaging agents. The steady-state level of each GFP fusion protein was assessed by immunoblotting whole cell lysates with a polyclonal anti-GFP antibody (1:5,000 dilution) (72). Anti-phosphoglycerate kinase (PGK) antibody (1:5,000 dilution, Invitrogen) was utilized to determine the relative levels of protein loaded per lane.

4. Results

4.1 Ntg1 and Ntg2 localization under normal growth and oxygen environments. To determine whether changes in the subcellular distribution of Ntg1 and Ntg2 occur in response to oxidative stress-induced DNA damage, the localization of Ntg1-GFP and Ntg2-GFP was evaluated in live yeast cells. Under normal growth conditions, Ntg1 is localized to both nuclei and mitochondria, while Ntg2 localization is exclusively nuclear (1, 90). This localization pattern was verified by analyzing cells expressing either Ntg1-GFP or Ntg2-GFP using direct fluorescence microscopy. As expected, Ntg1-GFP was localized to both nuclei and mitochondria (Figure 1A); whereas, Ntg2-GFP localization was strictly nuclear (Figure 1B). To biochemically confirm the organellar distribution of Ntg1 and Ntg2, sucrose gradient subcellular fractionation (Materials and Methods) was performed on lysates from cells expressing Ntg1-TAP or Ntg2-TAP to separate nuclear and mitochondrial fractions. Nuclear and mitochondrial protein lysate fractions were evaluated for purity using antibodies against a nuclear protein, Nop1, or a mitochondrial membrane protein, Por1 (62). Mitochondrial fractions were free of nuclear proteins as indicated by the detection of Por1 but not Nop1 (Figure 1C, D). Nuclear fractions were enriched for nuclear proteins with some mitochondrial contamination (Figure 1C, D). These results were expected as cytoplasmic contaminants have been routinely documented in conjunction with nuclear fractionation of *S. cerevisiae* (57, 65, 91). The localization of Ntg1-TAP and Ntg2-TAP was determined by probing nuclear and mitochondrial fractions. Sucrose gradient subcellular fractionation verified that Ntg1 was present in both nuclear and mitochondrial fractions (Figure 1C), and Ntg2 was detected only in nuclear fractions (Figure 1D).

4.2 Nuclear and mitochondrial oxidative stress induction by hydrogen peroxide (H_2O_2), antimycin, and methyl methanesulfonate (MMS). ROS levels increase in response to DNA damage in cells exposed to genotoxic agents including MMS, ultraviolet (UV) light, and H_2O_2 (19, 67, 68). In order to determine whether increased ROS levels influence the localization of Ntg1 and Ntg2, wild type cells were exposed to H_2O_2 to directly increase oxidative stress or the DNA alkylating agent, MMS, to indirectly increase ROS levels in response to DNA damage (68). In addition, cells were exposed to H_2O_2 plus antimycin to increase mitochondrial oxidative stress. Antimycin blocks oxidative phosphorylation (60), and exposure of cells to H_2O_2 plus antimycin increases oxidative stress, leading to induced oxidative DNA damage in yeast mitochondria (17). The relative levels of cellular ROS in different cellular compartments were determined following exposure to H_2O_2, H_2O_2 plus antimycin, and MMS using the fluorescent probes, dihydroethidum (DHEt) and MitoSox. DHEt is a general cellular superoxide probe (78); whereas, MitoSox accumulates in the mitochondrial matrix, allowing determination of superoxide levels specifically in mitochondria (38). Analysis of cells by flow cytometry revealed that H_2O_2 exposure resulted in elevated levels of cellular superoxide compared to unexposed cells (Figure 2A), but did not increase levels of mitochondrial superoxide (Figure 2B). Flow cytometry analysis also revealed that H_2O_2 plus antimycin exposure resulted in a general cellular increase in superoxide levels including an increase in mitochondrial superoxide, revealed by both DHEt and Mitosox fluorescent probes (Figure 2A, B). Exposure of cells to non-oxidative DNA damaging agents, such as MMS and UV light, can also increase cellular ROS levels (19, 67, 68). Consistent with this observation, exposure of cells to MMS resulted in a substantial

elevation in both total cellular and mitochondrial superoxide levels when compared to untreated controls (Figure 2C, D). Evaluation of mitochondrial superoxide levels following treatment with MMS revealed two subpopulations of cells, each containing levels of mitochondrial superoxide higher than those observed with no treatment (Figure 2D). These two subpopulations may represent cell stress and death response groups. Collectively, these results demonstrate that oxidative stress can be targeted to nuclei or mitochondria by exposure to specific agents. Importantly, a combination of H_2O_2 and antimycin or MMS exposure induces mitochondrial oxidative stress in a manner that is distinct from the primarily nuclear oxidative stress that results from exposure to H_2O_2 alone.

4.3 Relocalization of Ntg1 in response to increased nuclear and mitochondrial oxidative stress. In order to assess whether the steady state localization of Ntg1 is altered in response to nuclear oxidative stress, Ntg1-GFP localization was evaluated before and after a one hour induction of oxidative stress with various concentrations of H_2O_2 (Figures 3A, B). The cytotoxicities for H_2O_2 exposures of 0 mM, 5 mM, 10 mM, and 20 mM were 0%, 64%, 68%, and 75%, respectively (data not shown). The localization of Ntg1-GFP was assessed by direct fluorescence microscopy. As shown in Figure 3B, Ntg1-GFP appears more enriched in nuclei upon exposure to H_2O_2. In order to provide a quantitative measure of Ntg1-GFP localization, the subcellular localization of Ntg1-GFP was designated as nuclear only or nuclear plus mitochondrial based on colocalization with nuclear DAPI, mitochondrial DAPI, and MitoTracker staining in all cells displaying a GFP signal. The percentage of cells with nuclear only or nuclear plus mitochondrial localization of Ntg1-GFP was determined for several hundred cells for each treatment

group. A dose-dependent increase in nuclear only Ntg1-GFP was observed following H_2O_2-induced nuclear oxidative stress (Figures 3D). This result correlated with a dose-dependent decrease in the number of cells with a nuclear plus mitochondrial distribution of Ntg1-GFP, reflecting a decrease in mitochondrial localization of Ntg1. These results suggest that Ntg1 can be targeted to nuclei in response to nuclear oxidative stress.

Oxidative stress can be induced in mitochondria by exposing cells to H_2O_2 in combination with antimycin (See Figure 2B), resulting in increased mitochondrial oxidative DNA damage (17). We treated cells with H_2O_2 plus antimycin to determine whether elevated mitochondrial ROS triggers increased localization of Ntg1 to mitochondria. As shown in Figure 3C, localization of Ntg1-GFP to mitochondria increased following H_2O_2 plus antimycin-induced mitochondrial oxidative stress. The intensity of the GFP signal located in mitochondria of cells exposed to H_2O_2 plus antimycin was statistically greater than the intensity of the GFP signal located in mitochondria of H_2O_2-induced cells, as determined via image analysis using the software program, Metamorph 6.2. Specifically, the fraction of cells containing a mitochondrial GFP intensity score higher than 500 was significantly greater for cells exposed to H_2O_2 plus antimycin (0.78 ± 0.08) than for cells exposed to H_2O_2 (0.63 ± 0.09) (p value = 0.04). These data indicate that Ntg1 localization is influenced by mitochondrial ROS. In addition to increased mitochondrial localization, Ntg1 nuclear localization was increased following exposure to low doses of H_2O_2 plus antimycin. H_2O_2 plus antimycin not only induces oxidative stress in mitochondria, but also nuclei, thus increasing Ntg1 nuclear localization as well (Figure 2A, B).

Under normal growth conditions, Ntg2 is localized exclusively to nuclei (1, 90). Therefore, it was of interest to determine whether Ntg2 localization was affected by nuclear or mitochondrial oxidative stress. The localization of Ntg2-GFP was examined following exposure to nuclear (H_2O_2) or mitochondrial (H_2O_2 plus antimycin) oxidative stress. Ntg2-GFP localization remained exclusively nuclear following either nuclear or mitochondrial oxidative stress (data not shown), indicating that Ntg2 is not responsive to changes in either nuclear or mitochondrial oxidative stress.

4.4 Relocalization of Ntg1 in response to MMS exposure. To determine whether other DNA damaging agents that do not directly cause oxidative DNA damage are also capable of inducing a change in the localization of Ntg1, cells were exposed to the DNA alkylating agent, MMS, resulting in an increase in intracellular ROS (references (68) and Figure 2C, D). Survival of cells treated with 0 mM, 25 mM, and 55 mM MMS was 100%, 30%, and 3%, respectively (data not shown). An increase in nuclear only localization of Ntg1-GFP was observed following exposure to MMS (Figure 3D) (p values < 0.04 when comparing nuclear only localization for no treatment and MMS exposures). This result indicates that in addition to the ability of Ntg1 to respond to oxidative stress caused by H_2O_2 exposure, Ntg1 also responds to oxidative stress caused by DNA damaging agents, such as MMS, that do not directly introduce oxidative DNA damage.

4.5 Oxidative stress-induced relocalization of Ntg1 to mitochondria is due to a DNA damage response. Oxidative stress could provoke a change in localization of Ntg1 via a direct response to elevated levels of ROS or in response to the presence of oxidative DNA damage. In order to distinguish between these possibilities, rho^0 cells were

generated as described in Materials and Methods. rho^0 mitochondria do not contain DNA, whereas rho^+ mitochondria contain intact DNA (20). The absence of mitochondrial DNA in rho^0 cells was confirmed by direct fluorescence microscopy as evidenced by the absence of any extranuclear DAPI staining (Figure 4A). In rho^0 cells, a lack of Ntg1 mitochondrial localization following increased mitochondrial oxidative stress (exposure to H_2O_2 plus antimycin) would indicate that Ntg1 responds to the presence of mitochondrial oxidative DNA damage rather than ROS. Flow cytometric analysis of rho^0 cells revealed that mitochondrial superoxide levels increased in response to H_2O_2 plus antimycin exposure (Figure 4B). Regardless of exposure to ROS-generating agents, fewer rho^0 mitochondria contained Ntg1-GFP than rho^+ mitochondria as determined by colocalization of GFP with Mitotracker (Figure 4A) and quantification of cells with nuclear or nuclear plus mitochondrial GFP-Ntg1 localization (Figure 4C). Results indicate that rho^0 cells subjected to increasing levels of mitochondrial oxidative stress did not exhibit a change in Ntg1 localization (Figure 4C). In contrast, rho^+ cells subjected to the same mitochondrial oxidative stress conditions displayed a significant increase in mitochondrial localization of Ntg1. Exposure of rho^+ cells to H_2O_2 plus antimycin results in increased mitochondrial oxidative DNA damage (17) caused by increased mitochondrial ROS (See Figure 2). The difference in Ntg1 localization observed between rho^0 and rho^+ cells indicates that mitochondrial oxidative stress induces DNA damage that results in the relocalization of Ntg1 to mitochondria. Importantly, these data suggest that the mitochondrial localization of Ntg1 is directed by the presence of mitochondrial oxidative DNA damage and not simply by elevated levels of mitochondrial ROS.

4.6 Ntg1 and Ntg2 are post-translationally modified by sumoylation. Post-translational modification of various proteins via sumoylation can direct subcellular localization in response to environmental signals (27, 33, 42, 82). Several lines of evidence indicate that Ntg1 and Ntg2 may be post-translationally modified by SUMO. Ntg1 and Ntg2 contain seven and one putative sumoylation sites (Figure 5), respectively, as predicted using the SUMO prediction program, SUMOsp 1.0 (64, 85). Cell lysates from yeast that express Ntg1-GFP and Ntg2-GFP reveal a major species corresponding to the size of the fusion protein and a second, minor species of higher molecular size corresponding to the predicted size for mono-sumoylated Ntg1 and Ntg2 (90). In addition, a recent study cataloging sumoylated yeast proteins reported that Ntg1 interacts with Smt3 (26), which encodes the yeast SUMO (16, 40, 52); however, covalent modification of Ntg1 by Smt3 was not assessed in that study.

In order to test for sumoylation of Ntg1 and Ntg2, we looked for the presence of high molecular weight forms of Ntg1 and Ntg2. Western analysis of TAP purified Ntg1 and Ntg2 revealed species corresponding to the size of Ntg1-TAP (55 kDa) and mono-sumoylated Ntg1-TAP (70 kDa) as well as Ntg2-TAP (46 kDa) and mono-sumoylated Ntg2 (58 kDa) (Figure 6A, B). The size of the higher molecular weight species corresponds to the size predicted for addition of a single SUMO moiety (12 kDa) to both Ntg1 and Ntg2. To determine whether Smt3 is covalently attached to Ntg1 and Ntg2, GST-tagged Ntg1 and Ntg2 were purified from cells expressing both GST-tagged Ntg proteins and HA-tagged Smt3. Detection of the same high molecular weight species with both GST and HA antibodies would reveal covalent modification of Ntg1 and Ntg2. Western analysis confirmed the covalent modification of Ntg1 and Ntg2 by SUMO as

indicated by the co-detection of a high molecular weight species by both GST and HA antibodies (Figure 6C, D). Collectively, these results are consistent with the conclusion that both Ntg1 and Ntg2 are post-translationally modified by sumoylation.

4.7 Sumoylated Ntg1 accumulates in the nucleus following oxidative stress. To address whether sumoylation could play a role in the subcellular localization of Ntg1, cells expressing TAP-tagged Ntg1 were exposed to H_2O_2 (nuclear oxidative stress) or H_2O_2 plus antimycin (mitochondrial oxidative stress) and subjected to sucrose gradient subcellular fractionation (Materials and Methods). Mitochondrial fractions were free of nuclear proteins as determined by Western analysis using Por1 and Nop1 as mitochondrial and nuclear protein markers (62), respectively; whereas, nuclear fractions were enriched for nuclear proteins (Figure 7A, B). Sumoylated Ntg1 was detected in nuclei and increased in amount relative to unmodified Ntg1-TAP following both nuclear and mitochondrial oxidative stress (Figure 7A, B). Analysis of sumoylated and non-sumoylated Ntg1-TAP in nuclear fractions by chemiluminescence revealed that exposure to oxidative stress results in an approximately five-fold increase in nuclear sumoylated Ntg1 (Figure 7C). These results suggest that sumoylation of Ntg1 is associated with the nuclear localization of Ntg1 in response to oxidative stress.

4.8 Subcellular localization and function of mutant Ntg1 lacking a predicted SUMO site. The sumoylation prediction program, SUMOsp 1.0 (85), was utilized to determine lysine residues where Ntg1 is most likely to be sumoylated. SUMOsp 1.0 predicted that lysine 364 within the sequence, KREL, was most likely to be sumoylated among the 36 lysines present in Ntg1. To assess the possible requirement for lysine 364 in Ntg1 function, lysine 364 was replaced with arginine. This amino acid substitution

retains the positive charge of the residue, but blocks sumoylation (32). In order to determine whether sumoylation affects the subcellular distribution of Ntg1, the intracellular localization of Ntg1 K364R-GFP was compared to that of wild type Ntg1-GFP. Ntg1 K364R-GFP was localized to both nuclei and mitochondria (Figure 8A); however, the relocalization of Ntg1 K364R-GFP in response to H_2O_2 exposure (nuclear oxidative stress) or MMS exposure (Figure 8B) was markedly different from that of wild type Ntg1-GFP (See Figure 3). Specifically, the fraction of nuclear only Ntg1 K364R-GFP decreased in response to either H_2O_2 or MMS exposures, while the nuclear only localization of wild type Ntg1-GFP increased in response to both agents (compare Figures 3D and 8B). These results indicate that the predicted Ntg1 sumoylation site, K364, is important for relocalization of Ntg1 in response to nuclear oxidative stress, likely resulting in oxidative DNA damage, and provide further evidence of a role for SUMO in the dynamic localization of Ntg1.

In order to assess the function of K364R Ntg1, which cannot properly relocalize in response to oxidative stress, we exploited BER-/NER- (*ntg1 ntg2 apn1 rad1*) defective cells (75). These BER-/NER- defective cells are severely compromised for the repair of oxidative DNA damage and are highly sensitive to H_2O_2 (75). Importantly, these cells lack endogenous Ntg1 so that the function of K364R Ntg1 could be assessed as the only cellular copy of Ntg1. For this experiment, plasmids encoding wild type Ntg1-GFP or Ntg1 K364R-GFP were transformed into BER-/NER- cells, and the sensitivity of these cells to H_2O_2 was determined (Figure 8C). An episomal copy of wild type Ntg1 substantially increased cell survival following H_2O_2 exposure compared to BER-/NER- cells. In contrast, following H_2O_2 treatment, the survival of BER-/NER- cells expressing

an episomal copy of K364R Ntg1 was comparable to or less than the survival of the control BER-/NER- cells demonstrating that K364R Ntg1 is not properly localized *in vivo* to mediate its DNA repair function in the nucleus. To ensure that the compromised function of K364R Ntg1 was not due to decreased expression of the mutant protein, we assessed the steady-state level of both Ntg1-GFP and Ntg1 K364R-GFP in cell lysates by immunoblotting with an anti-GFP antibody (Figure 8D). This analysis revealed that the level of K364R Ntg1 was equivalent to wild type Ntg1. These results suggest that the predicted Ntg1 sumoylation site, K364, is important for the function of Ntg1 in conferring cellular survival following oxidative stress. In follow-up studies, BER-/NER- cells expressing an integrated copy of K364R Ntg1 displayed similar survival as BER-/NER- cells expressing wild type Ntg1 (data not shown). The difference between episomal and integrated K364R cells is likely due to a copy number effect, where K364R Ntg1 is expressed at higher levels in the cells expressing K364R Ntg1 from a plasmid. These results may indicate that K364 is not important for cellular survival in the presence of oxidative stress.

5. Discussion

To gain insight into the regulation of BER in response to oxidative stress-induced DNA damage, the localization and post-translational modification of *S. cerevisiae* Ntg1 and Ntg2 were evaluated. We demonstrate that Ntg1 relocalizes in response to both nuclear and mitochondrial oxidative DNA damage. In contrast, Ntg2 is exclusively nuclear, and this localization does not change in response to oxidative DNA damage.

Furthermore, sumoylation of Ntg1 is associated with nuclear localization in response to nuclear oxidative stress.

ROS are a byproduct of environmental factors and important cellular processes, including oxidative phosphorylation. Nuclear and mitochondrial oxidative stress occurs due to inefficiencies and malfunctions of these processes. Furthermore, increased nuclear and mitochondrial oxidative stress have been observed in cells with compromised nuclear and mitochondrial ROS scavenging systems (36, 50). Mitochondrial oxidative stress is increased when cellular oxidative phosphorylation activity is particularly high or disrupted (79). Furthermore, aging and various diseases have been associated with increased nuclear and mitochondrial ROS levels (13, 45, 81, 83). Under conditions where nuclear oxidative stress is high, nuclear oxidative DNA damage is elevated (19, 86). Likewise, conditions that increase mitochondrial oxidative stress are associated with high levels of mitochondrial oxidative DNA damage (17, 79). When oxidative stress is increased, it is essential for the cell to respond to oxidative DNA damage rapidly in order to prevent the detrimental consequences of unrepaired DNA, and a rapid response to oxidative DNA damage requires explicit regulation of BER components.

Regulating the localization of proteins is a way for cells to respond quickly to a stimulus without having to produce more protein. Because localization is a significant component of regulation for many processes (24, 29, 37, 58), we evaluated the localization of the BER proteins, Ntg1 and Ntg2, in response to oxidative stress and determined that the localization of Ntg1 is influenced by nuclear and mitochondrial oxidative stress, whether the stress is caused by an oxidizing agent (H_2O_2) or indirectly by MMS, a non-oxidative DNA alkylating agent (Figure 3). In addition to Ntg1, the

human transcription factor/ AP endonuclease, Ref1/ hAPE, was previously reported to translocate to nuclei and mitochondria following exposure to a DNA damaging agent (15, 21, 76, 77), adding further credibility to our claim that dynamic localization is a mechanism for regulation of BER in eukaryotic systems in general. In addition to demonstrating the dynamic localization of Ntg1, we were able to delineate the origin of the signal that results in targeting of Ntg1 to mitochondria by utilizing rho^0 yeast cells. Specifically, we demonstrated that Ntg1 responds to mitochondrial oxidative DNA damage and not simply elevated levels of ROS (Figure 4). To our knowledge, this is the first experimental strategy that has been able to distinguish between protein localization caused by ROS and its DNA damage products. We hypothesize that the nuclear localization of Ntg1 is similarly controlled by high levels of nuclear oxidative DNA damage. Because the localization of several human DNA repair proteins is affected by oxidative stress (15, 21, 49, 76, 77, 87, 88), and BER is highly conserved between *S. cerevisiae* and humans, we suggest that modulation of DNA repair protein localization is a general mechanism by which BER is regulated in eukaryotic cells. We propose a model in which BER proteins such as Ntg1 are located in nuclei and mitochondria in cells under normal growth and oxygen environments (Figure 9). When nuclear and mitochondrial oxidative stress occur, nuclear and mitochondrial oxidative DNA damage result. We hypothesize that specific signals are generated in response to oxidative DNA damage that target BER proteins such as Ntg1 to nuclei and mitochondria in order to increase the capacity to repair these lesions rapidly.

Nuclear oxidative DNA damage signals (NODDS) and mitochondrial oxidative DNA damage signals (MODDS) are likely to involve various proteins and pathways

including components of the BER pathway, components of other DNA damage management pathways, and other molecules that are involved in cellular stress responses. As oxidative DNA damage can be spontaneously produced, other types of spontaneous DNA damage, such as alkylation, methylation, deamination, and depurination, could alter the subcellular localization of BER proteins through signals similar to NODDS and MODDS. Our observation that Ntg1 relocalizes in response to MMS supports the idea that a variety of spontaneous DNA damage can trigger recruitment of BER proteins. The signals from non-oxidative species of spontaneous DNA damage could recruit BER proteins directly or indirectly. Using alkylation as an example, abasic sites generated during repair of the alkylation damage may signal for recruitment of BER proteins directly, or the ROS produced as a result of the alkylation damage may cause oxidative DNA damage which, in turn, recruits BER proteins through NODDS and MODDS.

We hypothesize that components of the sumoylation pathway are NODDS molecules. Several DNA repair and other DNA maintenance proteins are sumoylated (55), and sumoylation has been implicated in the nuclear localization of a number of proteins (59). Our results indicate that Ntg1 and Ntg2 are post-translationally modified by sumoylation and are consistent with a model where sumoylation plays a role in the localization of Ntg1 to the nucleus in response to increased nuclear oxidative stress (Figures 6 and 7). A 5-fold increase in nuclear sumoylated Ntg1 was observed following oxidative stress. We find that 1% of the Ntg1 pool is sumoylated in cells under normal conditions, increasing to 5% in cells exposed to oxidative stress. These results are consistent with data describing other sumoylated proteins where often less than 1% of the substrate can be detected as sumoylated at any given time (39). Sumoylation of a human

BER N-glycosylase, thymine-DNA glycosylase (TDG), has been hypothesized to occur in a cyclical pattern of sumoylation and de-sumoylation (28, 39). In this case, sumoylated TDG promotes a single event whose consequences persist after de-sumoylation (28). We hypothesize that sumoylation of Ntg1 occurs in order to concentrate Ntg1 within nuclei, but de-sumoylation occurs very quickly, making it very difficult to detect the pool of sumoylated Ntg1. Furthermore, we provide evidence that Ntg1 K364 is a potential target site of sumoylation that may contribute to the nuclear localization and function of Ntg1, although further experimentation is necessary to confirm this notion (Figure 8). We predict that other nuclear BER proteins may be sumoylated in order to allow intricate regulation of DNA repair protein localization. The function of sumoylated Ntg2 is unknown, but it is possible that SUMO plays a role in modulating the intranuclear localization of Ntg2.

SUMO could contribute to the localization of BER proteins in several ways. Sumoylation of Ntg1 and other BER proteins could modulate interactions with nuclear transport receptors, as sumoylation modulates interaction with the nuclear transport receptors for various proteins (59). Sumoylation could also block BER proteins from exiting the nucleus in the event of oxidative DNA damage (Figure 9). Sumoylation is also implicated in the regulation of sub-nuclear localization of numerous proteins. Localization of proteins to nucleoli, promyelocytic leukemia nuclear (PML) bodies, and other sub-nuclear locations is associated with sumoylation (22, 30). Therefore, sumoylation could allow Ntg1 and other BER proteins to accumulate in certain sub-nuclear, sub-genomic regions containing oxidative DNA damage.

As Ntg1 localizes to both nuclei and mitochondria, the proportion of the pool of Ntg1 that localizes to each organelle must be adjusted so that some level of repair is maintained in nuclei and mitochondria at all times. Various factors are likely to influence the localization of Ntg1 to nuclei or mitochondria. Yeast mitochondrial DNA contains two to three times more oxidative lesions than nuclear DNA following oxidative stress induced by various agents (69). When more Ntg1 is needed in mitochondria, relocalization diminishes nuclear Ntg1 pools. Nuclear DNA will not significantly accumulate DNA damage in the absence of Ntg1 because nuclear Ntg2, NER proteins, and Apn1 are available to repair baseline levels of oxidative DNA damage (75). Yeast mitochondria do not contain Ntg2 or NER proteins, leaving mitochondrial DNA vulnerable in the absence of Ntg1 (12). Because of the numerous factors influencing Ntg1 localization, a careful balance of NODDS and MODDS is required in order to increase repair capacity in one organelle without diminishing repair in the other. Such a balance of NODDS and MODDS is illustrated in rho^0 cells, where unexposed rho^0 cells display increased nuclear Ntg1 localization compared to unexposed rho^+ cells (Figure 4C). We speculate that the increased nuclear localization results from elimination of MODDS-mediated recruitment of Ntg1 to mitochondria that results in enhanced recruitment of Ntg1 to nuclei by NODDS.

Very few investigations have addressed the issue of dynamic localization of BER proteins in the process of initiating BER in response to oxidative stress. Our studies have uncovered what is likely to be a major component of the regulation of BER. By controlling the subcellular localization of BER proteins, cells can rapidly mobilize repair machinery to sites of oxidative DNA damage.

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Table 1: Strains and Plasmids Used in this Study

Strain or Plasmid	Description	References
FY86 (ACY193)	*MATa ura3-52 leu2Δ1 his3Δ200*	(90)
ACY737	*MATa ulp1ts ulp2Δ lys2 trp1*	(70)
SJR751 (DSC0025)	*MATα ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R*	(75)
SJR1101 (DSC0051)	*MATα ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1Δ::HIS3 rad1Δ::hisG*	(75)
DSC0221	*MATa ulp1ts ulp2Δ lys2 trp1 GAL-HA-SMT3 GAL-GST-NTG1*	This study
DSC0222	*MATa ulp1ts ulp2Δ lys2 trp1 GAL-HA-SMT3 GAL-GST-NTG2*	This study
DSC0282	*MATa ura3-52 leu2Δ1 his3Δ200 ntg1::KANAMYCIN bar1::HYG*	This study
DSC0283	*MATa ura3-52 leu2Δ1 his3Δ200 ntg2::BLASTICIDIN bar1::HYG*	This study
DSC0291	*MATa leu2Δ1 his3Δ200 ntg1::BLASTICIDIN bar1::HYG* pNTG1-GFP *rho^0*	This study
DSC0295	*MATa his3Δ1 leu2Δ0 met 15Δ0 ura3Δ0 TET-repressible C-terminal TAP tagged NTG1*	This study

DSC0296	*MATa his3Δ1 leu2Δ0 met 15Δ0 ura3Δ0 TET- repressible C-terminal TAP tagged NTG2*	This study
YSC1178-7499106 (DSC0297)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 C-terminal TAP tagged NTG1*	Open Biosystems
YSC1178-7502650 (DSC0298)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 C-terminal TAP tagged NTG2*	Open Biosystems
BY4147 (DSC0313)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
pNTG1-GFP	NTG1-GFP, *2μ, URA3,* AMP^R	(41, 84, 90)
pNTG2-GFP	NTG2-GFP, *2μ, URA3,* AMP^R	(41, 84, 90)
pNTG1K364R-GFP	NTG1 K364R-GFP, *2μ, URA3,* AMP^R	This study
pFA-KMX4	$KANAMYCIN^R$, *CEN,* AMP^R	(80)
pAG32	*Hygromycin B phosphor transferase* $MX4^R$, AMP^R	(23)
pCM225	$tet0_7$, *CEN*, *Kan* $MX4^R$, AMP^R	(7)
p1375	3HA, *CEN, TRP1,* AMP^R	(51)
p2245	pGAL1-GST, *CEN*, *TRP1*, AMP^R	(51)

Figure 1

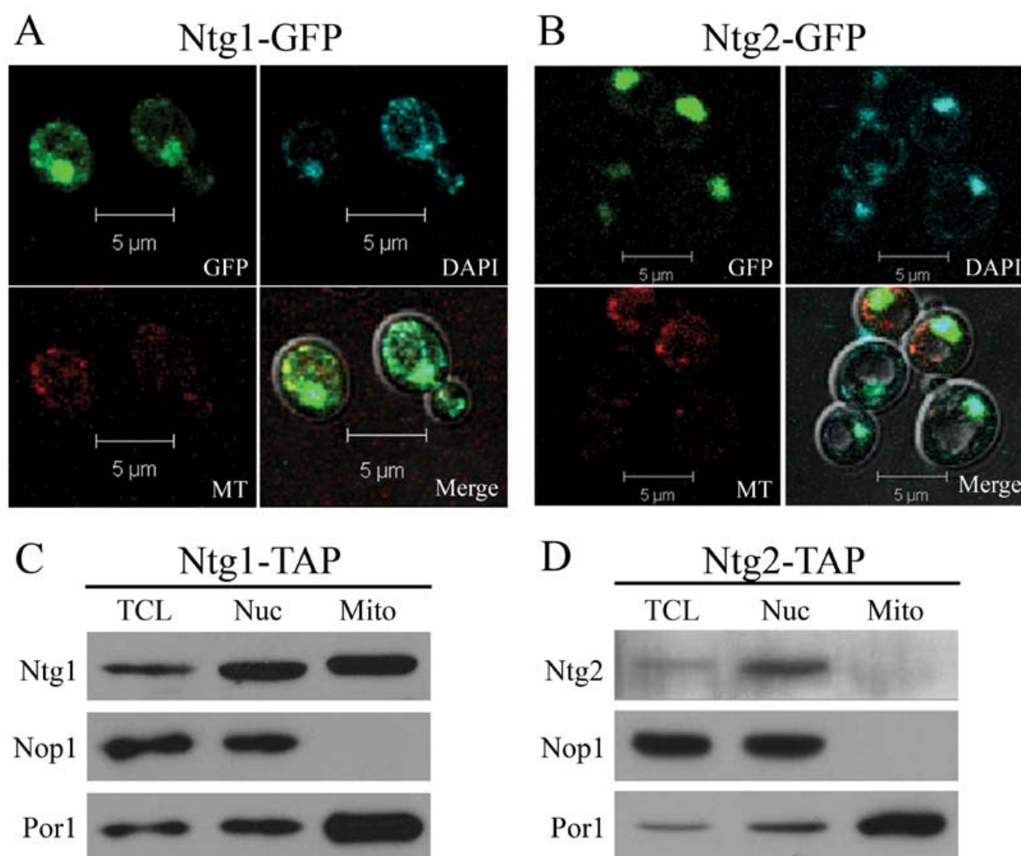


Figure 1. Subcellular Localization of Ntg1 and Ntg2 Under Normal Growth Conditions. A, B. Localization of GFP-tagged protein was assessed via direct fluorescence microscopy. GFP, DAPI, Mitotracker (MT), and merged images of cells expressing Ntg1-GFP or Ntg2-GFP are displayed. **C, D.** Sucrose gradient subcellular fractionation (Materials and Methods) and Western analysis was performed on Ntg1-TAP and Ntg2-TAP cells. Antibodies to Nop1 (nuclear marker protein), Por1 (mitochondrial marker protein), and the calmodulin domain of the TAP tag (to detect Ntg1 or Ntg2) were utilized to detect proteins present in total cell lysate (TCL), nuclear (Nuc), and mitochondrial (Mito) fractions.

Figure 2

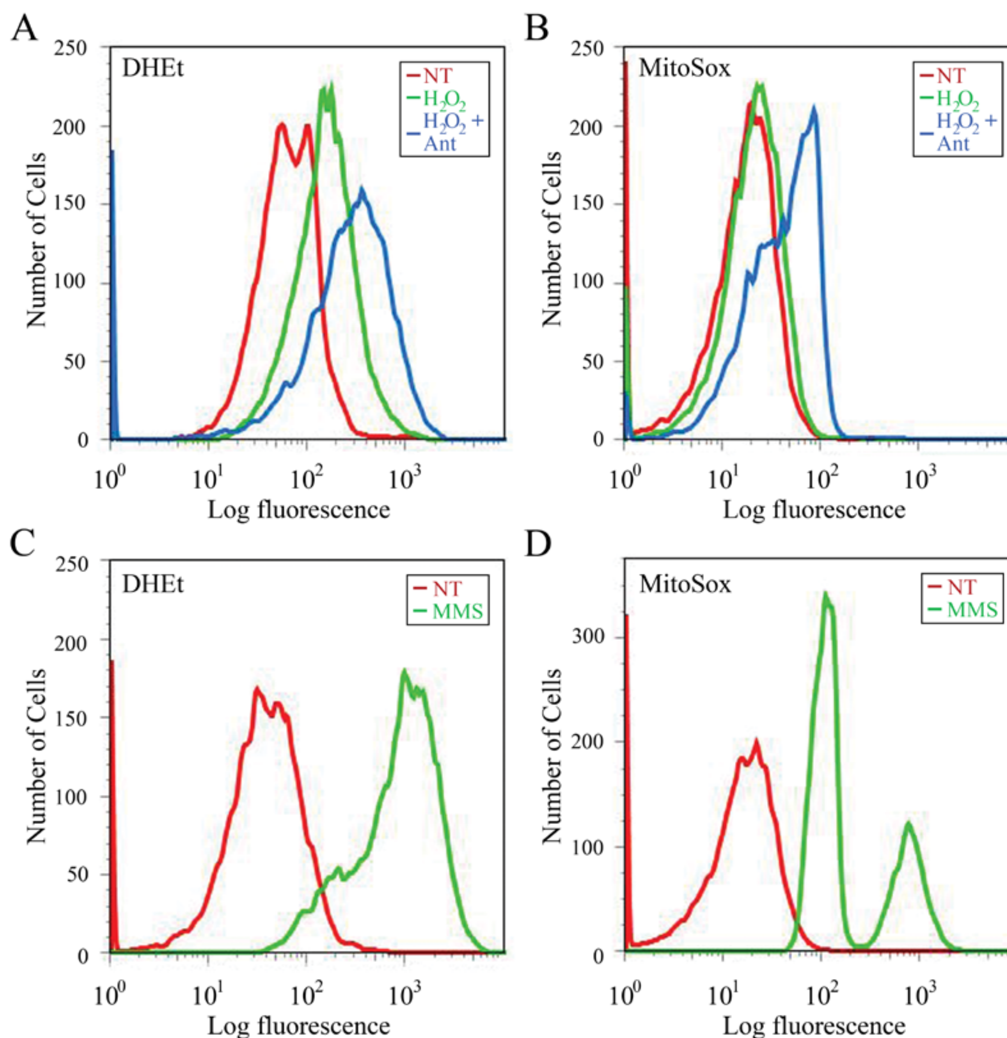


Figure 2. Flow Cytometric Analysis of Cells to Determine Intracellular ROS Levels Following Nuclear or Mitochondrial Oxidative Stress. A, B. Cells were left untreated (red, NT) or exposed to 20 mM H_2O_2 (green) or 20 mM H_2O_2 plus 10 μg/mL antimycin (blue) and incubated with dihydroethidium (DHEt) or MitoSox to assess relative levels of total cellular superoxide (DHEt) or mitochondrial superoxide (MitoSox). **C, D.** Cells were left untreated (red) or exposed to 55 mM MMS (green) and incubated with DHEt or MitoSox.

Figure 3

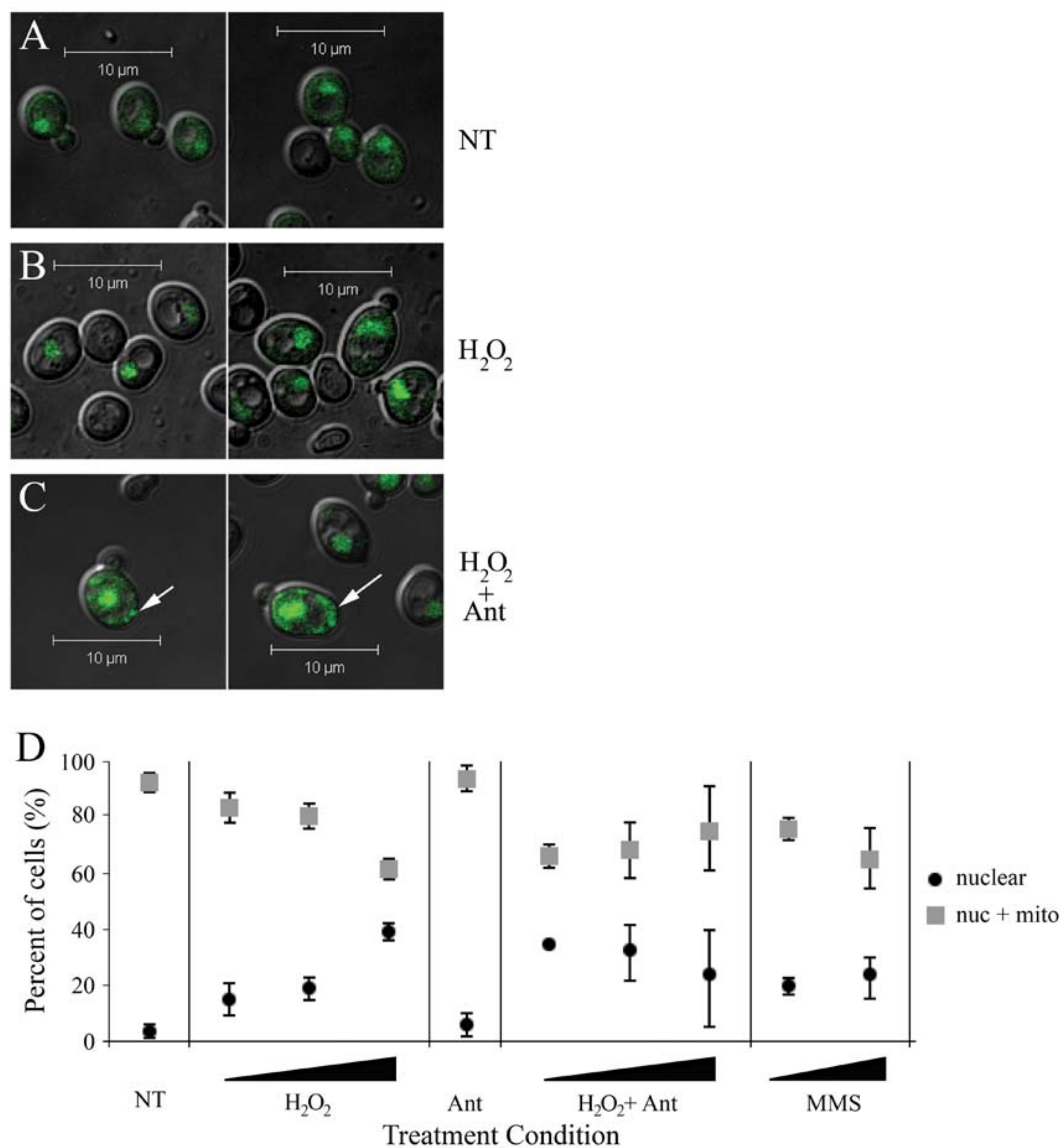
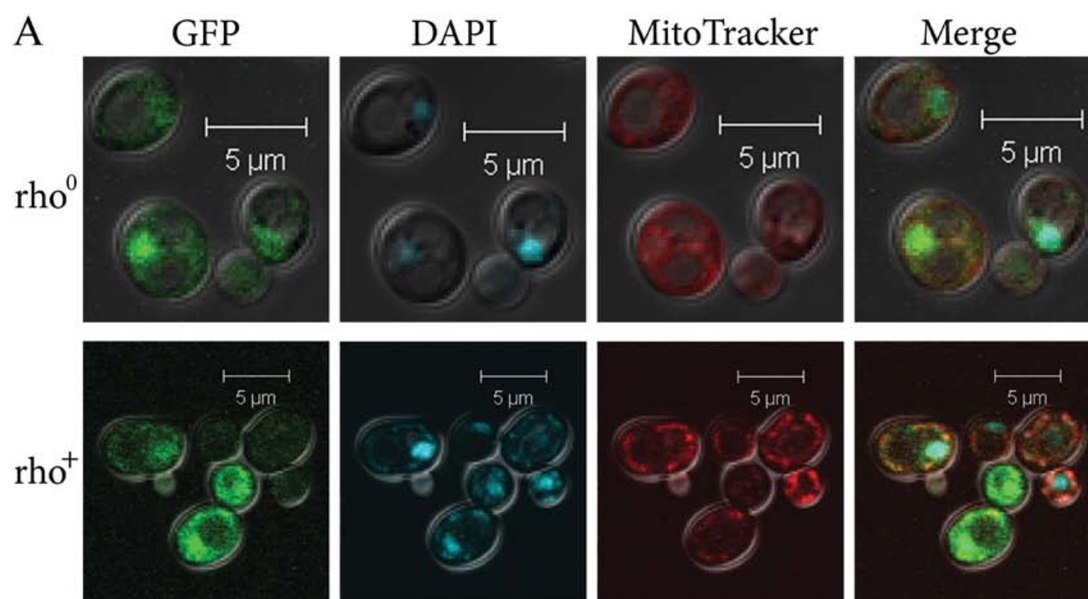
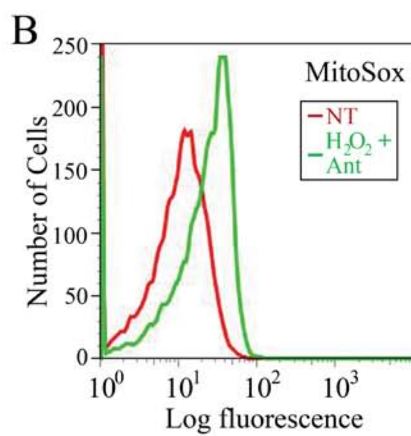


Figure 3. Subcellular Localization of Ntg1 Following Exposure to Nuclear and Mitochondrial Oxidative Stress. Localization of GFP-tagged Ntg1 was assessed via direct fluorescence microscopy following exposure to the indicated oxidative stress agent for 1 hour. **A**. GFP images of untreated cells expressing Ntg1-GFP. **B.** GFP images of cells exposed to 20 mM H_2O_2. **C.** GFP images of cells exposed to 20 mM H_2O_2 plus 10

μg/mL antimycin A. Arrows indicate increased mitochondrial Ntg1 localization observed by Metamorph image analysis. **D.** Ntg1-GFP localization analysis. Cells were left untreated (NT) or were exposed to H_2O_2, MMS, and/or antimycin (Ant) as indicated (Materials and Methods). Localization of Ntg1-GFP to nuclei only (nuclear) or nuclei plus mitochondria (nuc + mito) was determined for each cell and plotted as percentage of the total cells evaluated. Error bars represent standard deviation.

Figure 4





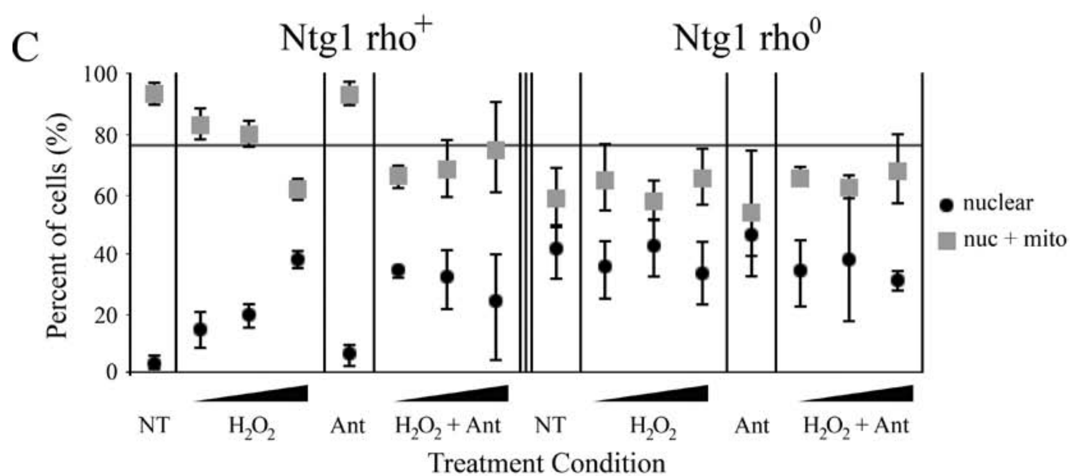


Figure 4. Mitochondrial Localization of Ntg1 is Influenced by Mitochondrial Oxidative DNA Damage. rho^+ cells and rho^0 cells were analyzed in order to assess the change in localization of Ntg1-GFP in the presence and absence of mitochondrial DNA in response to mitochondrial oxidative stress. **A.** Fluorescence microscopy was performed in order to confirm the rho status of the cells. Panels from left to right: GFP (Ntg1-GFP), DAPI (DNA), MitoTracker, Merge. **B.** Flow cytometry analysis of ROS levels in rho^0 cells. Cells were left untreated (red) or were exposed to 20 mM H_2O_2 plus 10 μg/mL antimycin (green) and incubated with MitoSox to assess the levels of mitochondrial superoxide. **C.** Quantification of Ntg1-GFP localization in rho^+ or rho^0 cells. Cells were left untreated (NT) or were exposed to H_2O_2 and/or antimycin (Ant) as indicated (Materials and Methods). Localization of Ntg1-GFP to nuclei only (nuclear) or nuclei plus mitochondria (nuc + mito) was determined for each cell and plotted as percentage of the total cells evaluated. Error bars represent standard deviation in the data. Gray line references overall higher percent localization of Ntg1-GFP to mitochondria in rho^+ cells compared to rho^0 cells (H_2O_2 plus antimycin).

Figure 5

```
Ntg1
  1 MQKISKYSSM AILRKRPLVK TETGPESELL PEKRTKIKQE EVVPQPVDID  50
 51 WVKSLPNKQY FEWIVVRNGN VPNRWATPLD PSILVTPAST KVPYKFQETY 100
101 ARMRVLRSKI LAPVDIIGGS SIPVTVASKC GISKEQISPR DYRLQVLLGV 150
151 MLSSQTKDEV TAMAMLNIMR YCIDELHSEE GMTLEAVLQI NETKLDELIH 200
201 SVGFHTRKAK YILSTCKILQ DQFSSDVPAT INELLGLPGV GPKMAYLTLQ 250
251 KAWGKIEGIC VDVHVDRLTK LWKWVDAQKC KTPDQTRTQL QNWLPKGLWT 300
301 EINGLLVGFG QIITKSRNLG DMLQFLPPDD PRSSLDWDLQ SQLYKEIQQN 350
351 IMSYPKWVKY LEGKRELNVE AEINVKHEEK TVEETMVKLE NDISVKVED

Ntg2
  1 MREESRSRKR KHIPVDIEEV EVRSKYFKKN ERTVELVKEN KINKDLQNYG  50
 51 GVNIDWIKAL KPIEYFEWIE SRTCDDPRTW GRPITKEEMI NDSGAKVPES 100
101 FLPIYNRVRL MRSKVKTPVD AMGCSMIPVL VSNKCGIPSE KVDPKNFRLQ 150
151 FLIGTMLSAQ TRDERMAQAA LNITEYCLNT LKIAEGITLD GLLKIDEPVL 200
201 ANLIRCVSFY TRKANFIKRT AQLLVDNFDS DIPYDIEGIL SLPGVGPKMG 250
251 YLTLQKGWGL IAGICVDVHV HRLCKMWNWV DPIKCKTAEH TRKELQVWLP 300
301 HSLWYEINTV LVGFGQLICM ARGKRCDLCL ANDVCNARNE KLIESSKFHQ 350
351 LEDKEDIEKV YSHWLDTVTN GITTERHKKK
```

Figure 5. **Amino Acid Sequences of Ntg1 and Ntg2.** The amino acid sequences of Ntg1 (top) and Ntg2 (bottom) are shown. The following domain structures are indicated: potentially sumoylated lysines with a [Hydrophobic] K x [ED] motif (red) as predicted by the SUMOsp 1.0 program (85), predicted nuclear localization sequence (green) as determined with the NUCDISC subprogram of PSORTII (56), predicted mitochondrial targeting sequence (bold, italicized) as determined with the MITDISC subprogram of PSORTII (56), predicted helix hairpin helix active site region (underlined) and active site lysine (blue) determined due to significant homology with endonuclease III and its homologs (4, 31), and the [4Fe-2S]-cluster (brown) characterized by the sequence, C-X_6-C-X-X-C-X_5-C (44).

Figure 6

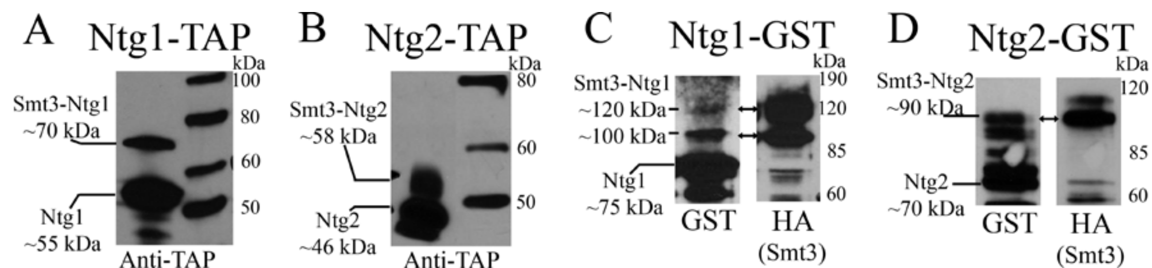


Figure 6. Post-translational Modification of Ntg1 and Ntg2 by SUMO. A, B. Western analysis of TAP-purified Ntg1-TAP and Ntg2-TAP utilizing an anti-calmodulin TAP antibody. Non-sumoylated and sumoylated species of Ntg1 and Ntg2 are indicated. Protein sizes are indicated in right margins. **C, D.** Western analysis of purified Ntg1-GST and Ntg2-GST detected with antibodies to GST (Ntg1 or Ntg2) and HA (Smt3). Non-sumoylated and sumoylated species of Ntg1 and Ntg2 are indicated. Protein sizes are indicated in the right margin. Double-headed arrows indicate sumoylated Ntg1 and Ntg2 detected simultaneously with GST and HA antibodies.

Figure 7

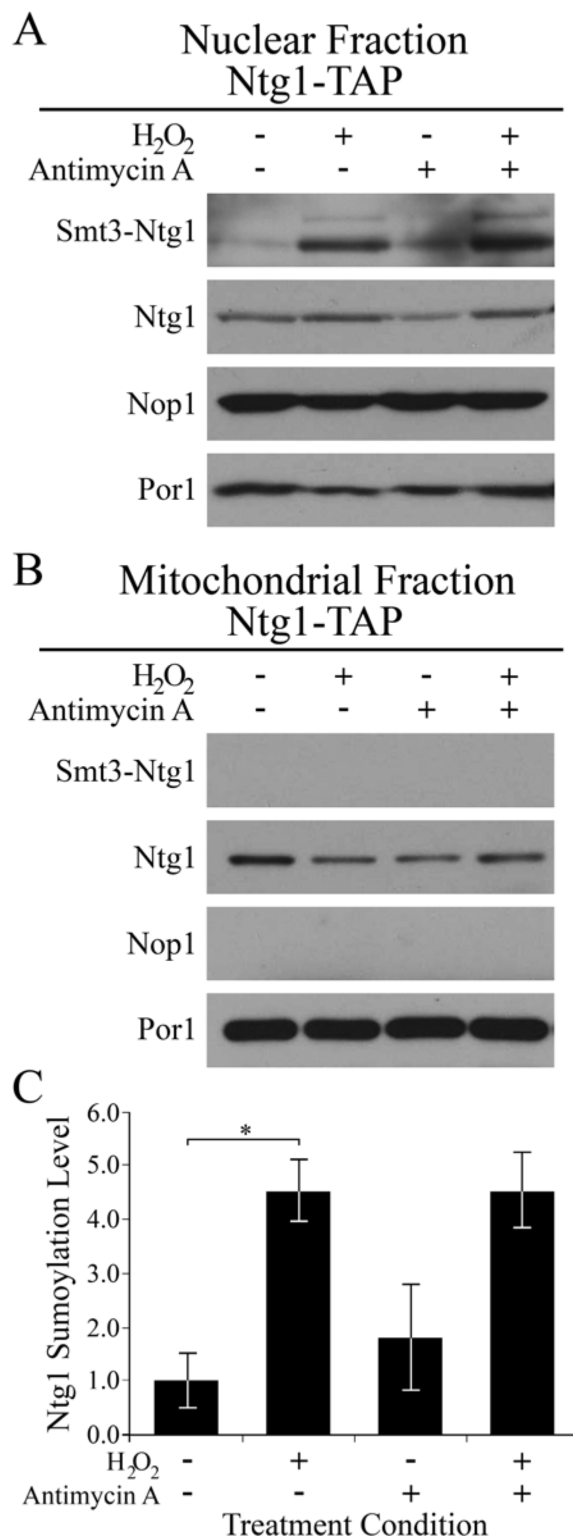


Figure 7. Sumoylation of Nuclear Ntg1 Increases in Response to Oxidative Stress. Cells were exposed to no treatment, 10 mM H_2O_2, 10 μg/mL antimycin, or 10 mM H_2O_2 plus 10 μg/mL antimycin. Sucrose gradient subcellular fractionation (Materials and Methods) was employed to assess the localization of Ntg1 to nuclei and mitochondria following exposure to nuclear (H_2O_2) or mitochondrial (H_2O_2 plus antimycin) stress-inducing agents. **A.** Western analysis of nuclear fractions utilizing antibodies to Nop1 (nuclear marker), Por1 (mitochondrial marker), and the calmodulin domain of TAP to detect Ntg1 and Smt3-Ntg1. **B.** Western analysis of mitochondrial fractions. **C.** Levels of nuclear sumoylated Ntg1 species detected by chemiluminescence in response to oxidative stress. Nuclear-enriched and mitochondrial subcellular fractions were generated (Materials and Methods) and evaluated by Western analysis. Following chemiluminescence evaluation of nuclear Ntg1, unmodified and sumoylated Ntg1 were quantified, and fold change in percent sumoylated Ntg1 was calculated. Error bars represent standard error of the mean. Asterisk indicates statistical significance (p value < 0.005).

Figure 8

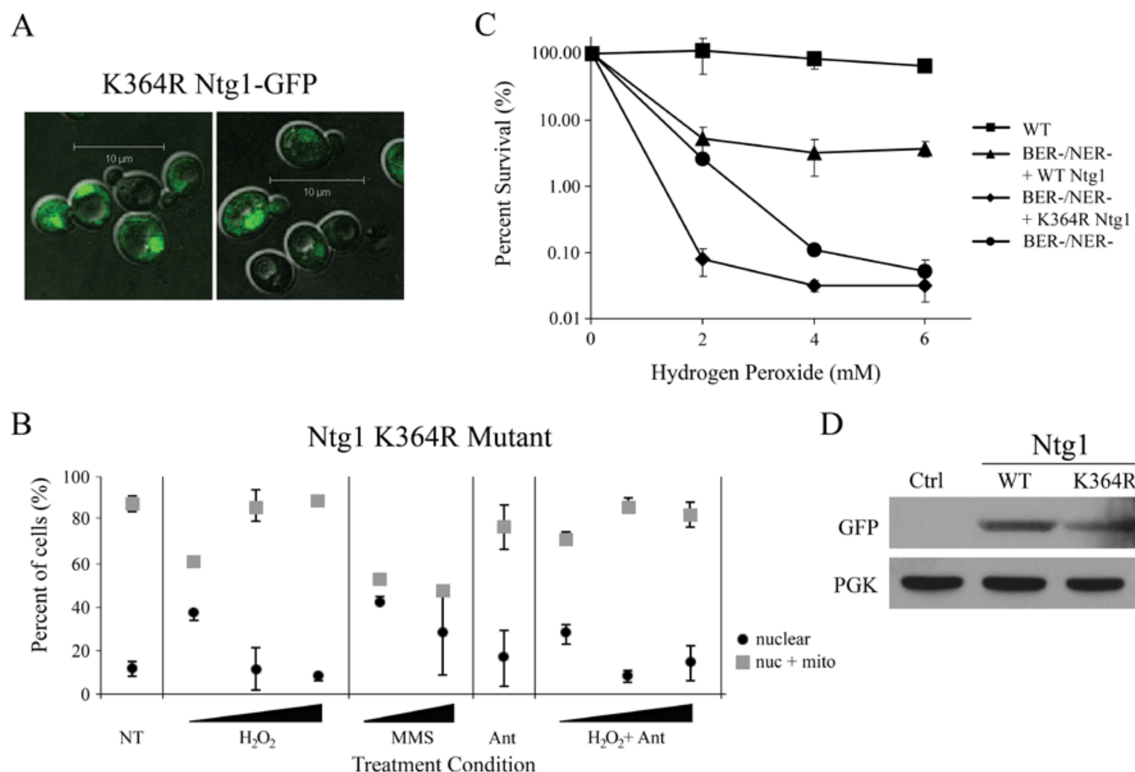


Figure 8. Subcellular Localization and Function of the Ntg1 K364R Mutant. A. GFP image of cells expressing Ntg1 K364R-GFP. **B.** Quantification of Ntg1 K364R-GFP localization. Cells were not treated (NT) or were exposed to the indicated oxidative stress inducing agent for 1 hour (Materials and Methods). Localization of Ntg1 K364R-GFP to nuclei only (nuclear) or nuclei plus mitochondria (nuc + mito) was determined for each cell and plotted as percentage of the total cells evaluated. Error bars represent standard deviation. Refer to Figure 3D for localization of wild type Ntg1. **C.** Functional analysis of K364R Ntg1. H_2O_2 sensitivity of wild type (WT), BER-/NER- deficient cells (BER-/NER-), and BER-/NER- deficient cells containing an episomal copy of wild type Ntg1-GFP (BER-/NER- + WT Ntg1) or Ntg1 K364R-GFP (BER-/NER- + K364R Ntg1) was assessed. Cells were exposed to 0, 2, 4, or 6 mM H_2O_2. The percent survival was set

to 100% for untreated samples. Error bars indicate standard deviations in the data. **D.** Steady-state expression levels of wild type Ntg1-GFP and mutant Ntg1 K364R-GFP in BER-/NER- deficient cells. Western analysis of whole cell lysates from BER-/NER- deficient (*ntg1 ntg2 apn1 rad1*) cells (Ctrl) and BER-/NER- deficient cells containing an episomal copy of wild type Ntg1-GFP (WT) or mutant Ntg1 K364R-GFP (K364R) was performed utilizing an anti-GFP antibody (to detect Ntg1) and anti-PGK antibody (to determine relative levels of protein loaded per lane).

Figure 9

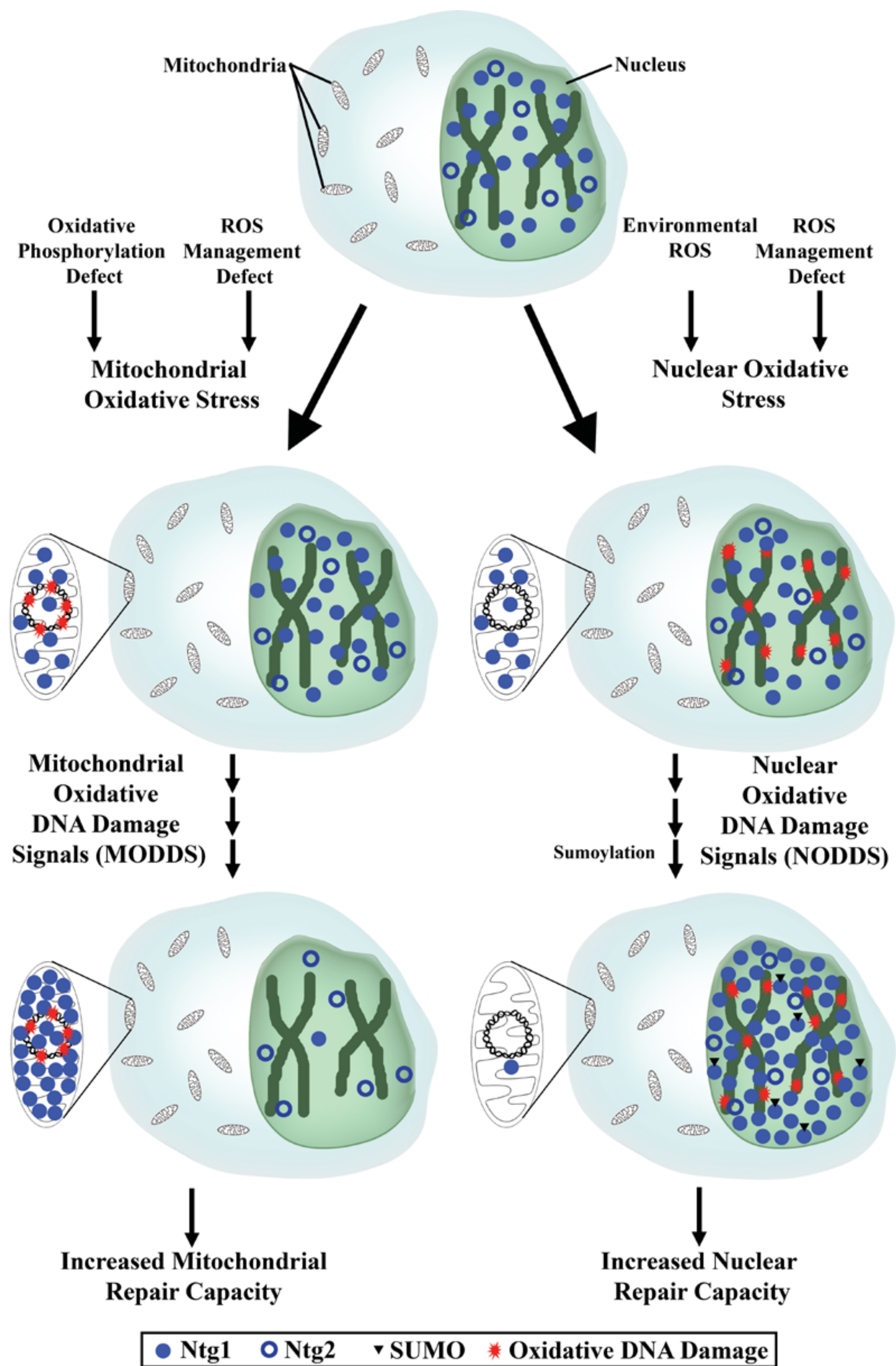


Figure 9. Proposed Model for Regulation of BER Proteins in Response to Oxidative Stress. The oxidative stress state of a cell is affected by its environment and metabolic processes. Nuclear and mitochondrial oxidative DNA damage (red stars) occurs as a result of nuclear and mitochondrial oxidative stress. Nuclear and mitochondrial oxidative DNA damage initiates signaling of BER proteins, such as Ntg1 (blue filled circles), to sites of damage. Nuclear oxidative DNA damage signals (NODDS) and mitochondrial oxidative DNA damage signals (MODDS) are responsible for recruiting BER proteins to nuclei and mitochondria, respectively. NODDS likely include the sumoylation machinery and influence nuclear protein localization. SUMO modification (black triangles) of Ntg1 concentrates Ntg1 in the nucleus following oxidative stress. When the target BER proteins are contacted by NODDS or MODDS, relocalization to nuclei and/or mitochondria occurs depending on the levels of oxidative DNA damage present in each organelle. Following recruitment of BER proteins into the nucleus and mitochondria, the capacity for repair of oxidative DNA damage increases accordingly. In order to maintain a steady state (baseline) level of BER proteins in nuclei, BER proteins such as Ntg2 (blue unfilled circles) do not relocalize in response to oxidative DNA damage.

APPENDIX 1

CELL CYCLE REGULATION OF LOCALIZATION OF NTG1

1. Abstract

Base excision repair (BER) is the primary repair pathway for the repair of one of the most frequently occurring classes of DNA damage, oxidative DNA damage. BER is known to occur in both nuclei and mitochondria of eukaryotic cells from yeast to humans. How BER proteins are localized to sites of nuclear and mitochondrial oxidative DNA damage in a timely manner following oxidative stress is unknown. These mechanisms of regulation are important for the efficient repair of nuclear and mitochondrial DNA that ensures genomic integrity. In an effort to determine mechanisms of regulation of BER proteins, cell cycle-dependence of the subcellular localization of the *Saccharomyces cerevisiae* DNA glysosylase/AP lyase, Ntg1, was evaluated. The results of this study indicate that Ntg1 localization is not regulated in a cell cycle-dependent manner.

2. Introduction

Oxidative DNA damage is deleterious to cells and can result in genomic mutations and cell death (15). Base excision repair (BER) is the primary repair pathway for oxidative DNA damage (22, 24). BER begins with the recognition and removal of a damaged base by a DNA glycosylase (20, 21). Next, either an AP lyase or AP endonuclease cleaves the DNA phosphate backbone, which opens up the DNA for the subsequent processes by trimming enzymes, DNA polymerase, and DNA ligases (2). Some BER proteins are bifunctional in that they are capable of DNA glycosylase and AP lyase function.

In *Saccharomyces cerevisiae*, Ntg1 is a bifunctional DNA glycosylase/AP lyase (29, 34). Ntg1 is the homolog of *Escherichia coli* endonuclease III. Ntg1 localizes to the nucleus and mitochondria of *S. cerevisiae* cells (1, 35). Previous observations from our lab noted that in nonsynchronous *S. cerevisiae* cultures, Ntg1 could be detected solely in mitochondria in approximately 43−47% of the cells (35). As 100% of the cells did not contain localization of Ntg1 in both nuclei and mitochondria, it is possible that there is a cell cycle-mediated aspect to the localization of Ntg1. It is estimated that *S. cerevisiae* cells which undergo a 90 minute cell cycle spend approximately 37 minutes in G1 and a total of 56 minutes in S/G2/M (4). The length of time of each cell cycle phase should be associated with the percentage of unsynchronized cells that exist in a certain cell cycle phase. If this is the case, then approximately 60% of unsynchronized *S. cerevisiae* cells should be in G1 phase, while approximately 40% should be in S/G2/M. Considering the approximately 60% of *S. cerevisiae* cells containing both nuclear and mitochondrial Ntg1, it is possible that Ntg1 localization is cell cycle-dependent in order to maintain nuclear Ntg1 BER activity in the nucleus prior to replication.

Regulation of subcellular localization in a cell cycle-dependent manner has been documented in several cases in *S. cerevisiae*. Many of the cell cycle regulated proteins are involved in the process of DNA replication. For example, several proteins involved in replication initiation and elongation are regulated in a cell cycle-dependent manner, including *S. cerevisiae MCM4/CDC54*, *MCM5/CDC46*, *MCM7/CDC47* (23). Among these, the localization of CDC46, was found to be regulated in a cell cycle-dependent manner, such that CDC46 enters the nucleus only during G1 (13). A second example is the double-stranded RNA-specific ribonuclease, RNase III, which is localized from

nucleolus to the nucleoplasm in the G2/M phase (6). Since the proteins whose localization is regulated in a cell cycle-dependent manner are associated with interactions with DNA, it is possible that Ntg1, as a protein that recognizes and repairs lesions in DNA, is regulated in a similar manner. Additionally, we hypothesize that it is advantageous for the cell to repair damaged nuclear DNA prior to replication in order to prevent possible DNA replication fork stalling. It is possible that Ntg1 is localized to the nucleus during G1 so that all oxidative DNA damage can be removed prior to S phase replication.

While the biochemical capabilities of BER proteins to recognize and repair DNA damage are well understood, the mechanisms of regulation of these proteins in yeast and humans are not. For example, how does a DNA glycosylase, like Ntg1, find oxidative DNA damage in such a large genome? To add an additional layer of complexity, both nuclear and mitochondrial DNA can be oxidatively damaged (28, 33). Certain BER proteins localize to both nuclei and mitochondria (19). How proteins like Ntg1 that localize to both nuclei and mitochondria are capable of maintaining repair in both organelles is unknown. As *S. cerevisiae* is a widely accepted model system for the study of DNA repair because its repair pathways are similar to human repair pathways, the *S. cerevisiae* Ntg1 was utilized in order to elucidate the mechanisms that regulate the function of BER proteins. It was the goal of these experiments to determine if Ntg1 localization is controlled by a mechanism that is cell cycle-dependent. *S. cerevisiae* cells expressing Ntg1-GFP were synchronized via alpha factor arrest and the subcellular localization of Ntg1 was monitored following release from alpha factor. The results revealed no evidence supporting a cell cycle-dependent compartmentalization of Ntg1.

3. Materials and Methods

3.1 Strains, media, and growth conditions. Haploid *S. cerevisiae* strains were utilized in this study. Yeast cells were cultured at 30° C in rich YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 0.005% adenine sulfate, and 2% agar for plates). In order to introduce plasmids or to disrupt the chromosomal locus, yeast cells were transformed by a modified lithium acetate method (16).

The pPS904 green fluorescent protein (GFP) expression vector (2 micron, *URA3*) was employed for generation of C-terminally tagged Ntg1-GFP fusion protein (17). The *S. cerevisiae* haploid strain FY86 was utilized for all localization studies (31). Δ*NTG1* strain (DSC0282) was generated by precisely replacing the *NTG1* open reading frame in FY86 with the kanamycin antibiotic resistance gene (pFA6-KMX4 (30), selected with 150 mg/L G418 [US Biological]). In order to successfully synchronize the cells, *BAR1* was replaced with the hygromycin resistance gene (pAG32 (11), selected with 300 mg/L Hygromycin B [Invitrogen]). Plasmids encoding Ntg1-GFP were introduced into Δ*NTG1* cells. The insertion events were confirmed by PCR and sequencing analysis.

3.2 Synchronization. 500 mL of cells were grown to a density of 5 x 10^6 cells/mL in YPD. 50 μg of alpha factor were added to the 500 mL culture, and the cells were incubated for 1.5 hours at 30°C. G1 arrested cells were then washed extensively with water and placed in YPD containing 25 μg/mL pronase. Cells were removed from culture every 15 minutes and fixed by adding formaldehyde to 4.4%.

3.3 Fluorescence microscopy. Cells were incubated with 25 nM MitoTracker (Invitrogen) in order to visualize mitochondria. Indirect immunofluorescence was performed as previously described (18). The cells were immuno-stained with anti-Nab2 (1:50,000) and anti-Texas Red-tagged Rabbit IgG (1:200) antibodies as markers for nuclei and mitochondria, respectively. Cells were then analyzed by fluorescence confocal microscopy, employing an Olympus BX60 epifluorescence microscope equipped with a Photometrics Quantix digital camera.

3.4 Flow cytometry. Cell cycle progression of yeast cells was monitored utilizing propidium iodide in conjunction with flow cytometry was performed as previously published (3), with the following exceptions: one mL of cells was removed from the culture every 15 minutes (in combination with the culture removed for microscopy). Flow cytometry analysis was performed for 10,000 cells at low speed on a BD FACSCalibur instrument (BD Biosciences).

4. Results

4.1 Synchronization of cells expressing Ntg1-GFP. In order to evaluate the localization during the cell cycle phases, cells were synchronized utilizing alpha factor arrest. Yeast MATa cells exposed to alpha factor arrest at the G1 phase (5, 8), and they can subsequently be released by removing the alpha factor from yeast media. In order to synchronize the Ntg1-GFP expressing strain, cells were incubated with alpha factor for 1.5 hours and then were incubated with pronase to degrade alpha factor and release cells from arrest. Flow cytometry analysis of cells incubated in pronase showed that cells

were no longer stalled at the G1 phase (1n), and the accumulation of cells with 2n chromosomal DNA occurred over time, indicating that the cells were undergoing synchronous progression through the cell cycle (Figure 1).

4.2 Localization of Ntg1 during cell cycle progression. In order to determine the localization of Ntg1 during each cell cycle phase, fluorescence microscopy of synchronized cells expressing Ntg1-GFP was evaluated. The localization of Ntg1-GFP was nuclear plus mitochondrial in the majority of the cells, regardless of cell cycle phase (Figure 2). In an effort to quantify the number of cells with mitochondrial or nuclear plus mitochondrial localization of Ntg1, the localization was determined for at least 100 cells (Table 1). Localization was scored as both nuclear and mitochondrial or mitochondrial alone. In all cell cycle phases, localization of Ntg1 was both nuclear and mitochondrial in greater than 80% of the cells, while localization of Ntg1 was mitochondrial alone in less than 20% of the cells. This is in contrast to the previously reported localization to mitochondria alone in 43−47% of nonsynchronous cells (35). Because of these divergent results, the localization of nonsynchronous cells expressing Ntg1-GFP was evaluated under our growth conditions in our strains. In nonsynchronous cells, Ntg1 localized to nuclei plus mitochondria in approximately 90% of cells, while Ntg1 localized to mitochondria only in less than 10% of cells. Perhaps differences in the growth conditions utilized in the two independent studies contributed to the discrepancy between studies.

5. Discussion

Yeast Ntg1 localizes to nuclei and mitochondria, and the integrity of nuclear and mitochondrial DNA is dependent upon Ntg1 (7, 9, 25, 27). As Ntg1 plays important roles in both nuclei and mitochondria, it is possible that Ntg1 localization is regulated so that Ntg1 can respond to oxidative lesions in both organelles. For this reason, we evaluated the possibility that Ntg1 localization is cell cycle-dependent. Synchronization of cells with alpha factor in conjunction with fluorescence microscopy revealed that the localization of Ntg1 to nuclei and mitochondria does not appear to be cell cycle-dependent.

Previous reports suggested that localization of Ntg1 could be regulated by cell cycle-related mechanisms. The observation that Ntg1 localized to mitochondria alone in 43−47% of nonsynchronous cells evoked this idea. The results presented in this study reveal some ambiguity between past and present studies as we were only able to calculate approximately 10% of nonsynchronous cells with mitochondrial only localization of Ntg1. The strain of yeast utilized for past and present studies was identical. It is possible that the difference in Ntg1 localization stems from the fact that growth conditions in the two experiments were different. You, et al. grew the cells to a density of 1×10^7 cells/mL in–URA synthetic media, while we grew the cells to a density of 5×10^6 cells/mL in rich YPD in order to prevent the introduction of additional stresses produced by nutrient deprivation and saturation of growth. Perhaps the different media types are capable of activating specific stress response pathways or are capable of handling ROS released from the cells to varying degrees, creating two divergent environments for detecting Ntg1 localization. Additionally, it is likely that logarithmic growth does not

have the associated nutrient deprivation and oxidative stresses as saturated growth, resulting in the expression of different groups of genes. The differences between the growth conditions of each experiment likely affected the outcome of the localization studies and explain why the results were not the same for both experiments.

As localization of Ntg1 does not appear to be cell cycle-dependent, it is evident that Ntg1 subcellular localization is controlled by other mechanisms. For example, Ntg1 contains seven putative post-translational modification sites for sumoylation, as predicted using the SUMO prediction program, SUMOsp 1.0 (26, 32). Sumoylation is associated with the nuclear localization of numerous proteins (10, 14). The nuclear localization of Ntg1 appears to be controlled by sumoylation of Ntg1 (12) and likely serves as a point of regulation for BER. Another mechanism of regulation for BER is that Ntg1 localization is recruited to nuclei or mitochondria in response to oxidative DNA damage or ROS (12). In this case, levels of mitochondrial and nuclear ROS or oxidative DNA damage are monitored by sensors in the cell that are capable of sending a signal to recruit Ntg1 to nuclei or mitochondria with DNA damage. Given its important role in maintaining nuclear DNA, it is likely beneficial for the cell that nuclear and mitochondrial localization of Ntg1 does not appear to be cell cycle-dependent. As oxidative DNA damage can occur spontaneously due to oxidative stress at any time throughout the cell cycle, utilizing a mechanism of cell cycle-dependent localization as a mode of regulation for Ntg1 could result in the accumulation of oxidative DNA damage during specific cell cycle phases, which could be deleterious to the cell. Therefore, it is more likely that Ntg1 is regulated by mechanisms that are cell cycle-independent.

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Table 1: Quantification of the Localization of Ntg1-GFP in Alpha Factor Synchronized Cells. N is the number of cells with mitochondrial specific localization or co-localization to nuclei and mitochondria. % is percent of total cells in that particular cell cycle stage representing the indicated localization.

	Mitochondria (N)	**Mitochondria (%)**	**Nucleus plus Mitochondria (N)**	**Nucleus plus Mitochondria (%)**
G1	23	13.1	153	86.9
S	19	11.3	149	88.7
G2	1	4.5	21	95.5
M	31	17.1	150	82.9

Figure 1

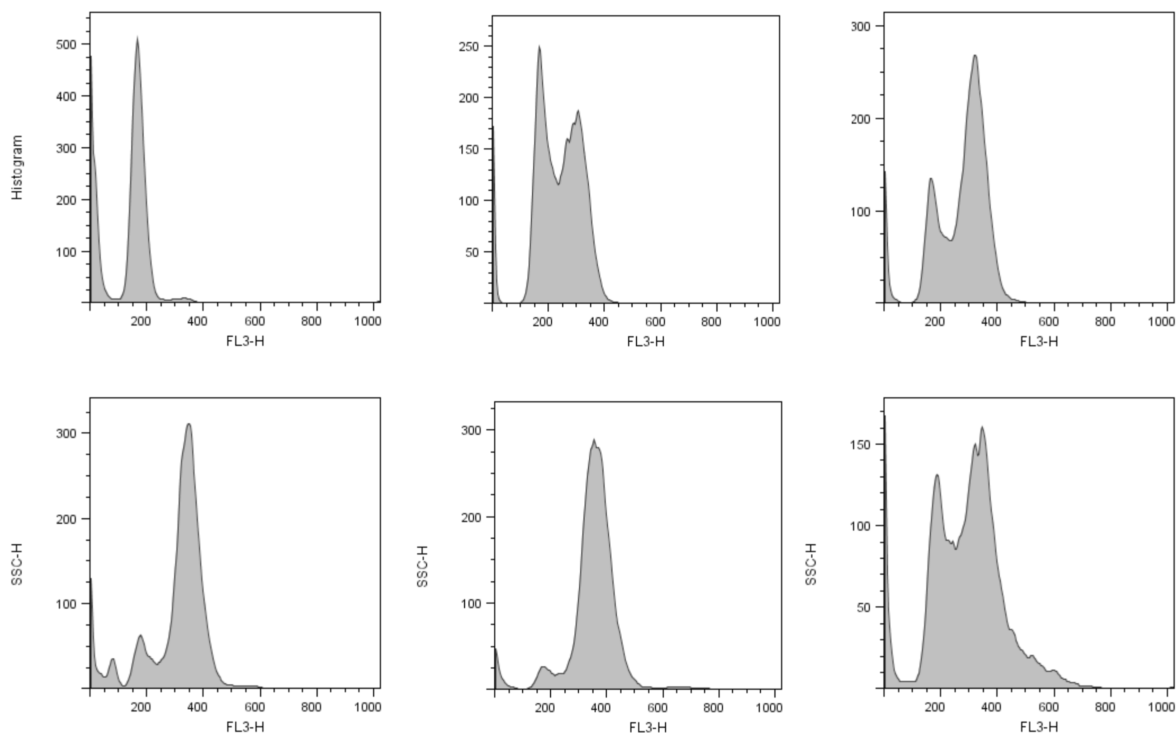

Figure 1. Flow Cytometry of Cells Released from Alpha Factor Arrest. Cells were incubated with alpha factor and released by incubation with pronase. Samples were taken at 0, 30, 45, 60, 90, and 105 minutes, and the cells were stained with propidium iodide. The cells were analyzed by flow cytometry. The peak around 200 FL3-H is the genome =1n peak, and the peak around 325 FL3-H is the 2n peak.

Figure 2

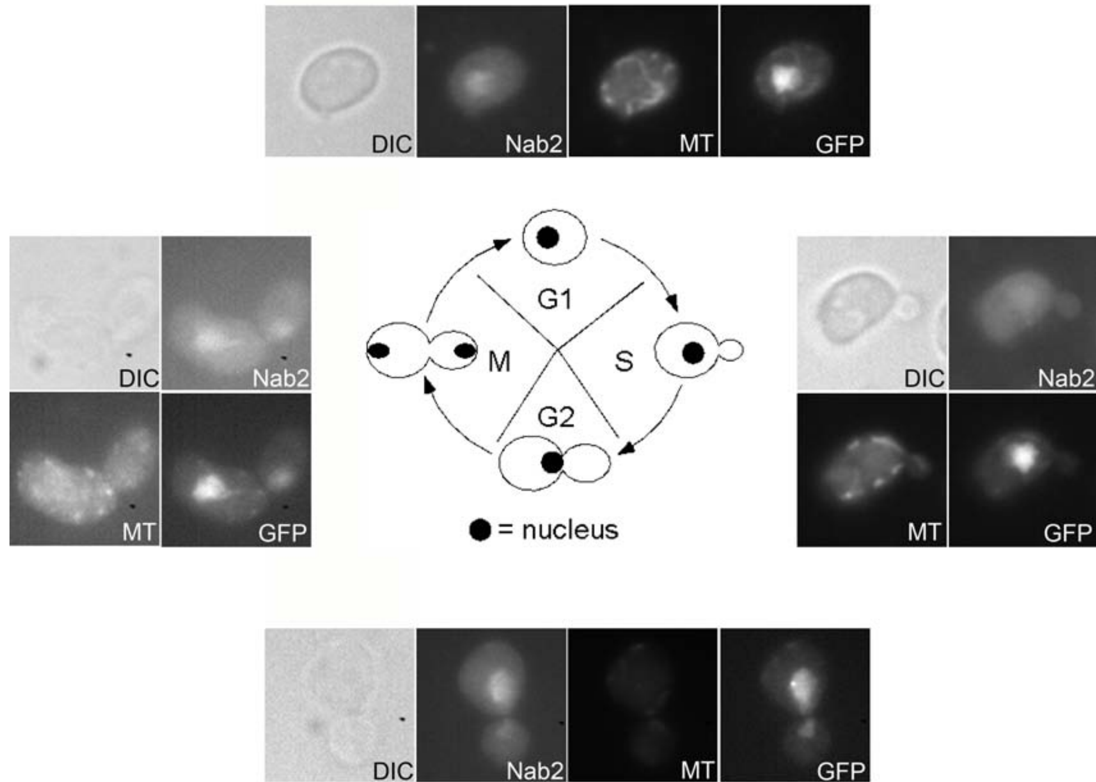

Figure 2. Localization of Ntg1 throughout the Cell Cycle. Cells were synchronized with alpha factor and released. Cells were collected from each cell cycle stage and assessed via microscopy. Representative DIC, anti-Nab2 (nuclear marker), Mitotracker, and GFP images from each cell cycle phase are presented.

CHAPTER 3

FUNCTIONAL SIGNIFICANCE OF NTG1 NUCLEAR AND MITOCHONDRIAL DYNAMIC LOCALIZATION

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The contribution by Lyra Griffiths includes significant written contribution and data presented in Table 3 and Figures 1-5.

1. Abstract

Numerous human pathologies are the result of unrepaired oxidative DNA damage. Base excision repair (BER) is responsible for the repair of both nuclear and mitochondrial oxidative DNA damage. The *Saccharomyces cerevisiae* BER protein, Ntg1, relocalizes to organelles with elevated oxidative DNA damage, indicating a novel mechanism of regulation for the BER pathway in eukaryotes. We propose that dynamic localization of BER proteins is regulated by constituents of stress response pathways. In an effort to define these regulatory elements, the components necessary for nuclear and mitochondrial localization of Ntg1 were determined, including a bipartite classical nuclear localization signal (cNLS), a mitochondrial matrix targeting sequence (MTS), and the classical nuclear protein import machinery. The bipartite cNLS and MTS of Ntg1 are necessary components for the dynamic localization of Ntg1, and without this mode of regulation, nuclear and mitochondrial mutations accumulate.

2. Introduction

DNA that is damaged and left unrepaired in either nuclei or mitochondria is linked to cancer, aging, and various degenerative diseases (3, 45, 59). The most frequently occurring type of DNA damage in eukaryotic cells is oxidative DNA damage, which is primarily repaired by the base excision repair (BER) pathway (65). Despite the physiological importance of the BER pathway, little is known about how BER proteins are regulated and optimally deployed to their targets within the cell in order to maintain genome stability.

Mechanisms for regulating only a small number of BER proteins have been described. These methods of regulation include transcriptional regulation, post-translational regulation, and targeted localization. Transcription of *Saccharomyces cerevisiae* 3-methyladenine DNA glycosylase (Mag1) is induced by DNA damaging agents, including methane methyl sulfonate (MMS), ultraviolet irradiation (UV), 4-nitroquinoline-1-oxide (4-NQO), and hydroxyurea (9, 10, 77). Post-translational regulation of repair proteins has been described widely, including post-translational modification by ubiquitin and the ubiquitin-like protein, SUMO (22, 32). In particular, the human BER thymidine DNA glycosylase (TDG) is sumoylated, which regulates the catalytic turn-over rate of this protein (29, 67). Finally, localization of AP endonuclease (APE) from the cytoplasm to the nucleus and mitochondria has been observed in human cells following oxidative stress (21, 71), supporting a role for localization in the regulation of BER proteins. Very little is known regarding how BER proteins are regulated beyond these examples, and it was a goal of this study to elucidate novel modes of regulation for BER in response to oxidative DNA damage.

In order to investigate new modes of regulation of BER, we focused on the *S. cerevisiae* BER protein, Ntg1. Ntg1 is a bifunctional DNA glycosylase with associated apurinic/apyrimidinic (AP) lyase function that allows Ntg1 to recognize oxidative DNA damage, create an AP site by removing the lesion from the DNA strand, and nick the DNA backbone on the 3′ side of the AP site (5, 28, 63, 79). Ntg1 is the functional homolog of the *Escherichia coli* BER protein endonuclease III (Nth), and the human BER protein hNTH1, all of which are critical for the repair of oxidative DNA damage (4, 24, 33, 80). As functional homologs are expressed from bacteria to human, and the BER

pathways are conserved between these species, we anticipate that our studies will allow us to identify mechanisms of regulation which are conserved in all eukaryotic Nth homologs.

Ntg1 and hNTH1 are localized to both nuclei and mitochondria (2, 27, 38, 80), where they repair oxidative lesions and maintain genomic stability of both nuclear and mitochondrial DNA (17, 19, 36). One recently described mode of regulation for Ntg1 is dynamic localization in response to nuclear or mitochondrial DNA damage caused by oxidative stress (27). Ntg1 is directed to nuclei or mitochondria in an organelle-specific oxidative DNA damage-mediated manner. This mode of regulation likely allows Ntg1 to rapidly respond to oxidative DNA damage in either nuclei or mitochondria. Relocalization of proteins in response to DNA damage and oxidative stress has been previously documented, suggesting that dynamic localization may be a general mode of regulation in response to DNA damage and oxidative stress. In addition to the human APE1 (21, 71), ribonucleotide reductase (50, 76) and Yap1 (44) are proteins regulated in a localization-dependent manner. Ribonucleotide reductase subunit R2 relocalizes from the nucleus to the cytoplasm in response to DNA damage (11, 49). Yap1, a transcription factor, accumulates within the nucleus in response to oxidative stress (44). Dynamic localization is a newly discovered mechanism of regulation for BER. As we hypothesize that other BER proteins are regulated in this manner, it is important to define the key determinants of this mode of BER regulation in order to understand how cells mediate a stress response. The response proteins and pathways identified are likely to comprise entire networks of factors that can receive signals for DNA damage and specify

localization of BER proteins to DNA damage loci, preventing the potential mutagenetic and cytotoxic effects of unrepaired DNA.

In order to determine the mechanism for Ntg1 dynamic localization, it is first necessary to delineate the basic mechanism by which Ntg1 is targeted to nuclei and mitochondria. The key components necessary for nuclear and mitochondrial import include nuclear localization sequences, mitochondrial targeting sequences, and nuclear or mitochondrial import machinery. Putative sequences directing nuclear and mitochondrial targeting of Ntg1 have been reported (6, 27). These putative sequences include two classical nuclear localization signal (cNLS) sequences and one mitochondrial matrix targeting sequence (MTS) (Figure 1A). cNLS motifs consist of a single cluster of basic residues (monopartite) or two neighboring clusters of basic residues (bipartite). These targeting sequences are recognized by the cNLS receptor, importin α, which binds the cargo protein in the cytoplasm and imports the cargo into the nucleus through nuclear pores in complex with importin β (23). MTSs are usually located in the N-terminal regions of proteins and consist of 10-80 amino acids which form amphipathic alpha helices that are recognized by the mitochondrial outer membrane translocase (7, 57, 74). Proteins with MTSs enter the mitochondrial matrix after being passed from the outer membrane translocase to the inner membrane translocase, Tim23 (15, 16).

We propose that dynamic localization allows targeting of Ntg1 to DNA damage loci, allowing for expedient response and repair of DNA damage in both nuclei and mitochondria. By identifying the key sequences and transport machinery that mediate Ntg1 dynamic localization, critical motifs and entire oxidative DNA damage response pathways can be uncovered that preserve genome stability. In this study, we functionally

define the specific cNLS and MTS sequences responsible for nuclear and mitochondrial localization as well as dynamic localization of Ntg1 in response to oxidative DNA damage. Furthermore, we determine the import pathway required for nuclear localization of Ntg1. In the absence of the cNLS, MTS, or classical nuclear import machinery, dynamic localization of Ntg1 does not occur, resulting in increased nuclear and mitochondrial mutation rates. Our results demonstrate that the cNLS, MTS, and classical nuclear import machinery are DNA damage response components that are important for maintaining the integrity of nuclear and mitochondrial DNA. In addition, our results identify the classical nuclear protein import machinery as novel DNA damage responders with potential to orchestrate the repair of all types of DNA damage, including oxidative DNA damage.

3. Materials and Methods

3.1 Yeast Strains, plasmids, and media. Haploid *S. cerevisiae* strains and all plasmids used in this study are listed in Table 1. Yeast cells were cultured at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 0.005% adenine sulfate, and 2% agar for plates), SD minimal medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% dextrose, 0.005% adenine sulfate, and 2% agar for plates), or YPG medium (1% yeast extract, 2% peptone, 3% glycerol, 0.005% adenine sulfate, and 2% agar for plates). In order to introduce plasmids or integrated chromosomal gene modifications, yeast cells were transformed by a modified lithium acetate method (40).

The pPS904 GFP expression vector (2 micron, *URA3*) was employed for generation of the C-terminally tagged Ntg1-GFP fusion protein (41). The *S. cerevisiae* haploid deletion mutant Δ*NTG1* (DSC0282) generated from wild type cells (FY86) was utilized to assess the localization of wild type and mutant Ntg1 (27, 75). All mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene), and resulting plasmids were sequenced to ensure the introduction of the desired mutation and the absence of any additional mutations (Table 2).

The pAC1069 vector was employed for generation of C-terminally tagged $NLS1_{NTG1}$-GFP_2, $NLS2_{NTG1}$-GFP_2, $NLS1/2_{NTG1}$-GFP_2, and MTS_{NTG1}-GFP_2 fusion proteins (34), creating the plasmids pD0386 – pD0389 (Table 1). The plasmids were introduced into Δ*NTG1* cells.

To express recombinant Ntg1, the *NTG1* open reading frame was cloned into pET-15b (Invitrogen) to generate the C-terminal His_6 epitope tagged Ntg1-His_6 (pD0390), (Table 1). Site directed mutagenesis of Ntg1-His_6 was performed to create $Ntg1_{nls1}$-His_6 (pD0391), $Ntg1_{nls2}$-His_6 (pD0392), $Ntg1_{mts}$-His_6 (pD0393), and $Ntg1_{catalytic}$-His_6 (pD0394) (Table1). Expression vectors were transformed into DE3 cells.

S. cerevisiae haploid wild type (DSC0367) and BER-/NER-defective (DSC707) cells were utilized to examine H_2O_2 sensitivity studies and mutation frequency studies. Site directed mutagenesis at the *NTG1* locus of the wild type (DSC0367) parent was performed via *delitto perfetto* protocol (68) in order to generate $Ntg1_{nls1}$, $Ntg1_{nls2}$, $Ntg1_{mts}$, and $Ntg1_{catalytic}$ encoded at the *NTG1* endogenous locus. The resulting mutants were then crossed with the BER-/NER-defective mutant (LAR023), creating diploids

which were then dissected to identify cells with the genotype *ntg2Δ apn1Δ rad1Δ*, *ntg1* which were selected for functional studies of Ntg1 (Table 1).

3.2 Exposure to DNA damaging agents. Cells were grown in 5 mL YPD or –URA SD minimal media to a density of 5 x 10^7 cells/mL, centrifuged, and washed with water. Cells were then resuspended in 5 mL water containing the appropriate DNA damaging agent: 2-20 mM H_2O_2 (Sigma); 55 mM MMS (Sigma); or 10 μg/mL antimycin A (Sigma). Cells were exposed to agent(s) for one hour at 30°C. The cytotoxicities of agents were evaluated by incubating cells in agent, washing cells with water, plating cells, and counting colonies.

3.3 Fluorescence microscopy. For all experiments, cultures were grown, exposed to DNA damaging agent, and stained with MitoTracker and DAPI as previously described (27). Cells were then analyzed by direct fluorescence confocal microscopy, employing a Zeiss LSM510 META microscope. Images were analyzed using the Carl Zeiss LSM Image Browser software. For quantitative studies of Ntg1 localization, cells were evaluated for nuclear only, mitochondrial only, or nuclear plus mitochondrial Ntg1-GFP localization. At least 200 cells were counted for each sample, and each analysis was repeated at least twice. Standard deviations of counts for three separate experimental repeats were calculated for each mutant and treatment condition.

3.4 Immunoblotting. The steady-state level of each Ntg1-GFP fusion protein variant was assessed by immunoblotting whole cell lysates probed with a polyclonal anti-GFP antibody (1:5,000 dilution) (62). Anti-3-phosphoglycerate (PGK) antibody

(1:10,000 dilution; Invitrogen) was utilized to determine the relative level of total protein lysate loaded in each lane.

3.5 Overexpression and purification of the recombinant Ntg1 variants. Recombinant Ntg1 was purified as previously described (53). Briefly, *E.coli* BL21 (DE3) cells containing each variant Ntg1-His_6 plasmids were grown in Luria broth (1% bacto-tryptone, 0.5% yeast extract, 1% sodium chloride) with ampicillin (100 µg/µL) to an OD600 of 0.5–1.0. Expression of Ntg1-His6 was induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 hours at 25°C. Pellets were collected by centrifugation at 7500 x g for 15 min and then lysed via sonication. The supernatants were applied to Ni+ affinity chromatography (Qiagen) to purify the Ntg1-His_6 variants and then further purified to apparent homogeneity by Mono S FPLC (Pharmacia).

3.6 Preparation of oligonucleotide and DNA strand scission assay. To assess the endonuclease activity of the Ntg1 variants, an oligonucleotide containing DHU at position 13 (DHU-31mer) was purchased from Midland Certified Reagent Company (Midland, TX). A complementary strand containing a guanine opposite the DHU position was obtained from Eurofins MWG/Operon (Huntsville, AL). The DHU-31mer was 5′-end-labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Promega) prior to annealing to the complementary strand (53). Single-stranded DHU-31mer was annealed in a 1:1.6 molar ratio to the appropriate complementary strand, heated to 80°C for 10 min, and cooled slowly to room temperature.

The endonuclease activity of the Ntg1 variants was assayed as previously described (73). Briefly, DNA strand scission assays were carried out in a standard reaction buffer (20 µL) containing 100 mM KCl, 10 mM Tris–HCl, pH 7.5, 1 mM

EDTA, 50 fmol of labeled DNA substrate, and 20 fmol of Ntg1 protein. Reactions were performed at 37°C for 15 min and then stopped by the addition of 10 μL of loading buffer (90% formamide, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) followed by heating at 90°C for 5 min. Reaction products were then resolved on a denaturing PAGE gel (15%) and analyzed with a STORM PhosphoImager (Amersham Biosciences).

3.7 Functional analysis of Ntg1 in vivo. To assess the biological function of the Ntg1, survival and mutagenesis experiments were carried out using the cells that express each Ntg1 variant encoded at the endogenous *NTG1* locus. The survival of wild type and repair-compromised or localization mutants was assessed by examining the sensitivity of cells to treatment with H_2O_2 as previously described (27). In order to assess the frequency of nuclear DNA mutation, an L-canavanine (CAN) resistance assay was employed (69). Wild type and repair-compromised cells were grown in 5 mL SD complete media or –URA SD minimal media to saturation. Dilutions of cells were plated onto YPD or CAN-containing medium (-ARG SD media with 60 μg/mL CAN) to identify forward mutations in the *CAN1* locus. The data from a minimum of 10 cultures were combined for each rate determination, and confidence intervals were determined. In order to assess the frequency of mitochondrial DNA mutation, an erythromycin resistance assay was employed. Erythromycin resistance assay conditions were adapted from (12). Cells were grown in 5 mL YPG and plated onto YPG and YPG plus 1 mg/mL erythromycin (Sigma) as previously described (17). Mutation frequencies were calculated as follows: number of erythromycin-resistant colonies/total number of

colonies. Reported average frequencies were calculated from 16 to 20 independent cultures, and confidence intervals were determined.

4. Results

4.1 Functional analysis of the targeting signals in Ntg1. Ntg1 is important for maintenance of both nuclear and mitochondrial genomes (17, 80). Consistent with this dual role, Ntg1 is localized to both nuclei and mitochondria (Figure 1B) (2, 80). Furthermore, oxidative DNA damage can trigger localization to organelles that harbor oxidative DNA damage (27). In order to determine how Ntg1 enters nuclei and mitochondria, it was first necessary to define the cis-acting sequences that target Ntg1 to each organelle. Ntg1 contains two putative monopartite cNLS sequences which were identified using the NUCDISC subprogram of PSORTII (Figure 1A) (55). The first predicted cNLS consists of amino acids 14-17 (RKRP) and the second consists of amino acids 31-37 (PEKRTKI) (27). Ntg1 also contains a putative MTS identified by the MitoProt II program as the first 26 amino acids (13). The putative MTS contains basic amino acids thought to be responsible for proper amphipathic alpha helix formation, including K3 and K6 (Figure 1A) (57). The putative active site sequence that mediates the DNA glycosylase/AP lyase activity of Ntg1 consists of amino acids 233-245 (ELLGLPGVGPKMA), and the key catalytic residue is proposed to be K243 (6, 27).

In order to assess the extent to which the predicted cNLS and MTS sequences direct localization of Ntg1 to nuclei and mitochondria, respectively, we created amino acid substitutions in key residues of Ntg1 and examined the localization of the resulting

proteins by creating C-terminal green fluorescent protein (GFP) fusion proteins (Table 2). cNLS1 (RKRP at residues 14-17) was changed to RAAP, creating $Ntg1_{nls1}$-GFP, while cNLS2 (PEKRTKI at residues 31-37) was changed to PEAATK. The localization of both $Ntg1_{nls1}$-GFP and $Ntg1_{nls2}$-GFP was primarily mitochondrial as compared to the dual nuclear and mitochondrial localization of wild type Ntg1-GFP (Figure 1B), indicating that both cNLS1 and cNLS2 are necessary for proper localization of Ntg1 to nuclei and suggesting that the two amino acid clusters represented by cNLS1 and cNLS2 could form two halves of a single bipartite cNLS. cNLS1 and cNLS2 were also altered in combination. The localization of $Ntg1_{nls1/2}$-GFP was primarily mitochondrial and similar to that of either cNLS mutant alone (Figure 1B). Despite the fact that cNLS1 overlaps with the mitochondrial cleavage site within the MTS, the changes made in $Ntg1_{nls1}$-GFP did not affect the localization of Ntg1 to mitochondria. Taken together, these results suggest that Ntg1 contains a bipartite cNLS with a longer linker sequence (16 amino acids) than most conventional bipartite cNLS motifs (46, 52) and is necessary for proper nuclear localization of Ntg1.

To experimentally define the MTS in Ntg1, we changed the third and sixth N-terminal basic amino acids in the sequence KISK to glutamic acid (EISE) to reverse the charge and reduce the potential to form the amphipathic alpha helix needed for mitochondrial entry (74). Localization of the resulting $Ntg1_{mts}$-GFP was primarily nuclear with little or no localization to mitochondria (Figure 1B). These results demonstrate that the N-terminal basic amino acids are important for mitochondrial localization of Ntg1.

In order to quantify the number of cells with nuclear or mitochondrial localization of Ntg1, we determined the number of cells containing nuclear only, mitochondrial only, or nuclear plus mitochondrial localization for cells expressing Ntg1-GFP, $Ntg1_{nls1}$-GFP, $Ntg1_{nls2}$-GFP, or $Ntg1_{mts}$-GFP (Figure 1C). $Ntg1_{nls1}$-GFP and $Ntg1_{nls2}$-GFP localization to nuclei was reduced approximately 60 percent, while $Ntg1_{mts}$-GFP localization to mitochondria was reduced approximately 40 percent. This quantitative analysis confirmed that cNLS1 and cNLS2, likely components of a bipartite cNLS, are major determinants of proper nuclear localization, and the MTS is important for efficient mitochondrial targeting of Ntg1. This analysis also indicates that Ntg1 may be able to localize to nuclei and mitochondria via nuclear protein import pathways other than those mediated through classical mechanisms since we do observe some residual localization. In order to verify that the localization of Ntg1 mutants was not due to a change in expression of Ntg1, we performed immunoblot analysis. This analysis revealed that the steady-state level of each Ntg1 variant was comparable to wild type Ntg1-GFP (Figure 1D, E).

To assess whether the cNLS and MTS motifs identified within Ntg1 are sufficient for targeting to nuclei and mitochondria, respectively, each targeting sequence was fused to a tandem GFP (GFP_2) (Experimental Procedures) and expressed in wild type cells. Two GFPs were employed to ensure that the cargo was too large (~52 kDa) to diffuse into and out of the nucleus (58). Direct fluorescence microscopy revealed that neither cNLS1-GFP_2 nor cNLS2-GFP_2 localized to the nucleus, while the sequence containing both cNLS1 and cNLS2 (cNLS1/2-GFP_2) was sufficient to target GFP_2 to the nucleus (Figure 2), indicating that both cNLSs are required for nuclear localization of Ntg1 and

confirming that cNLS1 and cNLS2, together, comprise a bipartite cNLS. These data suggest that the bipartite cNLS is important for the nuclear localization of Ntg1 and that Ntg1 interacts with the classical nuclear protein import machinery to access the nucleus.

4.2 The classical nuclear protein import machinery is required for nuclear localization of Ntg1. As Ntg1 contains a bipartite cNLS that is both necessary and sufficient for nuclear protein import of Ntg1 (Figures 1 and 2), we tested whether the classical nuclear protein import pathway is responsible for Ntg1 nuclear localization. The classical nuclear protein import pathway consists of the cNLS receptor subunit, importin α, and the nuclear pore targeting subunit, importin β (23). Both importin α and β are essential for classical nuclear localization, so we assessed the localization of Ntg1 in conditional mutants of importin α (*srp1-54*) and β (*rsl-1*) (42, 78). As controls, we also evaluated the localization of the previously characterized SV40 bipartite cNLS (34, 35) and non-cNLS containing protein, Nab2 (1), in these mutant cells. All proteins examined were properly localized to the nucleus in wild type cells both at the permissive and the non-permissive temperatures (Figure 3) and in both conditional mutants at the permissive temperature (data not shown). Both Ntg1-GFP and the SV40 bipartite cNLS were mislocalized to the cytoplasm in importin α mutant cells following a shift to the non-permissive temperature (Figure 3), indicating that importin α is required for proper nuclear localization of Ntg1. Similarly, Ntg1 and the SV40 bipartite cNLS were mislocalized in importin β mutant cells at the non-permissive temperature (data not shown). The control, Nab2-GFP, which is imported to the nucleus in an importin α-independent manner (1), was localized to the nucleus of importin α mutant cells at both the permissive and non-permissive temperatures, confirming that not all nuclear proteins

are mislocalized in this mutant (Figure 3). Sxm1 is a transport receptor that facilitates non-classical nuclear protein import (60). As a further control, Ntg1-GFP, SV40 bipartite cNLS, and Nab2-GFP all remained localized to the nucleus in Δ*sxm1* cells (Figure 3), indicating that Ntg1 is not mislocalized in all transport receptor mutant cells.

4.3 The NLS and MTS targeting signals are required for dynamic localization of Ntg1 in response to oxidative stress. Wild type Ntg1 is controlled by a mechanism of dynamic localization, in which nuclear oxidative DNA damage triggers recruitment of Ntg1 to the nucleus, and mitochondrial oxidative DNA damage triggers recruitment of Ntg1 to mitochondria (27). In order to determine whether mutants of Ntg1 containing defects in nuclear or mitochondrial targeting could respond to nuclear or mitochondrial oxidative DNA damage caused by increased oxidative stress, we assessed Ntg1 localization in cells exposed to hydrogen peroxide (H_2O_2), H_2O_2 plus antimycin, or methylmethane sulfonate (MMS). These exposures increase nuclear (H_2O_2, MMS) and mitochondrial (H_2O_2 plus antimycin, MMS) reactive oxygen species (ROS) and DNA damage (27). While nuclear localization of wild type Ntg1-GFP increased in response to nuclear oxidative stress (H_2O_2) and mitochondrial localization increased in response to mitochondrial oxidative stress (H_2O_2 plus antimycin) (27), the localization of $Ntg1_{nls2}$-GFP and $Ntg1_{mts}$-GFP was unchanged in the presence of oxidative stress (Figure 4). These results demonstrate that both the nuclear and mitochondrial targeting signals in Ntg1 are required for proper dynamic localization of Ntg1.

4.4 Dynamic localization of Ntg1 is required for response to DNA damage. In order to determine whether dynamic localization of Ntg1 is critical for proper response to DNA damage, we exploited isogenic wild type, BER-/NER- (*ntg1 ntg2 apn1 rad1*), and

ntg2 apn1 rad1 mutant strains (69) to assess the function of Ntg1 in vivo. While mutations accumulate at a higher rate in *ntg1* cells than wild type cells (17), BER-/NER-defective cells were utilized instead of *ntg1* cells in order to easily identify changes in mutation rates. BER-/NER- defective cells are severely compromised for the repair of oxidative DNA damage and are exquisitely sensitive to H_2O_2 (69). Loss of the BER activity provided by Ntg1 could result in two deleterious consequences: increased DNA mutation frequency and decreased survival in response to oxidizing agents (37, 54). To determine whether repair of oxidative DNA damage by Ntg1 plays a role in the prevention of nuclear and mitochondrial DNA mutation, we assessed the mutation rates of nuclear and mitochondrial DNA in the wild type, BER-/NER-deficient, and *ntg2 apn1 rad1* mutant cells. Nuclear mutation rates were assessed via an L-canavanine resistance assay (69), while mitochondrial mutation rates were assessed by an erythromycin resistance assay (56). BER-/NER-defective cells contain 68 times more nuclear DNA mutations than wild type cells, while *ntg2 apn1 rad1* cells harbor only 7 times more nuclear DNA mutations (Table 3), indicating that Ntg1 plays a very important role in preventing nuclear DNA mutations from occurring. In order to assess the impact of dynamic localization of Ntg1 on nuclear and mitochondrial mutation rates, *ntg2 apn1 rad1* $ntg1_{nls}$ and *ntg2 apn1 rad1* $ntg1_{mts}$ cells were analyzed. The mutation rates of *ntg2 apn1 rad1* $ntg1_{nls1}$ and *ntg2 apn1 rad1* $ntg1_{nls2}$ cells was significantly higher than the mutation rates of *ntg2 apn1 rad1* cells (Table 3), indicating that the nuclear localization of Ntg1 is important to prevent nuclear DNA mutations. The erythromycin resistance assay revealed that BER-/NER-deficient cells contain 46 times more mitochondrial mutations than wild type cells, while *ntg2 apn1 rad1* cells contained 6 fold more

mitochondrial mutations. While *ntg2 apn1 rad1* $ntg1_{nls1}$ and *ntg2 apn1 rad1* $ntg1_{nls2}$ cells did not contain more mitochondrial mutations than *ntg2 apn1 rad1* cells, *ntg2 apn1 rad1* $ntg1_{mts}$ cells contained 2.5 times more mitochondrial DNA mutations (Table 3), indicating that Ntg1 plays a role in reducing the number of incorporated mitochondrial mutations. In order to determine whether Ntg1 is important for cellular survival in the presence of oxidative stress, the survival of the same isogenic strains was assessed in the presence of H_2O_2. Survival of *ntg2 apn1 rad1* mutants in H_2O_2 was greater than that of BER-/NER-defective cells, but less than that of WT cells (Figure 5), indicating that the presence of Ntg1 partially rescues sensitivity to H_2O_2. The impact of dynamic localization of Ntg1 was assessed utilizing *ntg2 apn1 rad1* $ntg1_{nls}$ and *ntg2 apn1 rad1* $ntg1_{mts}$ cells, and the cytotoxicity of these cells in response to H_2O_2 was the same as that observed with *ntg2 apn1 rad1* cells (Figure 5). These results suggest that regulation of Ntg1 by dynamic localization prevents the accumulation of nuclear and mitochondrial DNA mutations, but this mode of regulation does not affect cellular survival in response to oxidative stress.

4.5 The DNA glycosylase/AP lyase activity of Ntg1 is not compromised by amino acid substitutions within the cNLS or MTS motifs. To confirm that the amino acid substitutions engineered to interfere with intracellular targeting of Ntg1 do not impair the catalytic activity of Ntg1, we performed DNA cleavage assays on Ntg1 substrate-containing oligonucleotides. For this experiment, we incubated purified recombinant Ntg1-His_6 (hexahistidine) variants with an oligonucleotide containing the Ntg1 substrate, dihydrouracil (DHU), and we detected Ntg1 endonuclease activity as cleavage of the oligonucleotide at the position of the DHU (79). $Ntg1_{nls1}$-His_6, $Ntg1_{nls2}$-

His_6, and $Ntg1_{mts}$-His_6 all exhibited robust enzymatic activity that is comparable to wild type Ntg1-His_6. To confirm that the cleavage activity detected is due to the endonuclease activity of Ntg1, we created a K243Q amino acid substitution within a key predicted catalytic residue (6, 27). As shown in Figure 6, cleavage of the DHU-containing oligonucleotide was not detected with this catalytic mutant Ntg1. This finding both confirms the specificity of the cleavage activity assay and provides the first experimental confirmation that K243 is required for the catalytic activity of Ntg1.

5. Discussion

5.1 Ntg1 as a model for the regulation of BER. BER is a critical process for the maintenance of both nuclear and mitochondrial genomic stability, which, in humans is significant for the prevention of disease. Little is known about the mechanisms that regulate BER in order to efficiently sustain genomic stability. The data in this study provide new insight into the regulation of BER by defining key components required for dynamic localization of Ntg1 that may function as part of a general mechanism for the regulation of BER. Specifically, we identified the bipartite cNLS, the MTS, and the catalytic site of Ntg1 and demonstrated that the classical nuclear protein import machinery, importin α/β, is necessary for nuclear localization of Ntg1. Furthermore, we determined that the bipartite cNLS and the MTS of Ntg1 are essential for proper dynamic localization of Ntg1 in response to nuclear or mitochondrial oxidative DNA damage. Importantly, we showed that in the absence of dynamic localization of Ntg1, nuclear and mitochondrial mutation rates increase. Given the role of BER in the prevention of human

disease, investigating this novel mode of BER regulation provides insight into how the cell prevents the deleterious consequences that result from oxidative DNA damage. Taken together, this data represents a major step forward in our understanding of the processes involved in the regulation of BER.

5.2 Components required for Ntg1 dynamic localization. Ntg1 is regulated by a mechanism of dynamic localization, which causes Ntg1 to localize to nuclei when nuclear oxidative DNA damage is elevated and to mitochondria when mitochondrial oxidative DNA damage is elevated. A major goal of this study was to define the key components that allow relocalization of Ntg1 to nuclei and mitochondria in response to oxidative DNA damage (27). The bipartite cNLS and MTS of Ntg1 were functionally defined, and in the absence of these signals, Ntg1 can no longer localize properly to nuclei or mitochondria, respectively, and can no longer participate in the process of dynamic localization (Figures 1 and 4). Previously, we proposed a model where the nuclear localization of Ntg1 is driven by nuclear oxidative DNA damage signals (NODDS), and mitochondrial localization is driven by mitochondrial oxidative DNA damage signals (MODDS) (27). We expand upon our previous model as our data suggests that NODDS and MODDS compete for the recruitment of Ntg1 to DNA damage loci (Figure 7). This concept is illustrated by the lack of dynamic localization of the MTS mutant Ntg1 to the nucleus during conditions of nuclear oxidative stress and of the cNLS mutant Ntg1 to mitochondria following mitochondrial oxidative stress. The inability to mobilize more Ntg1 into damage-containing organelles indicates that NODDS and MODDS compete with one another to recruit Ntg1, and the capacity for recruitment is exceeded under conditions where Ntg1 is already localized to one compartment or

another. Following the production of NODDS or MODDS, certain cellular components (responders) such as chaperones, nuclear import machinery, or sumoylation machinery are activated to interact with or modify Ntg1 in order to recruit it to the appropriate organelle to repair oxidative DNA damage. As a means of competing for Ntg1, we hypothesize that NODDS and MODDS activate DNA damage responders not only capable of facilitating recruitment, but also capable of preventing localization to the opposing organelle by interfering with crucial localization signals. Results from this study suggest that the classical nuclear protein import proteins, importin α/β, are novel DNA damage responders (Figure 7). As DNA damage responders, the classical nuclear protein import machinery associates with the bipartite cNLS of Ntg1 in order to facilitate recruitment of Ntg1 to nuclei in response to nuclear oxidative DNA damage.

In addition, it is likely that components of the sumoylation pathway are also DNA damage responders, potentially activated by NODDS, as previous studies from our group demonstrated that Ntg1 is sumoylated, and that sumoylated Ntg1 accumulates in nuclei in response to oxidative stress (27). The potential components of the sumoylation pathway that may play a role in nuclear localization of Ntg1 are the E2 conjugating enzyme, Ubc9, or the E3 ligating enzyme.

Identification of DNA damage responders that are activated by MODDS is a major objective of our future work, and as few mitochondrial import chaperones have been identified, there is a large opportunity for us to define the components required for mitochondrial import of Ntg1. We hypothesize that MODDS responders would interact with the MTS of Ntg1 in order to recruit Ntg1 into mitochondria in response to mitochondrial oxidative DNA damage (Figure 7).

5.3 Determining additional DNA damage responders. Identifying the remaining components of the dynamic localization process in response to NODDS and MODDS as well as the damage signals themselves is a major area of interest. One potential responder of particular significance is the stress response transcription factor, Yap1. Yap1 is sensitive to oxidative stress and responds by accumulating within the nucleus, where it upregulates genes that protect against cell stress-induced damage (44). Proteins capable of sensing the oxidative environment, such as Yap1, are DNA damage responders (61), and may function in pathways that regulate dynamic localization of Ntg1 or other BER proteins in response to oxidative DNA damage. We hypothesize that some of the downstream targets of Yap1 may be responders to NODDS and MODDS. Other potential NODDS or MODDS responders may include members of the DNA damage checkpoint pathways, Mec1/hATM (ataxia telangiectasia mutated), which is involved in the repair of double strand breaks (64), and Tel1/hATR (ataxia-telangiectasia mutated and rad3-related), which activates repair machinery in response to lesions generating single strand breaks (31).

5.4 Logistics of Ntg1 dynamic localization. A key question that arises from these results is: what is the pool of Ntg1 that relocalizes to organelles containing elevated levels of oxidative DNA damage? It is possible for proteins to relocalize from the cytoplasm to the nucleus or mitochondria as well as from nuclei to cytoplasm. To our knowledge, there is no evidence that proteins can exit mitochondria in order to relocalize to nuclei. It is thought that proteins targeted to mitochondria are unable to exit and are degraded when no longer needed; however, in some very specific instances, extramitochondrial localization of the mitochondrial forms of HSP60, HSP70, fumarase,

and a few other proteins have been demonstrated (66). As this is the case, we speculate that the pool of Ntg1 that directs localization likely resides in the nucleus and the cytoplasm. In the event of mitochondrial oxidative DNA damage, more Ntg1 would be released from nuclei into the cytoplasm and shuttled to mitochondria by responders that associate with the MTS of Ntg1. In the event of nuclear oxidative DNA damage, nuclear retention of Ntg1 is elevated, and Ntg1 is shuttled into nuclei via interactions between the bipartite cNLS of Ntg1 and importin α/β (Figure 7). A major focus of future studies is to address whether the pools of Ntg1 that relocalize are recruited from nuclei, cytoplasm, mitochondria, or a combination of these locations.

5.5 Putative BER proteins regulated by dynamic localization. We speculate that dynamic localization in response to DNA damage signals may be a mode of regulation that is employed by many BER proteins. Various BER proteins localize to both mitochondria and nuclei (48), all of which are candidates for regulation by dynamic localization. One particularly interesting candidate is the human AP endonuclease 1 (hAPE1), which relocalizes from the cytoplasm to nuclei and mitochondria following exposure to oxidative stress (H_2O_2) (21, 71). Other potential candidates can be identified by sequence homology to Ntg1, such as the *S. cerevisiae* BER protein uracil DNA glycosylase, Ung1, which contains a bipartite cNLS and MTS tantalizingly similar to that of Ntg1 (8). Additionally, both hNTH1 and mNTH1 contain bipartite cNLS and MTS signals that target them to both nuclei and mitochondria (38, 70), suggesting that the mode for regulating eukaryotic Nth-like proteins is conserved from yeast to humans. These findings suggest that dynamic localization of BER proteins in response to DNA damage signals may be a general mechanism for regulating BER.

5.6 Implications of dynamic localization as a regulator of genomic stability.

Regulation of BER by dynamic localization of Ntg1 plays a distinct role in maintaining genomic stability. Dynamic localization of Ntg1 protects both nuclear and mitochondrial DNA from mutation (Figure 5). As the accumulation of DNA mutations is associated with nuclear and mitochondrial genomic stability (14, 17), these results indicate that dynamic localization of Ntg1 plays a key role in maintaining the integrity of nuclear and mitochondrial DNA. Dynamic localization of Ntg1 does not appear to affect cellular survival in response to oxidative stress. While this result may appear contradictory, a few potential explanations for this result exist. First, preventing nuclear or mitochondrial localization of Ntg1 by mutating key localization signals may not fully preclude entry of Ntg1 into these organelles. Alternative pathways for Ntg1 nuclear and mitochondrial localization may exist so that in *ntg2 apn1 rad1* $ntg1_{nls}$ cells, for example, Ntg1 can enter the nucleus via pathways that are not mediated by importin α/β. In the case of BER-/NER-deficient cells, Ntg1 is not expressed and thus cannot localize to nuclei in order to repair replication or transcription blocks, resulting in cell death. In situations where Ntg1 can localize to nuclei and mitochondria via alternative pathways, it is possible that repair of potentially lethal damage is prioritized above the repair of potentially mutagenic lesions, as *ntg2 apn1 rad1* $ntg1_{nls}$ and *ntg2 apn1 rad1* $ntg1_{mts}$ cells accumulate nuclear or mitochondrial DNA mutations, respectively, but are not cytotoxic relative to *ntg2 apn1 rad1* cells in H_2O_2. Second, cellular survival may be influenced not only by blocks to transcription and replication but also by the total level of nuclear and mitochondrial mutations that accumulate in the absence of Ntg1. In this case, localization of Ntg1 to either nuclei or mitochondria may allow for sufficient reduction in the overall burden of

mutations in order to permit cell survival. In the case of BER-/NER-deficient cells, mutation rates in both nuclei and mitochondria are increased, and this is correlated with increased cell death in the presence of H_2O_2. Increased mutation rates in only one organelle, as seen in *ntg2 apn1 rad1* $ntg1_{nls}$ and *ntg2 apn1 rad1* $ntg1_{mts}$ cells, may not be sufficient to cause increased cell death. Third, although Ntg1 is involved in the repair of abasic sites, 8-oxo-guanine, 4,6-diamino-5-formamidopyrimidine (FapyAde), and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), all of which can pose blocks to replication or transcription (25, 47, 72), Ntg1 may not be essential for the repair of DNA lesions that are lethal to the cell by posing replication fork blocks and stalling of transcription machinery. Another DNA repair protein or pathway, such as recombination repair, translesion synthesis, or transcription coupled repair, may compensate in the absence of nuclear Ntg1, removing lesions that pose blocks to replication and transcription. This explanation is the least favored as alternative repair pathways were unable to rescue the survival of BER-/NER-deficient cells, which completely lack Ntg1.

Nuclear and mitochondrial mutation rates of *ntg2 apn1 rad1* $ntg1_{cat}$ were reduced compared to BER-/NER- defective cells. These results suggest that mutation of the catalytic activity of Ntg1 may not completely eliminate the catalytic activity of Ntg1; however, identical mutations at the active site of *E. coli* Nth inhibits all catalytic activity of Nth (20), suggesting that the catalytic activity of the Ntg1 catalytic mutant is also fully inhibited. Therefore the difference between *ntg2 apn1 rad1* $ntg1_{cat}$ cells and BER-/NER-defective cells is more likely due to significantly higher ROS levels in BER-/NER-defective cells or an unknown activity of Ntg1. For example, Ntg1 may be capable of recruiting other DNA damage response proteins to sites of DNA damage. In this

situation, the mutation rates would be increased in *ntg2 apn1 rad1* $ntg1_{cat}$ cells due to the absence of catalytic activity, but the rates would be lower than those of BER-/NER-defective cells due to the potential recruitment capability of Ntg1.

The fact that dynamic localization is important for thwarting DNA mutagenesis emphasizes the impact that this mode of regulation has on disease prevention. By preventing mutation of critical nuclear and mitochondrial genes, such as tumor suppressor genes and oncogenes in human cells, dynamic localization of BER proteins likely plays a crucial role in preventing human diseases such as cancer and neurodegenerative disorders. It is likely that other BER proteins are regulated in a similar manner via dynamic localization. As many BER proteins recognize lesions that pose replication fork and transcription blocks (18, 25, 47, 72), dynamic localization of these BER proteins will likely play a role in both maintaining nuclear and mitochondrial genomic stability and preventing cell death. This dual role of dynamic localization of BER proteins would then be responsible for protecting humans against diseases resulting from both gene mutations, such as cancer and neurodegenerative disorders, and cell death, as oxidative stress-related cell death of neuronal and muscle cells in humans is implicated in several degenerative disorders (39). By demonstrating the important consequences of Ntg1 regulation via dynamic localization in response to NODDS and MODDS, we have initiated the exploration of an important mode of regulation for BER. Given the probability that numerous BER proteins are regulated in this manner, dynamic localization likely confers the ability of BER to prevent numerous human disease and disorders.

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Table 1: Strains and Plasmids Used in this Study

Strain or Plasmid	Description	References
FY86 (ACY193)	*MATα ura3-52 leu2Δ1 his3Δ200*	(80)
PSY883 (ACY208)	*MATα ura3-52 trp1Δ63 leu2 his3-11 ade2-1 rsl1Δ::HIS3*	(43)
SJR751 (DSC0025)	*MATα ade2-101*$_{oc}$ *his3Δ200 ura3ΔNco lys2ΔBgl leu2-R*	(69)
SJR1101 (DSC0051)	*MATα ade2-101*$_{oc}$ *his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1Δ::HIS3 rad1Δ::hisG*	(69)
ACY443	*MATa leu2Δ1 his3Δ200 trp1Δ63 sxm1 ΔHIS3*	(62)
ACY1563	*MATa ura3 leu2 his3 trp1 ade2 can1 srp1–54*	Masayasu Nomura, UC Irvine
DSC0282	*MATa ura3-52 leu2Δ1 his3Δ200 ntg1::KANAMYCIN bar1::HYG*	(27)
DSC0367	*MATa his7- lys2Δ3'::LEU-lys2Δ5' ade5-1 trp1-289 ura3-52*	(14)
DSC0369	*MATa his7-1 lys2Δ3'::LEU-lys2Δ5' ade5-1 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1 rad1::kanMX*	(14)
DSC0371	*MATa his7-1 lys2Δ3'::LEU-lys2Δ5' ade5-1 ura3-52 ntg2::BSD apn1::TRP1 rad1::kanMX*	(14)
DSC0430	*MATa his7-1 lys2Δ3'::LEU-lys2Δ5' ade5-1 ura3-52 ntg2::BSD apn1::TRP1*	This study

	rad1::kanMX NTG1$_{nls1}$	
DSC0431	*MATa his7-1 lys2Δ3'::LEU-lys2Δ5' ade5-1 ura3-52 ntg2::BSD apn1::TRP1 rad1::kanMX NTG1*$_{nls2}$	This study
DSC0432	*MATa his7-1 lys2Δ3'::LEU-lys2Δ5' ade5-1 ura3-52 ntg2::BSD apn1::TRP1 rad1::kanMX NTG1*$_{mts}$	This study
DSC0433	*MATa his7-1 lys2Δ3'::LEU-lys2Δ5' ade5-1 ura3-52 ntg2::BSD apn1::TRP1 rad1::kanMX NTG1*$_{cat}$	This study
LAR023	*MATα his7-1 lys2Δ3'::LEU-lys2Δ5' ade5-1 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1 rad1::kanMX*	(14)
pAC719	*NAB2-GFP, 2μ, URA3*	(26)
pAC891	*SRP1-c-myc* (3X), *CEN, URA3, AMP*^{R}	(30)
pAC960	*ΔIBB-SRP1-c-myc* (3X), *CEN, URA3, AMP*^{R}	(30)
pAC1056	*BPSV40-NLS-* GFP_2, *CEN*, *URA3*, *AMP*^{R}	(34)
pAC1069	GFP_2, *CEN*, *URA3*, *AMP*^{R}	(34)
pAC1338	pGEX-4T *GST-ΔIBB-SRP1*	(51)
pAC1339	pGEX-4T *GST-ΔIBB-srp1*(D203K, E402R)	(51)
pNTG1-GFP pAC2669	*NTG1-GFP*, *2μ, URA3, AMP*^{R}	(41, 75, 80)
pD0386	*NLS1*$_{NTG1}$- GFP_2, *CEN*, *URA3*, *AMP*^{R}	This study
pD0387	*NLS2* $_{NTG1}$- GFP_2, *CEN, URA3, AMP*^{R}	This study
pD0388	*NLS1/NLS2* $_{NTG1}$- GFP_2, *CEN, URA3, AMP*^{R}	This study
pD0389	*MTS* $_{NTG1}$- GFP_2, *CEN, URA3, AMP*^{R}	This study
pD0390	pET-15b Ntg1-His_6, LacI ,AMP^R	This study

pD0391	pET-15b $Ntg1_{nls1}$-His_6, LacI ,AMP^R	This study
pD0392	pET-15b $Ntg1_{nls2}$-His_6, LacI ,AMP^R	This study
pD0393	pET-15b $Ntg1_{mts}$-His_6, LacI ,AMP^R	This study
pD0394	pET-15b $Ntg1_{catalytic}$-His_6, LacI ,AMP^R	This study
pD0395	*$NTG1_{nls1}$-GFP, 2μ, URA3, AMP^R*	This study
pD0396	*$NTG1_{nls2}$-GFP, 2μ, URA3, AMP^R*	This study
pD0397	*$NTG1_{nls1/nls2}$-GFP, 2μ, URA3, AMP^R*	This study
pD0398	*$NTG1_{mts}$-GFP, 2μ, URA3, AMP^R*	This study
pD0399	*$NTG1_{nls1/nls2/mts}$-GFP, 2μ, URA3, AMP^R*	This study

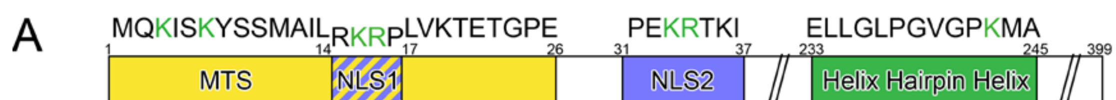
Table 2: Ntg1 Localization Motifs

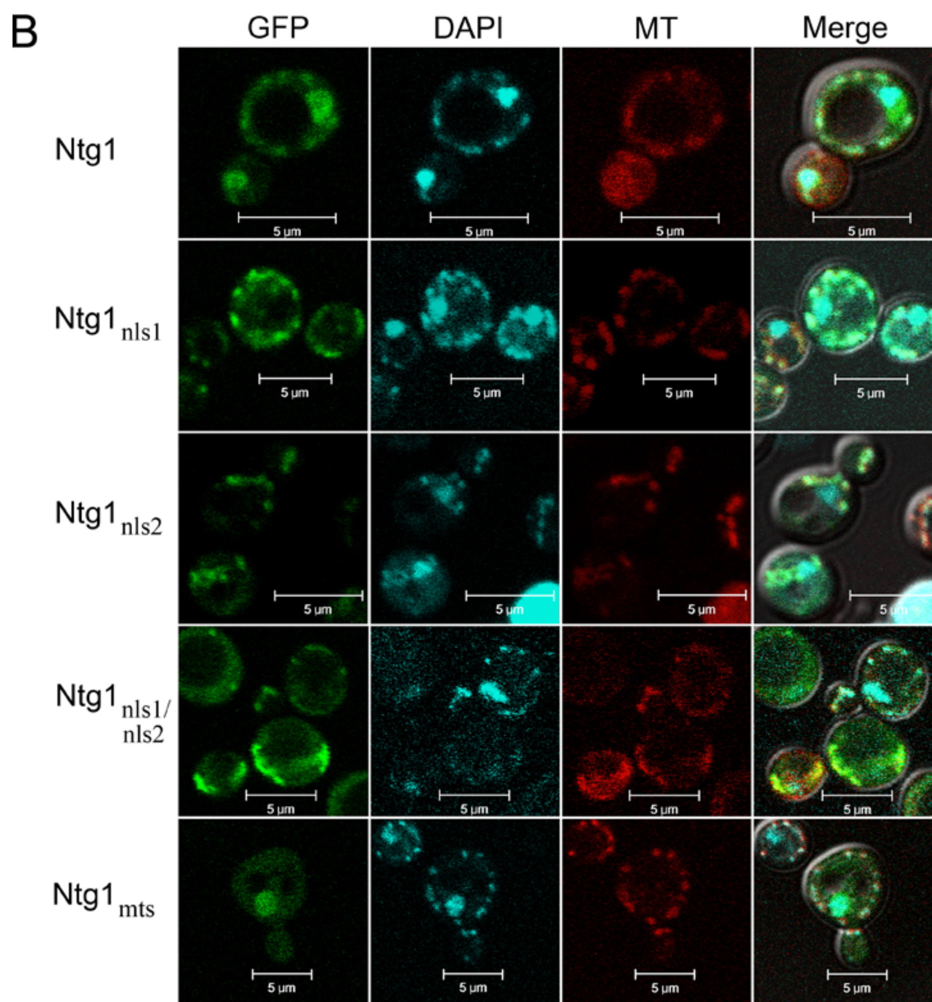
Ntg1 localization sequence name	Residue number	Wild type amino acid sequence	Mutant amino acid sequence
cNLS1	14-17	RKRP	RAAP
cNLS2	31-37	PEKRTKI	PEAATKI
MTS	1-26	MQKISKYSSMAILRKRPL VKTETGPE	MQEISEYSSMAILRKRPL VKTETGPE

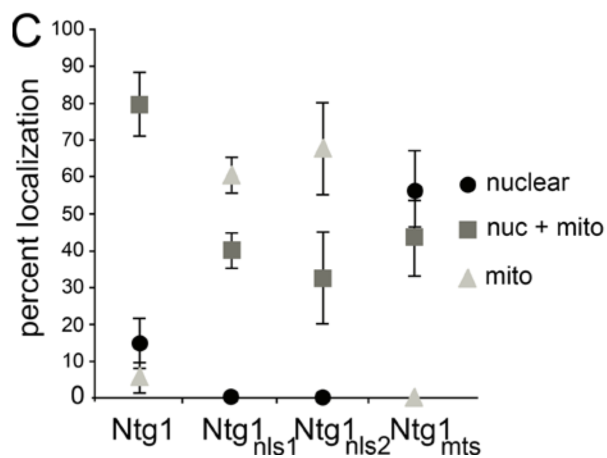
Table 3: Rates of Nuclear and Mitochondrial Mutations with Different DNA Excision Repair Capacities

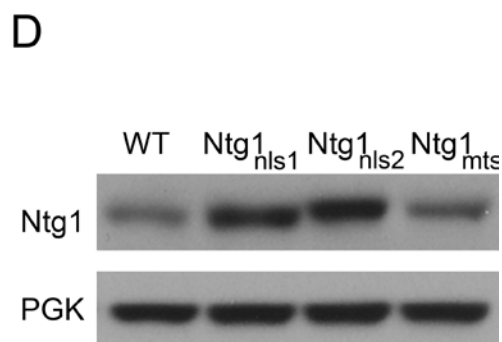
DNA Repair Background	Nuclear Mutation Rate (m) (10^{-7}) (95% Confidence Limits)	Fold Change	Mitochondrial Mutation Frequency (*f*) (10^{-8}) (95% Confidence Limits)	Fold Change
WT	6 (1 - 43)	1	2.0 (1.0 - 4.7)	1
BER-/NER-	413 (212 - 623)	68	53.6 (28.9 - 117)	28
apn1 ntg2 rad1	44 (29 - 58)	7	5.9 (4.6 – 13.8)	3
apn1 ntg2 rad1 $ntg1_{nls1}$	156 (103 - 684)	26	11.0 (7.1 - 20.8)	6
apn1 ntg2 rad1 $ntg1_{nls2}$	83 (60 - 175)	14	5.5 (4.0 - 14.3)	3
apn1 ntg2 rad1 $ntg1_{mts}$	37 (25 - 59)	6	15.0 (10.7- 16.9)	8
apn1 ntg2 rad1 $ntg1_{cat}$	274 (117-1190)	45	16.2 (3.9 - 22.9)	8

Figure 1









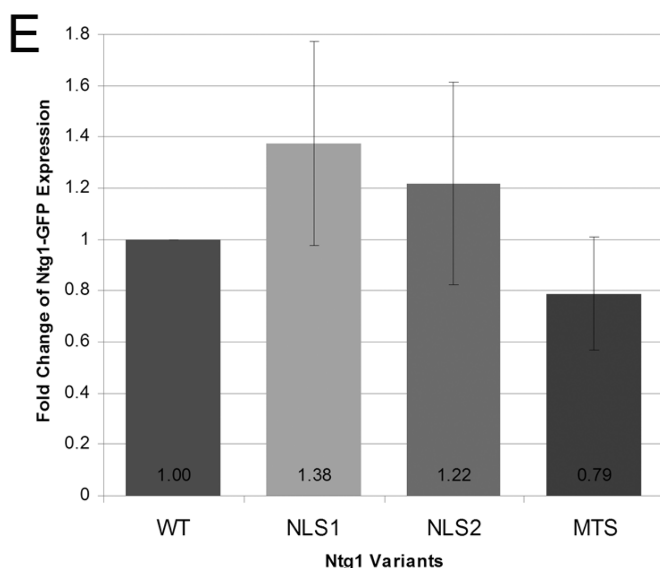


Figure 1. Definition of Functional Intracellular Targeting Signals within Ntg1. **A.** Schematic of Ntg1. Residues predicted to be critical for nuclear and mitochondrial localization and catalytic activity of Ntg1 are indicated. The basic amino acids predicted to be important for mitochondrial localization (K3 and K6), two putative cNLSs (residues 14-16 and 31-37), and the putative active site lysine (K243) within the helix hairpin helix active site motif (residues 233-245: ELLGLPGVGPKMA) are shown. Green residues were altered in this study in order to examine function. The green lysine (K243) is the putative active site lysine. **B.** The localization of GFP-tagged proteins was assessed via direct fluorescence microscopy. GFP (green), DAPI (blue), Mitotracker (red), and merged images of cells expressing Ntg1 wildtype (WT), $Ntg1_{nls1}$, $Ntg1_{nls2}$, $Ntg1_{nls1/2}$, and $Ntg1_{mts}$ variants of Ntg1-GFP are shown. **C.** Quantification of WT, NLS mutant, and MTS mutant Ntg1-GFP localization. Localization of Ntg1-GFP variant to nuclei only (nuclear), mitochondria only (mito), or nuclei plus mitochondria (nuc + mito) was determined for each cell examined and plotted as percentage of the total cells evaluated. Error bars represent standard deviation. **D.** Steady state expression levels of Ntg1

variants. Immunoblots of whole cell lysates from cells expressing Ntg1-GFP, $Ntg1_{nls1}$-GFP, $Ntg1_{nls2}$-GFP, or $Ntg1_{mts}$-GFP. The immunoblot was probed with anti-GFP antibody to detect Ntg1, and anti-PGK antibody as a loading control. **E.** Quantification of WT, NLS mutant, and MTS mutant expression. The normalized expression levels were quantified for each sample from five separate immunoblot experiments. Error bars represent standard deviation.

Figure 2

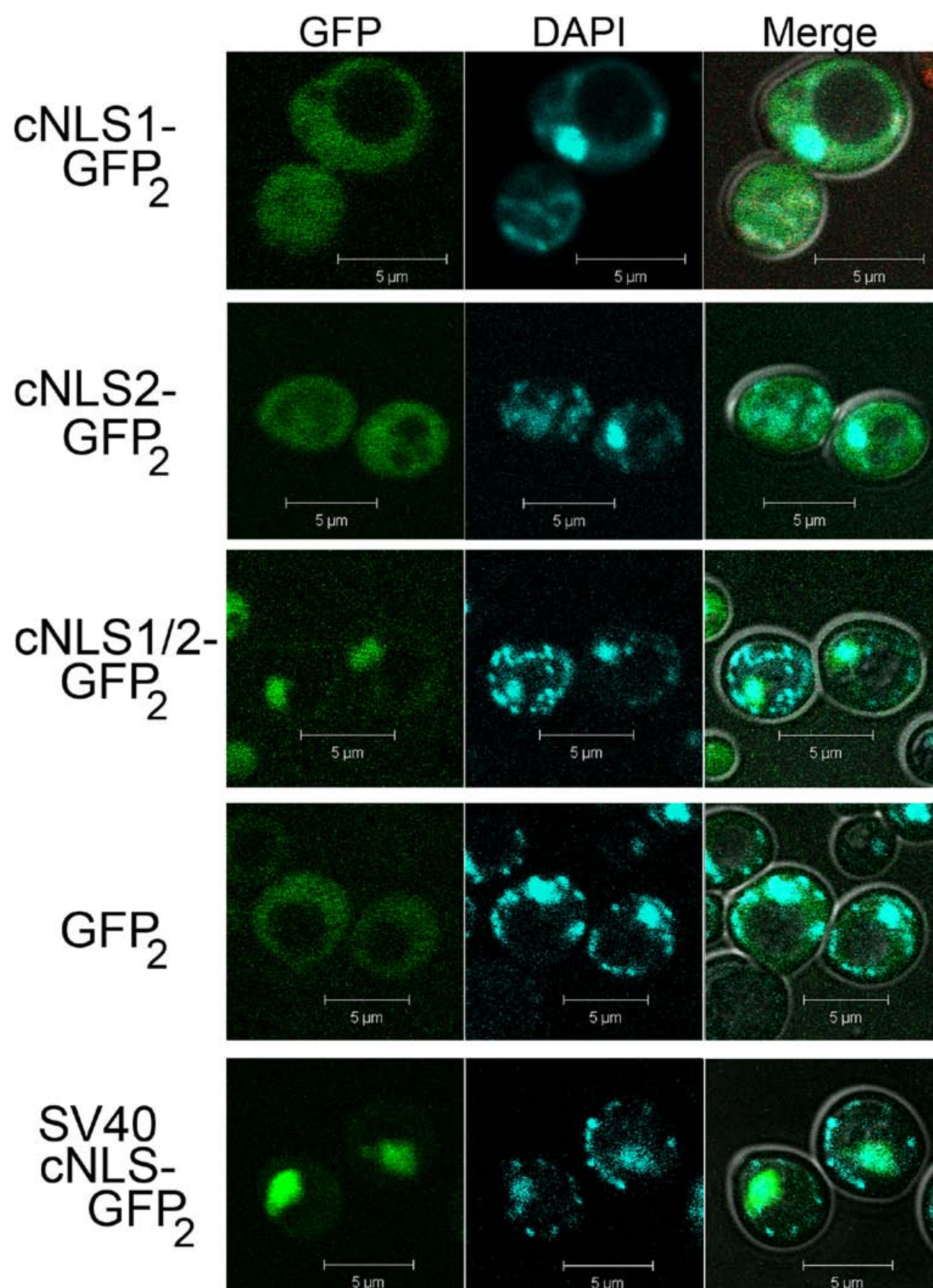


Figure 2: The Bipartite cNLS of Ntg1 is Sufficient to Direct Nuclear Localization of Ntg1. GFP (green), DAPI (blue), and merged images of cells expressing $Ntg1_{cNLS1}$-GFP_2, $Ntg1_{cNLS2}$-GFP_2, $Ntg1_{cNLS1/2}$-GFP_2, and the control proteins, GFP_2 alone (empty vector) and $SV40_{cNLS}$-GFP_2 (positive control).

Figure 3

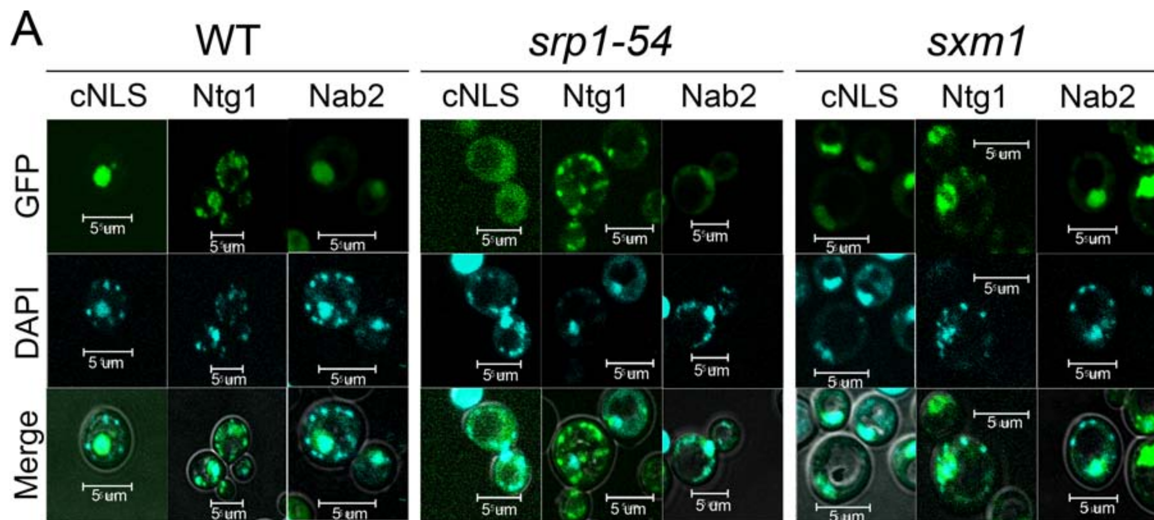


Figure 3: The Classical Nuclear Protein Import Pathway is Required for Nuclear Localization of Ntg1. **A.** Localization of Ntg1-GFP (Ntg1), and two control proteins, a cNLS cargo, SV40 bipartite cNLS (cNLS), and a non-cNLS cargo, Nab2-GFP (Nab2), was assessed via direct fluorescence microscopy. GFP (green), DAPI (blue) to indicate the position of the nucleus, and merged images of wild type (WT), importin α (*srp1-54)*, and control *sxm1Δ* mutant cells are shown.

Figure 4

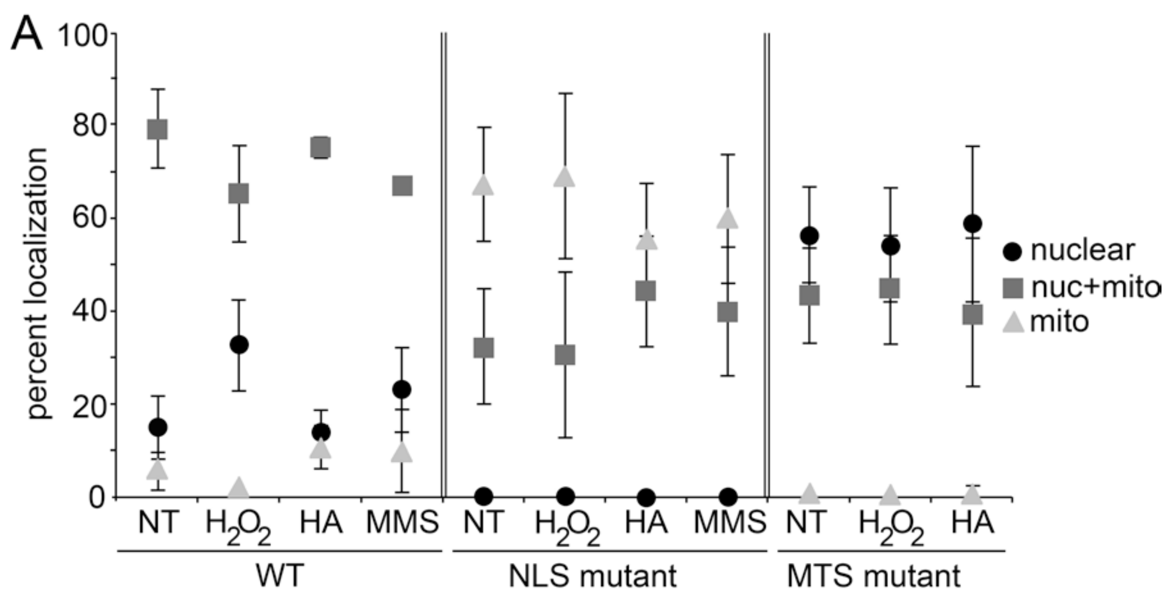


Figure 4: Functional Intracellular Targeting Signals are Required for Dynamic Localization of Ntg1 in Response to Oxidative DNA Damage. Quantification of the localization of wild type Ntg1-GFP, $Ntg1_{nls2}$-GFP , and $Ntg1_{mts}$-GFP following cellular stress. Cells were not treated (NT) or were exposed to 20 mM H_2O_2, 55 mM MMS, or 20 mM H_2O_2 plus 10 µg/mL antimycin (HA) as described (See Experimental Procedures). The localization of Ntg1-GFP variants to nuclei only (nuclear), mitochondria only (mito), or nuclei plus mitochondria (nuc + mito) was determined for each cell and plotted as percentage of the total cells evaluated for at least 100 cells per variant and condition. Error bars represent standard deviation.

Figure 5

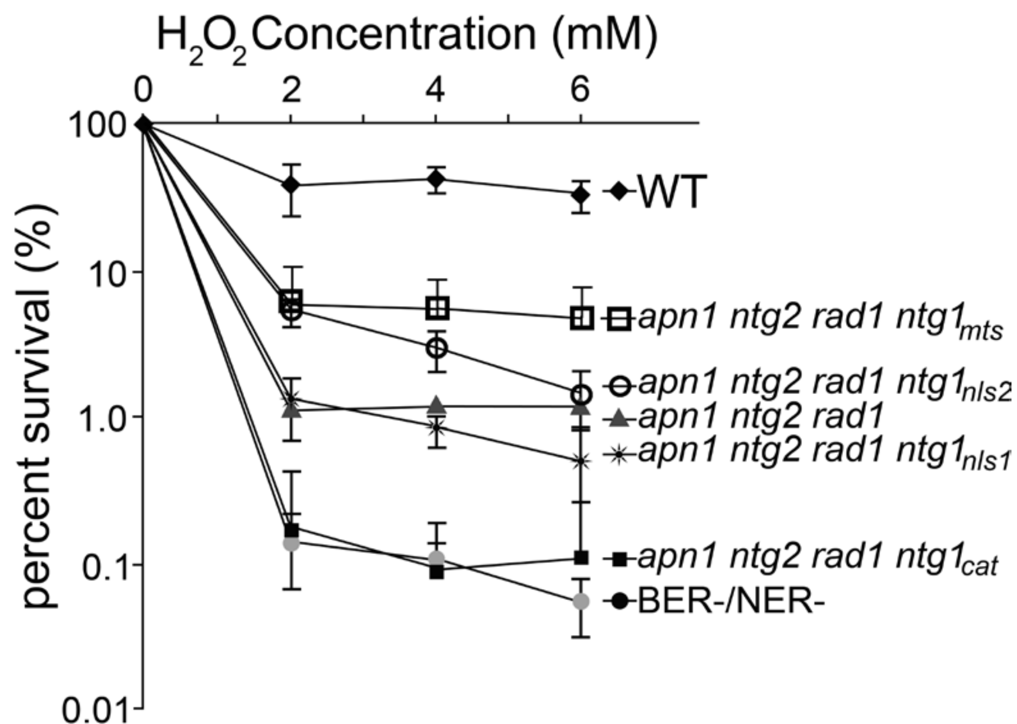


Figure 5: Functional Analysis of the Dynamic Localization of Ntg1. The H_2O_2 sensitivity of wild type (WT), BER-/NER- defective (*apn1 ntg1 ntg2 rad1*), *apn1 ntg2 rad1* and *apn1 ntg2 rad1 ntg1$_{mutant}$* cells was assessed. The percent survival was set to 100% for untreated samples and was determined for 0, 2, 4, and 6 mM H_2O_2 doses. Error bars indicate standard deviations in data.

Figure 6

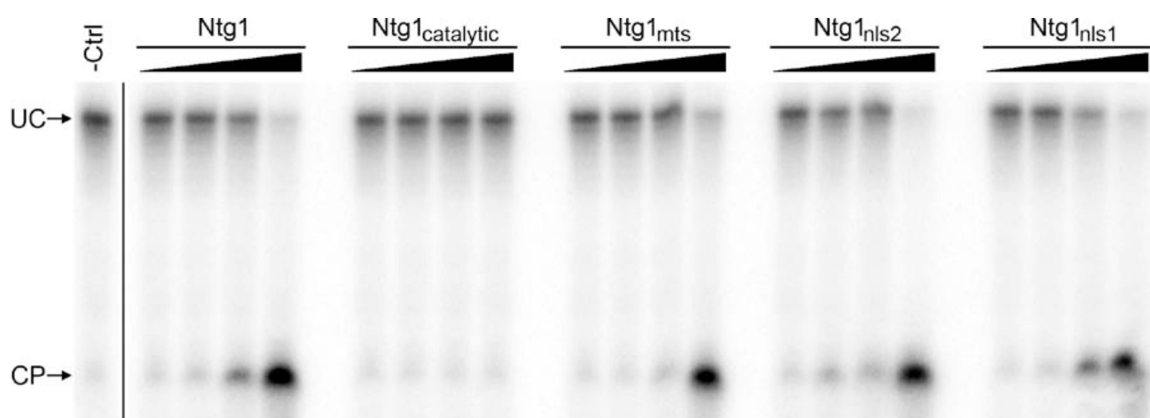


Figure 6: Amino Acid Substitutions within Intracellular Targeting Signals do not Affect the Catalytic Activity of Ntg1. Ntg1 DNA glycosylase/AP lyase activity was assessed by monitoring cleavage of a ^{32}P 5′-end-labeled oligonucleotide (31mer) containing dihydrouracil by the Ntg1 variant proteins, Ntg1, $Ntg1_{nls1}$, $Ntg1_{nls2}$, $Ntg1_{mts}$, or $Ntg1_{catalytic}$. The positions of the uncleaved 31mer oligonucleotide (UC) and the cleaved 13mer oligonucleotide (CP) are indicated. No enzyme was added to the negative control lane (-Ctrl). Protein concentrations are as follows from left to right: 1.85 ng/μL, 5.5 ng/μL, 16.6 ng/μL, and 50 ng/μL. All lanes are from the same gel at the same exposure.

Figure 7

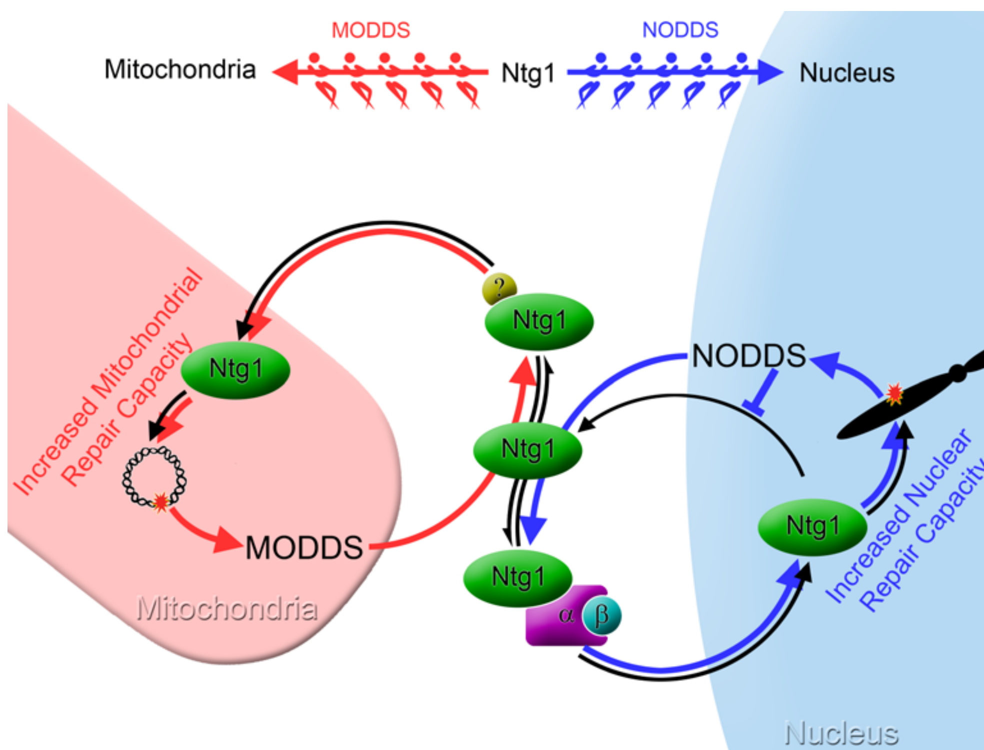


Figure 7: Model of Ntg1 Dynamic Localization in Response to Nuclear and Mitochondrial Oxidative DNA Damage. Nuclear oxidative DNA damage signals (NODDS) and mitochondrial oxidative DNA damage signals (MODDS) compete for the recruitment of Ntg1 to sites of oxidative DNA damage from the cellular pool of Ntg1. The cellular pool is comprised of Ntg1 in constant flux between the nucleus and the cytoplasm. NODDS (blue) promote the association between Ntg1 and DNA damage responders in the cell. These responders are members of networks of stress response pathways. The classical nuclear protein import machinery, including importin α/β, is one such DNA damage responder that is activated by NODDS. MODDS (red) activate another class of DNA damage responders. The response to NODDS and MODDS by Ntg1 and DNA damage responders results in appropriate concentrations of Ntg1 in nuclei

and mitochondria, given the oxidative DNA damage levels in each organelle. Once localized to these organelles, Ntg1 facilitates the repair of nuclear or mitochondrial oxidative DNA damage, thus preventing cell death and promoting genomic stability. Black arrows represent localization under steady state conditions.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

The generation of reactive oxygen species (ROS) during cellular metabolism causes oxidative stress in cells that can damage DNA, proteins, and lipids (34). Unrepaired oxidative DNA damage leads to cell death and mutagenesis, which is associated with aging and numerous pathologies, including cancer and other degenerative disorders (1, 4, 21, 35, 36). As base excision repair (BER) is the primary pathway for repairing oxidative DNA damage, it is a very important pathway for the prevention of various human diseases (22, 24), and mutations in numerous BER proteins are associated with multiple cancer types (31).

Nuclear and mitochondrial DNA accumulate oxidative damage, and BER occurs in both organelles in order to alleviate the potential deleterious consequences of cell death and mutagenesis. Despite this important role of BER, very little is known about how the process of BER is regulated. Certain BER proteins, such as Ntg1, Ogg1, Ung1 and Ape1, localize to nuclei and mitochondria, and it is thought that their primary role is to reduce levels of oxidative DNA damage in both organelles (20). As proteins such as these must be capable of detecting oxidative lesions in two subcellular compartments, the localization of these proteins appeared most promising as a potential target for BER regulation.

This dissertation delineates ways in which the *S. cerevisiae* BER protein, Ntg1, is regulated in response to nuclear and mitochondrial oxidative DNA damage. Several important findings regarding the regulation of Ntg1 via localization were reported in this dissertation: 1) Ntg1 is present in both nuclei and mitochondria. Increases in nuclear or mitochondrial ROS levels induce the accumulation of Ntg1 in each of these organelles. 2) Mitochondrial oxidative DNA damage, and not ROS levels, *per se*, stimulates the

localization of Ntg1 to mitochondria, and we hypothesize that nuclear oxidative DNA damage produces the signal for Ntg1 nuclear localization. 3) Ntg1 is sumoylated, and sumoylation appears to affect the nuclear localization of Ntg1. 4) Ntg1 localization is not regulated in a cell cycle-dependent manner. 5) The bipartite cNLS and MTS are important for the nuclear and mitochondrial dynamic localization of Ntg1, and the classical nuclear localization proteins, importin α and β are DNA damage responder proteins that allow Ntg1 to localize to the nucleus in response to nuclear oxidative DNA damage. 6) Dynamic localization of Ntg1 contributes to the collective effort by repair proteins to prevent the accumulation of nuclear and mitochondrial mutations. We consider these findings to be a significant contribution relating to the regulation of BER as we hypothesize that other BER proteins are regulated in a similar manner.

Dynamic localization is a major mode of regulation for the S. cerevisiae BER protein, Ntg1. The data presented in Chapter 2 illustrate how the localization of Ntg1 is regulated. Central to this dissertation, the localization of Ntg1 changes when cells are exposed to nuclear versus mitochondrial oxidative stress (Chapter 2, Figures 2, 3). By increasing oxidative stress in nuclei, nuclear oxidative DNA damage is induced, requiring a response by Ntg1. Similarly, inducing mitochondrial oxidative stress results in increased mitochondrial oxidative DNA damage, which requires repair by Ntg1. In each of these cases, Ntg1 is recruited to the appropriate organelle to initiate BER.

Additionally in Chapter 2, we were able to distinguish whether it is ROS or oxidative DNA damage that produces a signal to recruit Ntg1. With the use of rho^0 cells,

we determined that Ntg1 mitochondrial localization is mediated by DNA (Chapter 2, Figure 4), and we hypothesize that Ntg1 nuclear recruitment works in a similar manner. The caveat to this interpretation is that not only does ROS cause oxidative DNA damage, but oxidative DNA damage results in the production of ROS (10, 27). It is possible that the initial ROS create the oxidative DNA damage in the first place, while the latter ROS produce the signal that recruits Ntg1 to the proper organelle. Therefore, we hypothesize that the recruitment of Ntg1 to nuclei and mitochondria is not ROS-dependent, *per se*, but is mediated by oxidative DNA damage, instead.

Regulation of proteins by changing localization in response to a stimulus, such as that of Ntg1 in response to oxidative DNA damage, implies a process that is capable of rapid response. If new protein production was necessary every time an oxidative DNA lesion occurred, the response time for removal of the lesion would be much longer than if the cell could simply localize a protein that already exists. Ntg1 and many other BER proteins recognize and excise lesions that can result in either nuclear and mitochondrial DNA mutagenesis or can pose blocks to replication and transcription machinery (7, 8, 10, 12, 19, 33). Therefore, regulation of BER proteins at the level of localization is advantageous in order to remove DNA damage in a short amount of time so as to reduce the incorporation of nuclear and mitochondrial mutations and reduce the risk of cell death posed by replication and transcription blocks.

The data presented in Appendix 1 suggest that the localization of Ntg1 is not cell cycle-dependent. As we saw no evidence that nuclear or mitochondrial Ntg1 localization changed depending on the cell cycle phase, we hypothesize that the localization of Ntg1 is predominantly regulated by changes in levels of nuclear and mitochondrial oxidative

DNA damage. It is likely that utilizing a mechanism of regulation other than cell cycle-dependent localization is advantageous to the cell because this mode of regulation would likely result in the accumulation of oxidative DNA damage during certain cell cycle phases, which could result in genomic instability.

The localization of Ntg2 is not dynamic. The fact that the localization of Ntg2 is nuclear only and does not change in response to oxidative DNA damage levels (Chapter 2, data not shown) implies that Ntg2 resides solely in the nucleus to ensure nuclear genomic integrity. Therefore, if Ntg1 is in the process of responding to major mitochondrial DNA damage and is depleted from nuclei, Ntg2 is present to guarantee that the nuclear DNA will remain stable.

The finding that only one of the two Ntg DNA glycosylases in *S. cerevisiae* responds to increased oxidative DNA damage with a change in localization is notable. Budding yeast possess two *Escherichia coli* Nth homologs, indicating that Ntg1 and Ntg2 may have resulted from a gene duplication event during the evolution of *S. cerevisiae* (28). It is conceivable that Ntg1 evolved as a protein capable of responding to both nuclear and mitochondrial oxidative DNA damage, requiring regulated localization between the nucleus and mitochondria following oxidative stress. In contrast, Ntg2 may have evolved to function in repair of nuclear DNA damage exclusively as Ntg2 remains nuclear in all circumstances. *S. cerevisiae* may simply prioritize nuclear BER over mitochondrial BER by maintaining Ntg2 in the nucleus due to the capability of yeast cells to respire anaerobically in the absence of mitochondrial DNA (29). Further evidence in

support of regulation of Ntg1 by nuclear and mitochondrial oxidative DNA damage is the fact that Ntg2 contains an iron-sulfur center, but Ntg1 does not (9). The absence of the iron-sulfur center may allow Ntg1 to remain active when recruited to the high redox environment of the mitochondria, a situation believed to be detrimental to proteins containing an iron-sulfur center (34, 38). We speculate that certain BER proteins with dual organellar localizations may be regulated in a manner similar to Ntg1, where localization is influenced by levels of nuclear and mitochondrial oxidative DNA damage, whereas other BER proteins, such as Ntg2, are responsible for maintaining baseline repair capacities in nuclei and/or mitochondria at all times. There is at least one other eukaryotic species that contains two Nth homologous gene sequences, *Arabadopsis thaliana* (6). It is probable that *A. thaliana* and any other eukaryotes with two Nth homologs will be managed in a similar manner as Ntg1 and Ntg2, as these organisms have both nuclei and mitochondria (and chloroplasts in plants), all containing DNA. It is also possible that other eukaryotic DNA repair proteins which are the products of a duplication event are also regulated in a localization-mediated manner. Interestingly, two copies of the alkyladenine glycosylase (AlkA) gene are present in numerous eukaryotic species (6). Therefore, it is valuable to consider the possibility that other eukaryotic DNA glycosylases and repair proteins resulting from a duplication event could be regulated at the level of localization.

Post-translational modification by SUMO. Results from Chapter 2 indicate that Ntg1 and Ntg2 are sumoylated (Chapter 2, Figure 6). Our results indicate a potential role for sumoylation of Ntg1 in dynamic localization. Sumoylated Ntg1 is detected only in the

nucleus following the occurrence of oxidative DNA damage, indicating that sumoylation may influence the nuclear localization of Ntg1 (Chapter 2, Figure 7). Additionally, one of the potential sumoylation sites of Ntg1 was determined at lysine 364, and mutation of this lysine resulted in mislocalization of Ntg1 (Chapter 2, Figure 8). Together, these data suggest that sumoylation allows Ntg1 to interact with the nuclear pore to allow protein entry, or sumoylation prevents Ntg1 from exiting the nucleus, creating a pool of nuclear Ntg1 in the presence of nuclear oxidative DNA damage.

Investigating the sumoylation of Ntg1 and Ntg2 is a future direction suggested by this dissertation. Ideally, the sites of sumoylation should be mapped in Ntg1 and Ntg2, which will allow us to determine if all seven predicted sumoylation sites of Ntg1 and one sumoylation site of Ntg2 are utilized and if there are others that cannot be identified with the consensus motif. Once the sumoylation sites are determined, the function of modification for each protein can be elucidated by making point mutants at sumoylation sites and determining how Ntg1 and Ntg2 are affected via microscopy, enzyme activity assays, and biochemical analysis. Potential functions of sumoylation include regulation of localization, prevention of degradation, and modification of enzymatic activity. The major limitation of determining the sumoylation sites of Ntg1 and Ntg2 is that these BER proteins are expressed at low levels and only a small proportion of Ntg1 and Ntg2 are sumoylated in whole cell lysate. Therefore, generating the substantial quantities of protein necessary to make these analyses is time-consuming and cumbersome.

Identification of response elements that regulate the dynamic localization of Ntg1. As dynamic localization in response to oxidative DNA damage appears to be a novel mode of regulation for a BER protein, we were particularly focused on determining how Ntg1 is recruited to organelles in response to oxidative DNA damage. Thus, in Chapter 2 (Figure 4) we determined that relocalization is DNA damage-mediated. This implies that oxidative DNA damage must produce a signal (NODDS and MODDS) to recruit Ntg1 to either the nucleus or mitochondria. The next step was to determine how NODDS and MODDS are able to affect localization of Ntg1. In particular, it was a goal to identify proteins which act as DNA damage responders, facilitating the recruitment of Ntg1 to nuclei or mitochondria. In an effort to identify such factors, the bipartite cNLS and MTS of Ntg1 were determined, and mutations in these sequences prevent localization of Ntg1 to nuclei and mitochondria, respectively (Chapter 3, Figure 1). Additionally, the transport machinery that is responsible for translocating Ntg1 from the cytoplasm into the nucleus was determined to be the classical nuclear protein transport machinery, importin α/β (Chapter 3, Figure 3). These results indicate that the classical nuclear import machinery plays a very important role in the response to oxidative DNA damage by escorting Ntg1 into the nucleus in response to NODDS and identify importin α/β as novel DNA damage response proteins.

It is quite likely that that we have not yet determined all of the NODDS and MODDS responders that are central to the dynamic localization of Ntg1. Several likely candidates for responders to both NODDS and MODDS exist. Putative NODDS responders include components of the SUMO pathway, components of the classical nuclear protein import pathway, and components of the well-characterized ATM/ATR

DNA damage response pathways. As we observed the sumoylation of Ntg1 only in yeast nuclei following oxidative stress (Chapter 2, Figure 7), it is possible that E2 conjugating enzyme, Ubc9, or the E3 ligating enzyme responsible for Ntg1 sumoylation may respond to NODDS and sumoylate Ntg1. This sumoylation event may act to prepare Ntg1 to interact with classical nuclear import machinery and the nuclear pore or prevent Ntg1 from being able to interact with nuclear export machinery. As importin α/β is involved in Ntg1 nuclear localization, other proteins in this pathway, such as other karyopherins, unknown scaffolding proteins, or unknown stress responders, may also be involved in the localization of Ntg1 to the nucleus. Perhaps those karyopherins which interact with sumoylation machinery are stronger candidates for a role in nuclear localization. Support for this idea is that importin α/β (Kap60-Kap95) along with Kap121 are necessary for targeting of Ulp1, which is involved in SUMO processing and deconjugation, to the nuclear pore complex (26). Interestingly, Kap121 is the karyopherin responsible for Yap1 nuclear import (17). As Yap1 is involved in the oxidative stress response (5, 11, 18, 25), this suggests that importin α/β, Kap121, Yap1, Ntg1, and Ulp1 may be members of an oxidative stress response pathway involved in regulating nuclear protein transport. Other potential NODDS responders include yeast Mec1 (human ATM, ataxia telangiectasia mutated), which is involved in the repair of double strand breaks (30), and yeast Tel1 (human ATR, ataxia-telangiectasia mutated and rad3-related), which activates repair machinery in response to lesions generating ssDNA (14). It is possible that these DNA damage sensors may also play a role in the response to oxidative DNA damage. In the case of MODDS responders, mitochondrial transport chaperones, of which little is known, could facilitate the import of Ntg1 into mitochondria. Other possibilities exist as

responders to NODDS and MODDS; however, we hypothesize that the identification of a responder for the recruitment of Ntg1 is likely to be a responder for the recruitment of other proteins that are regulated by a mechanism of dynamic compartmentalization in response to DNA damage or oxidative stress.

We hypothesize that regulation of subcellular localization in response to levels of nuclear and mitochondrial DNA damage is a control mechanism for other DNA repair proteins that reside in both nuclei and mitochondria. In an effort to identify additional yeast nuclear and mitochondrial BER proteins that might be regulated by a process of dynamic localization, we identified the NLS and MTS sequences of a set of candidate BER proteins with dual localization. We hypothesize that the localization of proteins with a similar N-terminal NLS/MTS sequence motif as Ntg1 are likely to be regulated in a similar fashion. We analyzed the NLS/MTS sequences of the following proteins: hNTH, Ntg1, Ung1, Ogg1, Apn1, Abf1, Abf2, Msh1, Pif1, Cdc9, Mag1, and Mag2. Of these proteins, only uracil DNA glycosylase 1 (Ung1) has a potential bipartite NLS and an N-terminal MTS, which appears to be in the same orientation as the NLS and MTS sequences of Ntg1 (Figure 1 and (2)). It is a future direction of this study to determine if Ung1 is regulated by a process of dynamic localization. Not only will this analysis show that dynamic localization is a general mode of regulation, but it will also demonstrate whether a number of DNA damage types (uracil and alkylation products, in this case) can cause the production of NODDS or MODDS in order to generate a localization response as ROS are produced in response to various types of DNA damage (27). Additionally, other nuclear/mitochondrial BER proteins in both yeast and humans will need to be tested

for the possibility of regulated localization in order to determine if dynamic localization is a widely utilized mode of regulation for BER proteins across species.

The role of dynamic localization of Ntg1 on DNA mutagenesis. We determined that Ntg1 plays a significant role to prevent the accumulation of nuclear and mitochondrial DNA mutations (Chapter 3, Table 3). The accumulation of mutations in genes, such as tumor suppressor genes and oncogenes, influences the maintenance of nuclear and mitochondrial genomic stability, indicating that Ntg1 plays a role in preventing microsatellite and chromosomal instability. Many human diseases, including cancer and other degenerative disorders, are associated with the accumulation of nuclear and mitochondrial DNA mutations that lead to genomic instability and disease progression (13, 35). The fact that Ntg1 plays an important role in the prevention of nuclear and mitochondrial mutations indicates the significance of understanding how Ntg1 and other BER proteins are regulated.

While dynamic localization of Ntg1 influences nuclear and mitochondrial DNA mutagenesis, dynamic localization of Ntg1 did not appear to play a role in H_2O_2-induced cytotoxicity (Chapter 3, Figure 7). A number of explanations are possible for why dynamic localization is important for preventing mutations but not for cellular survival in oxidative stress. It is likely that recombination, translesion synthesis, and transcription coupled repair processes are upregulated in the absence of Ntg1, Apn1, Rad1, and Ntg2 and are capable of repairing the oxidative lesions that pose replication and transcription blocks, allowing alternative repair to occur in the absence of dynamic localization of

Ntg1. It is also possible that dynamic localization of BER proteins in response to oxidative DNA damage is not the only mechanism by which Ntg1 enters nuclei and mitochondria, meaning that proteins like Ntg1 can protect the cell from replication and transcription stalling via pathways independent of dynamic localization (importin α/β, for example). As mutation rates are influenced by dynamic localization, it is possible that proteins like Ntg1 are capable of prioritizing repair of lesions that can result in cell death over lesions that result in mutagenesis. Finally, a threshold level of DNA damage may dictate cellular survival following oxidative stress, such that the level of accumulation of nuclear mutations or mitochondrial mutations that result from mislocalization of Ntg1 is not sufficient to drive the level of cell death that is observed in Ntg1 null cells.

A model for the regulation of BER by the dynamic localization of Ntg1. Figure 2 depicts a model of how Ntg1 localization is regulated and how this contributes to the overall health of the cell. In our model, Ntg1 localization is affected by nuclear and mitochondrial oxidative DNA damage. We have designated the signals that are produced in response to nuclear or mitochondrial oxidative DNA damage as nuclear or mitochondrial oxidative DNA damage signals, respectively (NODDS or MODDS, respectively). These NODDS and MODDS could be sent directly to Ntg1 or to a mediator protein in the Ntg1 recruitment process, and these signals ultimately compete for the nuclear or mitochondrial localization of Ntg1 to DNA damage loci. Various proteins are likely to interact with Ntg1 in order to facilitate the recruitment of Ntg1 into damage-containing nuclei and mitochondria. These proteins are considered DNA damage responders, and the classical nuclear protein import machinery, importin α/β,

was identified as Ntg1 NODDS responders, indicating a novel function for importin α/β in the DNA damage response. As Ntg1 is sumoylated, and sumoylation of Ntg1 appears to affect its nuclear localization in response to oxidative stress, members of the sumoylation pathway are likely Ntg1 NODDS responders as well. Following recruitment of Ntg1 into nuclei or mitochondria, Ntg1 performs its DNA glycosylase/AP lyase activity in order to repair oxidative DNA damage, and this promotes genomic stability, indicating the importance of Ntg1 dynamic localization.

Implications for the regulation of human BER proteins and human disease. The human homolog of Ntg1, hNTH1, has also been studied in regards to its localization. hNTH1 localizes to both nuclei and mitochondria (15, 32). In addition, the N-terminal region of hNTH1 contains two NLSs and one potential site for cleavage of a mitochondrial presequence (Figure 1) (16). Ikeda, et al. demonstrated in 2002 that hNTH1 contains a bipartite NLS and an MTS and that the bipartite cNLS is dominant over the MTS of hNTH1 so that the majority of cells demonstrate only nuclear localization of hNTH1 (16). The fact that both hNTH1 and Ntg1 contain similar localization sequences (bipartite NLS and MTS), indicates that the regulatory mechanisms that influence hNTH1 and Ntg1 are likely to be similar. If this is the case, hNTH1 mitochondrial localization is likely to occur in response to mitochondrial oxidative stress and DNA damage. This situation should be addressed in future studies, as the regulation of hNTH1 by dynamic localization is likely to play a key role in the prevention of nuclear and mitochondrial DNA mutation and, ultimately, human disease. In order to determine whether misregulation of hNTH1 is associated with cancer

occurrence, the sequence of hNTH1 could be evaluated from DNA samples in cancer and normal tissues. It may be possible to detect sequence variants in the bipartite NLS or MTS of hNTH1 that affect localization of hNTH1 in cancer cells specifically. Cancer-specific variations in the sequences that dictate localization of hNTH1 would indicate that hNTH1 localization is important and that hNTH1 plays an important role in preventing the genomic instability that can result in tumorigenesis. As genomic instability is thought to be an early event in the progression of cancer, misregulation of hNTH1 could be a valuable tool for early diagnosis of cancer, allowing for earlier treatment and better prognosis.

BER is important for prevention of the cell death and mutagenesis that is associated with aging, various degenerative disorders, and cancer (1, 4, 21, 35, 36). Human cells do not rely exclusively on hNTH1 for BER to function in nuclei and mitochondria, preventing a situation that could be deleterious if hNTH1 were to become impaired or have its capacity exceeded. Instead, there are numerous BER, and even NER, proteins that can remove and repair oxidative lesions, which for the most part maintain a healthy human being. If we consider the case of human cancer, tumorigenesis could begin with one cell containing only a couple of mutations. If this one cells acquires mutations in genes that give this cell a growth advantage, then the cell could result in cancer. As oxidative stress is a major source of DNA damage, the likelihood of the incorporation of mutations at sites of oxidative DNA damage in human cells is relatively high; however, with intact BER proteins, such as hNTH1, the DNA damage is removed prior to the occurrence of mutation. In the case that BER proteins are suddenly misregulated and can no longer participate in dynamic localization, the accumulation of

mutations will occur in both nuclei and mitochondria, and this will likely result in transformation into a cancer cell. Associated with this BER misregulation and the accumulation of mutations are microsatellite instability and chromosomal instability. The negative impact that misregulation of dynamic localization of BER proteins could have on the health of human cells indicates how important it is to understand the details of this type of regulation. The significant impact of this mode of BER regulation also implies the possibility of utilizing BER proteins in preventative medicine. Like antioxidants, BER proteins could be induced in patients so as to prevent the accumulation of deleterious mutations and prevent human disorders. Additionally, the activity of BER proteins could be monitored in order to verify that an appropriate level of DNA repair is taking place to prevent disease. Potential cancer, neurodegenerative disorder, neuromuscular disorder, and, even aging, patients would benefit from this type of preventative medicine. It is also possible that cancer patients could be treated with chemotherapeutic drugs that inhibit BER proteins in cancer cells and induce BER proteins in normal cells in order to facilitate the elimination of cancer, while promoting the health of normal cells.

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Figure 1

A. Ung1

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1     MWCMRRLPTN SVMTVARKRK QTTIEDFFGT KKSTNEAPNK KGKSGATFMT
51    ITNGAAIKTE TKAVAKEANT DKYPANSNAK DVYSKNLSSN LRTLLSLELE
101   TIDDSWFPHL MDEFKKPYFV KLKQFVTKEQ ADHTVFPPAK DIYSWTRLTP
151   FNKVKVVIIG QDPYHNFNQA HGLAFSVKPP TPAPPSLKNI YKELKQEYPD
201   FVEDNKVGDL THWASQGVLL LNTSLTVRAH NANSHSKHGW ETFTKRVVQL
251   LIQDREADGK SLVFLLWGNN AIKLVESLLG STSVGSGSKY PNIMVMKSVH
301   PSPLSASRGF FGTNHFKMIN DWLYNTRGEK MIDWSVVPGT SLREVQEANA
351   RLESESKDP
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B. hNTH1

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1     MCSPQESGMT ALSARMLTRS RSLGPGAGPR GCREEPGPLR RREAAAEARK
51    SHSPVKRPRK AQRLRVAYEG SDSEKGEGAE PLKVPVWEPQ DWQQQLVNIR
101   AMRNKKDAPV DHLGTEHCYD SSAPPKVRRY QVLLSLMLSS QTKDQVTAGA
151   MQRLRARGLT VDSILQTDDA TLGKLIYPVG FWRSKVKYIK QTSAILQQHY
201   GGDIPASVAE LVALPGVGPK MAHLAMAVAW GTVSGIAVDT HVHRIANRLR
251   WTKKATKSPE ETRAALEEWL PRELWHEING LLVGFGQQTC LPVHPRCHAC
301   LNQALCPAAQ GL
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Figure 1. The Amino Acid Sequence of Ung1 and hNTH1. The full amino acid sequences of *S. cerevisiae* Ung1 **(A)** and hNTH1 **(B)** are displayed, with putative NLS (green), MTS (red), and SUMO sites (blue) highlighted. The sites were predicted with the NUCDISC subprogram of PSORTII (23), MitoProt II program (3), and the SUMOsp 1.0 program (37).

Figure 2

Cancer
Degenerative Disorders
Aging
Cell Death
Mutagenesis
Chromosomal Instability (CIN)
Healthy Individual
Cellular survival
DNA DAMAGE
Without Proper Localization
BER
Proper Localization
Nuclear Oxidative DNA Damage
Mitochondrial Oxidative DNA Damage
Nuclear oxidative DNA damage signal (NODDS)
Mitochondrial oxidative DNA damage signal (MODDS)
Nuclear Localization
Mitochondrial Localization
NODDS Responder
cNLS
Ntg1
MODDS Responder
MTS
Ntg1
NODDS Responder
MODDS Responder
Ntg1
Importin α/β
SUMO machinery
Mitochondrial import chaperone?

Figure 2. A Model for Dynamic Localization of Ntg1 as a Major Mode of BER Regulation. Nuclear and mitochondrial oxidative DNA damage occurs as a result of oxidative stress in each organelle. This resulting oxidative DNA damage creates a signal, nuclear oxidative DNA damage signal (NODDS) or mitochondrial oxidative DNA damage signal (MODDS) that initiates the recruitment of Ntg1 to nuclei or mitochondria, respectively. DNA damage responders, including importin α/β and sumoylation machinery, recruit or facilitate accumulation of Ntg1 in the nucleus or mitochondria in response to NODDS and MODDS. Once in the nucleus or mitochondria, Ntg1 increases oxidative DNA repair capacity, which can prevent cell death, mutagenesis, chromosomal instability (CIN), and microsatellite instability (MIN). By preventing relocalization of BER proteins that are regulated by a process of dynamic localization, yeast cells could undergo processes that display phenotypes similar to human aging and cancer (10). This gives us reason to believe that dynamic localization of BER proteins will be very important for maintaining a healthy human individual.